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Environmental Enrichment Reveals Effects of Genotype on Hippocampal Spine Morphologies in the Mouse Model of Fragile X Syndrome

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Fragile X Syndrome (FXS) and the Fmr1 knockout (KO) mouse model of this disorder exhibit abnormal dendritic spines in neocortex, but the degree of spine disturbances in hippocampus is not clear. The present studies tested if the mutation influences dendritic branching and spine measures for CA1 pyramidal cells in Fmr1 KO and wild-type (WT) mice provided standard or enriched environment (EE) housing. Automated measures from 3D reconstructions of green fluorescent protein (GFP)-labeled cells showed that spine head volumes were ~40% lower in KOs when compared with WTs in both housing conditions. With standard housing, average spine length was greater in KOs versus WTs but there was no genotype difference in dendritic branching, numbers of spines, or spine length distribution. However, with EE rearing, significant effects of genotype emerged including greater dendritic branching in WTs, greater spine density in KOs, and greater numbers of short thin spines in KOs when compared with WTs. Thus, EE rearing revealed greater effects of the Fmr1 mutation on hippocampal pyramidal cell morphology than was evident with standard housing, suggesting that environmental enrichment allows for fuller appreciation of the impact of the mutation and better representation of abnormalities likely to be present in human FXS.

Keywords: 3D reconstruction, dendritic spine, enriched environment, fragile x syndrome, hippocampus

Introduction

Fragile X syndrome (FXS) is the most common form of inherited mental retardation. It is mainly caused by an expansion of CGG repeats in the Fmr1 gene that encodes Fragile X Mental Retardation Protein (FMRP); the expansion leads to hypermethylation that blocks FMRP expression thereby resulting in the disorder (Penagarikano et al. 2007). FMRP regulates protein synthesis within dendritic spines and its absence results in abnormal levels of a number of synaptic proteins (Todd et al. 2003; Zalfa et al. 2005; Muddashetty et al. 2007; Schütt et al. 2009; Chen et al. 2010). Mice lacking FMRP due to knockout (KO) of Fmr1 expression exhibit many features of human FXS including impaired learning (Consortium TD-BFX 2000; Segal et al. 2003; Grossman et al. 2005; Zhao et al. 2005; Li et al. 2001). Because the hippocampus is responsible for new declarative memory formation, one would expect that cellular abnormalities underlying impairments in encoding would be readily evident in this structure. Therefore, a major goal of this study was to use 3D reconstructions and automated size measures to characterize spine abnormalities in hippocampus of adult Fmr1 KO mice. Using mice expressing GFP in scattered hippocampal neurons, confocal imaging and 3D reconstructions of field CA1 apical dendrites, we assessed 1) dendritic branching, and 2) the densities and morphometric measures of spines on secondary dendrites. With an interest in the prospect that rearing in an EE might normalize spine morphologies and, as a consequence, synaptic plasticity in the KOs (Restivo et al. 2005; Meredith et al. 2007), we also evaluated dendrite and spine measures for mice reared in standard laboratory cages.

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versus an EE. The analyses revealed only modest differences between hippocampal spine measures for Fmr1 KO and WT mice provided standard housing with the exception of spine head volumes which were reliably lower in the mutants. Rearing in the EE did not attenuate this difference; rather, it revealed greater effects of the FXS mutation on dendritic arbors and spines of adult hippocampal neurons.

Materials and Methods

All experiments were carried out in using protocols approved by the Institutional Animal Care and Use Committee at the University of California at Irvine, and consistent with National Institutes of Health guidelines. Fmr1 KOs on the C57BL/6 background were crossed with C57BL/6 mice expressing GFP under the Thy1 promoter in scattered neurons (i.e., the GFP-M line (Feng et al. 2000)). Mice were genotyped, and male Fmr1 KOs and WT expressing the GFP transgene were used.

Enriched Environment

At postnatal day 21, male Fmr1 KO GFP and WT GFP mice were placed in EE housing 5 per cage by genotype. The EE consisted of one large (53 × 30 × 20 cm) and one medium-sized (44 × 25 × 20 cm) rodent cage that were connected by a tube allowing for passage between the 2 "rooms," each with ground corncob bedding. The larger cage had a habit trail system, small toys, food, and water. The medium-sized cage had a running wheel, small balls, and small chew toys. Mice were continuously housed in this EE from the time of weaning. At 3 months of age, all EE animals were killed with an overdose of Euthasol (Western Medical Supply) and then intracardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) along with age- and sex-matched KO GFP and WT GFP mice that had been group-housed in standard mouse cages (45 × 17 × 13.5 cm) containing bedding, food, and water (standard housing, or SH). Tissue was cryoprotected for 2 days in 20% sucrose/4% paraformaldehyde, frozen, and stored at −80 °C.

Immunocytochemistry and Microscopy

Coronal sections (40–50 μm) through the hippocampus were prepared on a freezing microtome and processed for the immunofluorescence localization of GFP to enhance labeling of spine profiles. The free-floating tissue sections were incubated in chicken anti-GFP (1:1000; Abcam *ab13970) in PB containing 0.3% triton-X 100 and 4% bovine serum albumin overnight at room temperature. Following rinses in PB, tissue was incubated with Alexafluor488 anti-chicken IgG (1:1000; Invitrogen) for 1 h, rinsed, mounted onto slides, and coverslipped with Vectashield (Vector Laboratories).

Image z-stacks of GFP-labeled field CA1 pyramidal cells, and their apical dendrites, were collected using a Zeiss 510 confocal microscope and a 63 × 1.4 NA objective. For apical dendrite analyses, image z-stacks were collected with 1 μm steps through the target dendrite and with minimal zoom to capture a broad dendritic span (Fig. 1A). For spine analyses, images were collected from secondary dendrites in the apical field using 0.15-μm step spacing which is the Nyquist rate for the objective used (Fig. 1B). To minimize variations in spine measures reflecting distance from the cell body, images were collected from the most proximal secondary dendrites (i.e., the first or second relative to the cell soma): each z-stack included the first 90–100 μm of the secondary dendrite from the point of emergence from the primary apical dendrite. Image stacks were deconvolved using AutoQuant Version 2.2.1 (Media Cybernetics), and then 3D builds were made using Imaris software Version 7.0.0 and the Filament Tracing module (Bitplane).

Dendrite Branching Analysis

The branching of GFP-labeled dendrites was measured using Imaris’ automated Sholl (1953) analysis that quantifies the number of intersections between dendritic branches and concentric Sholl rings placed at even distances from the cell body. Numbers of branches and total dendritic lengths for secondary and tertiary dendrites were also determined using Imaris’ automated Dendrite Branch Level analysis. The branch level was assessed according to diameter calculations of the individual dendrite segments at each branching point; thus, at each branching point the dendritic segment with the smaller mean diameter was assigned the higher order branch level. For each data point, measures were determined for each cell and then the individual cell values were averaged to determine mean values per animal.

Spine Measures

The 3D renderings of individual dendrites were prepared as illustrated in Figure 1. The automatic seed point detector function in Imaris, which uses thresholding of labeling intensity to demarcate labeled structures, and incorporates a Mexican Hat Filter to eliminate background and enhance object detection, was used to identify spines and define both heads and necks. Spine length was defined as the sum of

Figure 1.
the radius of the spine terminal point (i.e., the center of the head to the tip) and spine segment (or “neck”) length extending from the head to the point of attachment on the dendrite. The maximum spine length and minimum spine end diameter were set to 5 and 0.2 µm, respectively; objects that did not fit within these size constraints were excluded from analysis. A recent study using Imaris and these same parameters to generate automated measures of hippocampal spines demonstrated, in a side-by-side comparison with manual measures, that this automated method yielded more precise data for spine heads, necks, and length (i.e., less variability), and better accuracy relative to estimated population statistics based on ultrastructural data (Swanger et al. 2011). Automated measures of the lengths and head volumes for spines, and the numbers of spines per dendritic span, were generated from the 3D renderings and data were exported to Excel (Microsoft). An in-house Matlab (Mathworks) script was used to bin measures for each 10 µm span of the target dendrites. Measures of spine head volume versus spine length were plotted using SigmaPlot Version 11.0 (Systat Software, Inc.); for these analyses, spine measures for each experimental group were expressed as a percent of the total population for that group. Then, for the 2 groups being compared, the spine-profile populations that overlapped between the 2 groups were subtracted leaving the nonoverlapping, or “unique,” population for each group in the comparison.

Statistical Analysis
To compare the effect of 2 variables (e.g., genotype and housing), a 2-way ANOVA was used, followed by post hoc comparisons of all groups in Prism 5.0 (GraphPad Software, Inc.); post hoc comparisons are presented for some analyses to show the source of significance between data groups. Best-fit curve analyses for spine head volume and length measures were done using Minitab version 16.1.0 (Minitab, Inc.). For these analyses, each dendrite was assessed separately for a “best” fit distribution using the Anderson-Darling goodness of fit test (Anderson and Darling 1952) which determines if the sample (all spines per dendrite) fits the 95% confidence interval of the following distributions: normal, lognormal, 3-parameter lognormal, exponential, 2-parameter exponential, Weibull, 3-parameter Weibull, smallest extreme value, largest extreme value, gamma, 3-parameter gamma, logistic, log logistic, and 3-parameter log logistic. After a best-fit was identified for each dendrite, the overall best-fit for each group was determined based on which distribution was consistently fit for >50% of the dendrites. Thus, if over 50% of the dendrites had a Weibull distribution of spine measures, then this distribution was assigned to the animal group as the overall best-fit. However, if more than one curve met this criteria then the curve with the lowest (i.e., more significant) Anderson–Darling value was used. Where appropriate, the calculated kurtosis and shape values for each dendrite’s curve were averaged to generate an individual animal’s mean and then group means were compared.

Results

EE Increases Dendritic Arborization
The effects of genotype and housing on apical dendritic arbors of CA1 pyramidal cells were evaluated using Sholl concentric ring and Dendrite Branch Level analyses. For neurons from mice reared in standard group housing (SH) for the 2 months post-weaning, the number of dendritic intersections with Sholl rings, the number of dendritic branches, and total lengths of secondary and tertiary dendritic branches, were similar between genotypes (P > 0.05, for all measures) (Fig. 2). For WT mice, housing in the EE modestly increased numbers of Sholl intersections (Fig. 2A) but significantly increased numbers of tertiary dendritic branches (P < 0.001 for EE vs. SH groups) (Fig. 2B). By contrast, EE housing did not significantly influence these measures of pyramidal cells from Fmr1 KO mice. Finally, the length of tertiary dendrites was greater in EE than in SH mice for both genotypes (P < 0.01 and 0.05 for WTs and KOs, respectively) (Fig. 2C); this effect was selective for tertiary dendrites as numbers and lengths of secondary dendrites were comparable across groups (Fig. 2B,C). Overall, due to greater effect of housing environment on the WT mice, placement in the EE revealed effects of genotype on dendritic arbors that were not evident for mice given standard housing.

Rearing Effects on Spine Density
The analysis of dendritic spines focused on secondary dendrites to avoid potential confounds arising from changes in the structure of higher order dendritic branches. To simplify presentation of the results, the term “spine” is used throughout to describe all protrusions extending at least 0.2 µm off of the dendritic shaft, regardless of morphological features.

The numbers of spines were quantified for 10 µm bins across an 80-µm span of the secondary dendrites. As shown in Figure 3A, there was no effect of genotype on the incidence or distribution of spines across these bins for mice in standard housing. For EE-housed mice, there was a small, but significant, effect of genotype on spine density (P = 0.0252, 2-way ANOVA) with Fmr1 KOs displaying somewhat greater numbers of spines in more distal bins than the WTs (Fig. 3B). As with the dendritic analysis, the presence of differences in spine numbers between genotypes for EE-housed mice reflected a greater influence of housing on WT mice: for the full dendritic span evaluated, there was a trend for effect of housing in WT mice (WT SH vs. WT EE; P = 0.0627, 2-way ANOVA) but not in Fmr1 KOs (KO SH vs. KO EE; P = 0.4279, 2-way ANOVA).

Spine Head Volumes Are Smaller in KOs
To evaluate effects of genotype and housing environment on spine morphology, we measured the length and head volume for spines on secondary dendrites of field CA1 pyramidal cells. The Fmr1 KOs had significantly smaller spine head volumes than did the WTs for both housing conditions (P < 0.0001 for effect of genotype, 2-way ANOVA): for KOs, the mean spine head volume was 40.7% and 42.6% lower than in WTs for SH and EE groups, respectively (Fig. 4A,B). Average spine length was significantly different between genotypes housed under standard conditions (P < 0.0185 for effect of genotype, 2-way ANOVA), with KO spines exhibiting greater lengths than WT spines (Fig. 4C). However, there was no effect of genotype in EE-reared animals (Fig. 4D); of note, while spines situated closer to the primary apical dendrite (bins 1 and 2) were somewhat shorter for mice raised in the EE (Fig. 4D) when compared with SH (Fig. 4C), this effect was not statistically significant [so, this is one measure “normalized” in the KOs by EE].
The preceding analyses demonstrated marked effects of genotype on spine head volume but did not provide information on the distribution of spines with different measures across individual dendrites in the various groups. To address this issue, we conducted a best-fit curve analyses for head volumes and lengths of spines on individual dendrites, using the Anderson–Darling goodness of fit test (Anderson and Darling 1952). To determine the “best-fit” curve for each experimental group, 2 criteria were used in sequence: 1) measures from >50% of the dendrites in each group had to be fit to a curve, and 2) if more than one curve met this criteria then the curve with the lowest (i.e., more significant) Anderson–Darling value was used.

The best-fit analyses for spine head volumes revealed both differences between genotypes within a given housing condition, and an effect of housing for each genotype. For WT mice reared in SH, the majority (71.4%) of dendrites had spine head volumes that fit a gamma distribution (Fig. 5A). In contrast, measures from Fmr1 KO mice reared in SH did not fit just one distribution: one-third fit either a gamma or 3-parameter Weibull distribution whereas the rest did not fit any distribution.

Rearing in the EE altered the best-fit curves for head volume in both genotypes: for WT EE mice, the majority (70.8%) of dendrites had spine head volumes that fit a Weibull distribution, whereas for KO EE mice, the majority (75%) fit a gamma distribution (Fig. 5A).

The best-fit distribution for KO EE mice differed from both WT groups in having a greater proportion of spines with small...
head volumes (Fig. 5A). To test the significance of this difference, kurtosis values (i.e., measures of “peakedness”) of the fit-distributions for individual dendrites were averaged to calculate individual animal means, and then group mean values were compared. As expected, the KO EE group exhibited higher kurtosis values than did either WT group but this only approached significance for the KO EE versus WT EE comparison (P = 0.0541, Student’s t-test).

The analyses of spine length distribution determined that the 3-parameter Weibull provided the best fit for dendrites in all 4 groups, although the percentage of dendrites fitting this curve varied: 88% for WT SH, 66% for KO SH, 62.5% for WT EE, and 80% for KO EE. As shown in Figure 5B, the best-fit distributions for spine lengths were nearly identical for the 2 genotypes in SH, but differed with housing in the EE. Notably, for EE mice, the WT distribution was left-shifted (toward smaller lengths) relative to that for the Fmr1 KOs. Analyses of each dendrite’s best-fit curve for spine length confirmed this observation. Average kurtosis values were greater for WTs than for KOs, and this difference was significant after rearing in the EE. Finally, Weibull distributions can be defined by the parameters of shape, scale, and threshold: a shape value of 3 indicates a near normal distribution whereas much lower or higher values (e.g., 1 or 10) indicate a right or left skew, respectively. As shown in Figure 5C, among groups the WTs reared in the EE had the lowest shape value indicating that exposure to the more complex environment increased the rightward skew in the spine length distribution of the WTs but not the mutants.

### Housing and Genotype Effects on Spine Morphology Distributions

To identify differences in spine morphology between groups, we considered both head volume and length together for each spine. Specifically, for each group, 3D density plots (spine length vs. head volume) were generated (Fig. 6A–D). All 4 groups had similar spine distributions with the majority of spines in each group having head volumes of ≤0.1 µm³ and lengths of ≤1 µm. However, analysis of spine measures on a “per animal” basis demonstrated a significant effect of genotype. In particular, for each mouse, we determined the percent of the spine population with measures placing that element in 1 of the 4 zones enumerated in Figure 6D. There were no group differences in the proportion of spines within zones 1 and 4 (Fig. 6E). However, the Fmr1 KOs housed in the EE had more short and narrow spines (zone 2) than did the other groups, and fewer spines with large head volume (zone 3) than WT mice with the same housing.

To further isolate differences between groups, we used a subtractive method: spines with measures that were shared between 2 groups (e.g., the same volume and length measures for WT SH and KO SH groups) were subtracted leaving the nonoverlapping, or “unique,” populations for a given group in the comparison. These unique spine populations represented ~16–20% of the total for each group depending on the comparison (Table 1). Using this approach, we first evaluated the effects of housing on spine distributions within a genotype. As shown in Figure 7A, and summarized in the top paired comparisons in Table 1, WT mice reared in either housing condition had nonoverlapping populations of spines that predominantly had head volumes of ≤0.1 µm³ and lengths of ≤1 µm. However, for the WTs, housing in the EE increased the incidence of short spines with larger head volumes (zone 3). The effect of EE was different for Fmr1 KOs. Mutants given SH exhibited greater numbers of nonoverlapping spines with narrow heads (≤0.1 µm³) but with lengths ranging up to 2 µm (Fig. 7B, left) thereby extending well into zone 1. In contrast,
Environmental Rearing Effects on Fmr1 KO Hippocampal Spines

Discussion

The present studies demonstrate that there are consistent effects of genotype on spine measures for mice reared in the same housing condition. With SH, nonoverlapping spines in the KOs had very narrow heads (largely ≤0.05 µm) but variable lengths thus distributing across zones 2 and 1 whereas those in the WTs extended across all 4 zones with greatest numbers in zones 2 and 3 (Fig. 7C; Table 1). The zone distributions of nonoverlapping spines were similar for mice housed in the EE: for WTs, they were distributed across all 4 zones with greatest numbers in zones 2 and 3 whereas, in the KOs, they were clustered in zone 2 and, to lesser extent, zone 1 (Fig. 7D; Table 1).

Effects on Dendritic Branching

Rearing genetically normal animals in an EE is known to increase dendritic arborizations of cerebellar, neocortical, and hippocampal neurons (Volkmar and Greenough 1972; Greenough et al. 1973; Faherty et al. 2003; Kozorovitskiy et al. 2005; Leggio et al. 2005; De Bartolo et al. 2011). It also increases brain-derived neurotrophic factor expression (Ickes et al. 2000;
Franklin et al. 2006; Angelucci et al. 2009; Kazlauckas et al. 2011; Kuzumaki et al. 2011) and neurogenesis (Kempermann et al. 1997; Nilsson et al. 1999) which likely contribute to the changes in dendritic architecture. In line with this, we found that in WT mice rearing in an EE increased field CA1 apical dendritic branching as determined by numbers of tertiary dendritic branches; this effect was absent in the mutants. For both genotypes, tertiary dendrites were longer with 2 months in the EE when compared with SH. The consequences of these changes in tertiary branches to CA1 pyramidal neurons are likely to be seen at the level of signal integration. Strong stimulation of first or second order pyramidal cell dendritic branches reliably elicits action potentials whereas the same stimulation applied to higher order terminal dendritic segments reportedly elicits a shift from graded (with lesser stimulation) to all-or-none responses that are still subthreshold for triggering somatic action potentials (Wei et al. 2001). This feature of distal dendrites allows the cells to have multiple signal

Figure 6. Housing in an enriched environment increases effects of genotype on spine size measures. (A–D) Histograms show spine population distributions for WT SH (A), KO SH (B), WT EE (C) and KO EE (D) groups; for comparison between groups and to control for differences in absolute numbers of spines between groups, data from all animals in each group were combined and normalized to 100% of the population. Four zones were delineated in each graph as identified by bold lines marking a head volume boundary at 0.1 µm³ and a length boundary at 1.0 µm (D). Note that in general the distributions are very similar across groups, although the KO groups tend to have a higher proportion of spines in zone 2. (E) Bar graph shows quantification of the proportion of spines in zones 1–4 on a per animal (±SEM) basis for each group (n = 5 for WT SH, and 3 for all other groups): For zones 2–4, 2-way ANOVA revealed significant effects of genotype: P = 0.0129, zone 2; P = 0.009, zone 3; P = 0.045, zone 4. Post hoc comparisons were done between genotypes in each rearing condition, and for each genotype across housing conditions. As shown, KO EE mice had greater numbers of spines in zone 2 than either WT EE or KO SH groups (**P < 0.01, Student’s t-test), and fewer spines in zone 3 than WT EEs (**P < 0.01, Student’s t-test).
processing capabilities including both linear and nonlinear operations. Importantly, the amplitude of the terminal dendritic all-or-none response has been suggested to reflect both the

physical dimensions of these dendritic segments and their number of voltage-gated channels (Wei et al. 2001). These findings suggest that EE effects on third-order branch lengths described here would be likely to alter local responses to intense afferent activity and, therefore, the processing power of distal dendritic arbors.

Our results show that although environmental enrichment influences the dendritic arbors of hippocampal neurons of both genotypes, it has lesser effect in Fmr1 KO when compared with WT mice. A previous study of effects of housing environment on layer V occipital cortical neurons led to a different conclusion. That study found no effect of the Fmr1 KO genotype or environmental enrichment on apical dendrites (Restivo et al. 2005). Rather, 2 months in an EE increased basal dendritic branching in both Fmr1 KO and WT mice but to greater extent in the KOs thereby eliminating the effects of genotype on this measure (Restivo et al. 2005). Although the enrichment paradigm in the cortical study was somewhat different from that used here (i.e., in the former, mice were intermittently exposed to novel environments in addition to enriched housing), differences in the nature of structural responses to enrichment in the 2 works likely reflect the specific neuronal populations evaluated. Relevant to this point, cortical neurons in different fields and layers have been shown to have distinct EE-induced changes in dendritic branching, including differences between effects in apical and

| Zone | WT SH | WT EE | KO SH | KO EE |
|------|-------|-------|-------|-------|
| sl > 1.0 | 3.88 | 2.62 | 6.16 | 15.73 |
| sl ≤ 1.0 | 8.83 | 7.77 | 6.54 | 12.43 |
| shv < 0.1 | 2.36 | 4.52 | 3.22 | 1.38 |
| shv ≥ 0.1 | 1.08 | 1.09 | 0.62 | 0.46 |

Note: The “Total %” is the overall percent of nonoverlapping spines for each group in each of the 4 paired comparisons; columns to the right of this indicate the % of nonoverlapping spines within each of the zones identified in Figure 6A and 7A. The difference (Diff.) values were generated by subtracting SH from EE values for the top 2 comparisons, and subtracting WT from KO values for the second 2 comparisons; bold numbers indicate a difference >4% of the total. sl, spine length (μm); shv, spine head volume (μm^3).

Figure 7. Subtraction analysis shows effects of housing and genotype on the physical proportions of dendritic spines. Histograms showing the differences in unique spine populations between housing conditions for each genotype (A and B) and for each genotype under the different housing conditions (C and D). For each pair-comparison, the overlapping distributions were subtracted away leaving only the spine population that is unique to each condition plotted. The 4 size zones, identified in A (left) are defined by boundaries at 1 μm head volume and 1 μm spine length (bold lines). (A) The distributions of nonoverlapping spines for WT SH and WT EE groups were similar although the EE group had fewer long-thin spines (zone 1) and spines with larger head volumes (zone 3; see Table 1 for values), (B) For the KOs, housing in the EE reduced the scatter in spine head volume and length, relative to profiles from SH mice thereby increasing the proportion of nonoverlapping spines that were both short and narrow (zone 2). (C) Population distributions show clear effects of genotype in both housing conditions. With SH, nonoverlapping spines for the WTs are clustered in zones 2 and 3 whereas for the KOs they are clustered in zones 1 and 2 and have particularly small head volume. (D) The nonoverlapping spines for WT and KO mice in the EE are similar to that seen after SH (compare to panel C) although for the KOs fewer spines have very small heads (<0.05 μm^3) and for the KOs unique spines are less numerous in zone 1. See Table 1 for further zone analyses.
basal fields (Gelfo et al. 2009). Moreover, other features of the mutant phenotype (e.g., a greater response to stress) might limit effects of environmental enrichment on hippocampal neurons as discussed below.

**Hippocampal Spine Abnormalities are Modest for KOs Raised in Standard Housing**

Studies of neocortical pyramidal cells reliably find dendritic spine abnormalities in Fmr1 KOs. Typically, cortical spines are described as being more numerous, and both longer and thinner, in KOs than in WTs (Comery et al. 1997; Galvez and Greenough 2005; McKinney et al. 2005; Hayashi et al. 2007; Su et al. 2011), with the latter morphological features inviting speculation that the KO profile includes spines that are relatively immature. Similarly “immature” spines are not consistently described for hippocampal pyramidal cells in the KOs, and reported effects of genotype on numbers of spines for hippocampal neurons are mixed (Braun and Segal 2000; Segal et al. 2003; Grossman et al. 2006; de Vrij et al. 2008; Pfeiffer et al. 2010; Levenga, Hayashi, et al. 2011; Su et al. 2011; He and Portera-Cailliau 2012; Pop et al. 2012). Only 2 studies have evaluated spines of CA1 neurons in situ with an emphasis on head morphology. The first concluded that adult KO apical dendrites had more stubby or mushroom-shaped spines suggesting larger spine heads than in WT (Grossman et al. 2006). The second found spine head area was smaller in 7-day old KOs when compared with WT’s (Bilousova et al. 2009); spine heads were also reportedly smaller for pyramidal cells in culture (Bilousova et al. 2009; Swanger et al. 2011). Our measures of 3D reconstructions of adult secondary dendrites accord with the later study. Specifically, for adult mice reared in standard cages, the Fmr1 KOs had smaller mean spine head volumes when compared with WT, and KO head volume distributions did not conform to the gamma profile that predominates in WT.

In contrast to reliable effects on spine head size, we did not detect an effect of genotype on numbers of spines for mice reared in standard housing. These findings are in line with several reports that the numbers of hippocampal spines in the KOs are normal (Grossman et al. 2006; de Vrij et al. 2008; Levenga, Hayashi, et al. 2011; Su et al. 2011; Pop et al. 2012), but differ from others reporting density as being abnormally high (Gross et al. 2010; Levenga, de Vrij, et al. 2011; Swanger et al. 2011) or low (Braun and Segal 2000; Segal et al. 2003) in the mutants. In addition, although we did not detect an effect of genotype on spine length distribution, there was an effect of genotype on average spine length for mice reared in standard housing with KOs exhibiting longer spines than WT. These findings are consistent with a recent report (Pop et al. 2012) but differ from others describing normal spine lengths on KO hippocampal neurons (Braun and Segal 2000; Segal et al. 2003; Bilousova et al. 2009). The inconsistencies across studies may reflect differences in experimental variables such as the age and environment (in vitro vs. in vivo) of the neurons evaluated, the dendritic branch order analyzed and, perhaps more importantly, methods of quantification including techniques for dendrite and spine labeling, imaging, and analysis (for further discussion, see He and Portera-Cailliau 2012; Pop et al. 2012). While most studies, including the present work, have used labeling methods that appear to randomly label neurons (e.g., Golgi staining, DiOlistic labeling, etc.), it is possible that the sampled populations of neurons differ between studies even for the same hippocampal subfield. Finally, regarding analytical techniques, it is noteworthy that the present study is the first to measure hippocampal spines in adult Fmr1 KO and WT mice using a standardized computer-based approach that was shown to have a higher degree of precision and accuracy for spine measures than manual quantification (Swanger et al. 2011).

**Rearing in an Enriched Environment Revealed Effects of Genotype on Spine Measures**

As noted, EE rearing resulted in more pronounced effects of genotype on field CA1 spine measures than did standard housing. Average spine counts were higher in mutants than in WT for EE rearing groups only. Moreover, there were differential effects of housing on spine size measures. This was not evident for “average group measures” of spine head volume; KOs reared in both housing conditions had smaller mean spine head volumes when compared with WT. However, differential responses to housing became evident as curves that best-fit spine length and head volume distributions were identified. Among mice reared in the EE, best-fit distributions for lengths of spines on individual dendrites revealed a leftward shift of central tendencies, toward smaller lengths, and significant effects on kurtosis and shape values that were limited to the KOs. These effects of EE on the WT’s gave rise to differences between the genotypes that were not evident for mice reared in standard cages. Best-fit curves also revealed differential effects of housing on head volume distributions in the 2 genotypes. WT spine head measures conformed to a gamma distribution with SH and to a Weibull distribution with EE housing. For KOs reared in SH, head volume distributions did not conform to one particular curve whereas those reared in the EE had spine heads that fit a gamma profile with significantly greater kurtosis, in the small head range, than was evident for WT’s in either housing condition.

In an effort to integrate spine length and head volume distribution results, we constructed 3D plots that further highlighted effects of genotype and housing on spine population distributions. For animals raised in the EE, the KOs had a greater proportion of spines in our Zone 2 and less in Zone 3 when compared with WT indicating that for short spines (<1 µm in length) the mutants have more with small head volumes and fewer with large head volumes. The same trends were evident for mice raised in SH but effects were not statistically significant. Subtractive analysis was then used to reveal populations of spines (defined by both length and head volume measures) that were unique to a genotype within a particular housing environment and unique to a housing environment for a particular genotype. Results of these subtractive analyses, summarized in Table 1, confirmed that the KO groups (SH and EE) exhibited more unique spines with smaller head volumes than did same-environment WT’s but that differences between genotypes were greater for mice raised in the EE. Integrating these findings with the observation that, with EE rearing, KOs had greater spine numbers than WT’s, it appears that differences in spine morphology between genotypes are not influenced by the density of spines. This is an important point to consider for studies aimed at correcting spine defects in the Fmr1 KOs and in FXS. In addition, as head volume is positively correlated with postsynaptic density area (Harris and Stevens 1989;
ties and, thus, function at the synapse. Second, there is evidence that the excitability of CA1 pyramidal neurons by reducing spiking threshold and after-hyperpolarization amplitude, causing cells to fire more action potentials with LTP-inducing stimuli (Malik and Chattarji 2012). This increased excitability would be expected to augment effects of a given amount of afferent drive and thereby facilitate synaptic plasticity (Duffy et al. 2001; Artola et al. 2006; Malik and Chattarji 2012). Clearly, future studies are needed to determine if EE rearing can restore normal LTP in both hippocampus and neocortex of Fmr1 KO mice, and if so, to elucidate the mechanisms involved.

The question remains as to why Fmr1 KO cortical and hippocampal spines differ in their response to rearing conditions. It is possible that longer periods in the EE, or greater diversity in environmental conditions, may be needed to elicit structural changes in hippocampus, than in neocortex, of the mutants. Alternatively, it may be the case that factors known to have preferential effects on hippocampal as opposed to cortical neurons lead to regional differences in effects of environmental enrichment in the mutants. For example, stress hormones (e.g., glucocorticoids) act on hippocampal neurons and have direct effects on dendritic spines (Shors et al. 2001; Komatsu et al. 2005; Jafari et al. 2012). Multiple studies have shown that Fmr1 KO hippocampal synaptic function are not known although computational analyses indicate that features such as spine volume and neck size influence the degree of biochemical compartmentalization and voltage amplification within the spine head, as well as the level of cooperativity between neighboring spines (Harnett et al. 2012). Studies of EE effects on cortical neurons in the Fmr1 KOs have found that in addition to normalizing spine measures (Restivo et al. 2005) enrichment can rescue the otherwise impaired cortical LTP (Meredith et al. 2007). From the present evidence that EE housing does not normalize spine measures for KO hippocampal CA1 pyramidal cells, we anticipate that this conditioning will not correct LTP deficits observed in this same field in the mutants (Lauterborn et al. 2007; Hu et al. 2008; Shang et al. 2009; Lee et al. 2011; Yun and Trommer 2011). However, there are reasons to be cautious about this prediction. Plasticity impairments in the mutant may arise from functional abnormalities that do not reflect spine shape (e.g., receptor signalling, protein trafficking) (Hu et al. 2008; Gross et al. 2010; Sharma et al. 2010; Seese et al. 2012); housing in the EE could normalize specific protein levels and/or signaling activities and, thus, function at the synapse. Second, there is evidence that Fmr1 KO neurons have an elevated threshold for induction of stable LTP relative to WTs (Lauterborn et al. 2007; Meredith et al. 2007). It has been reported that EE rearing increases the excitability of CA1 pyramidal neurons by reducing spiking threshold and after-hyperpolarization amplitude, causing cells to fire more action potentials with LTP-inducing stimuli (Malik and Chattarji 2012). This increased excitability would be expected to augment effects of a given amount of afferent drive and thereby facilitate synaptic plasticity (Duffy et al. 2001; Artola et al. 2006; Malik and Chattarji 2012). Clearly, future studies are needed to determine if EE rearing can restore normal LTP in both hippocampus and neocortex of Fmr1 KO mice, and if so, to elucidate the mechanisms involved.

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enrichment effects in the WTs leads to greater changes in hippocampal spine measures than is the case in the mutants.

Independent of mechanism, evidence that rearing in an EE reveals effects of genotype that are not evident when using standard housing conditions suggests that EE-reared mice should be used to anticipate the effects of reduced FMRP expression in persons with FXS. Humans with this condition, and other developmental disorders associated with autism and intellectual disability, live in a relatively complex environment with varied sensory and social input and at least modest day-to-day stressors. For mice, EE rearing is more akin to this naturalistic setting. Thus, findings from EE-reared mice may be more predictive of the human FXS condition, and useful in evaluating therapies aimed at reversing neurobiological features underlying cognitive dysfunction in this syndrome.
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