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Effects of Chronic Immobilization Stress on Anxiety-like Behavior and Basolateral Amygdala Morphology in Fmr1 Knockout Mice

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Abstract

Several lines of clinical evidence support the idea that fragile X syndrome (FXS) may involve a dysregulation of hypothalamic-pituitary-adrenal axis function (Wisbeck et al, 2000; Hessl et al, 2002). We had tested this idea in a mouse model of FXS (*Fmr1* KO) and found that the hormonal response to acute stress was similar to that of wild type (WT) mice (Qin and Smith, 2008). We report here responses to chronic stress (CS) in *Fmr1* KO mice. Following restraint for 120 min/day, 10 consecutive days, we assessed dendrite and spine morphology in basolateral amygdala (BLA). We also monitored behavior in an elevated plus maze (EPM) and the hormonal response to this novel spatial environment. After CS, mice of both genotypes underwent adrenal hypertrophy, but effects were greater in WT mice. Behavior in the EPM indicated that only WT mice had the expected increase in anxiety following CS. Serum corticosterone and ACTH levels were both increased following the spatial novelty of EPM, and there were no differences between genotypes in the hormonal responses. BLA dendritic branching increased proximal to the soma in WT, but in *Fmr1* KO mice branching was unaffected close to the soma and slightly decreased at one point distal to the soma. Similarly, spine density on apical and basal dendrites increased in WT but decreased in *Fmr1* KO mice. Spine length on apical and basal dendrites increased in WT but was unaffected in *Fmr1* KO mice. These differences in behavioral response and effects on neuron morphology in BLA suggest a diminished adaptive response of *Fmr1* KO mice.

Keywords

fragile X syndrome; FMRP; chronic stress; anxiety; amygdala; dendritic spines

Introduction

Fragile X syndrome (FXS) is the most common known cause of mental retardation and a leading genetic cause of autism. It is due to the silencing of a single gene, *FMR1*, resulting in the absence of the gene product, fragile X mental retardation protein (FMRP). Symptoms of FXS include a reduction in intellectual ability (Rousseau et al., 1994) and behavioral dysfunction such as hyperactivity, anxiety, attention problems and autistic-like behavior

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(Miller et al., 1999). Some clinical studies suggest that boys with FXS have dysregulation of hypothalamic-pituitary-adrenal (HPA) axis function (Wisbeck et al., 2000; Hessl et al., 2002). Boys with FXS exhibited higher salivary cortisol levels when exposed to a social stressor and a prolonged recovery back to baseline (Hessl et al., 2002).

In the mouse model of FXS (*Fmr1* knockout (KO)), there is also evidence of a dysregulation of HPA axis function. Glucocorticoid receptor immunoreactivity in *stratum radiatum* of the hippocampus was lower in *Fmr1* KO mice (Miyashiro et al, 2003) suggesting that feedback regulation of CORT levels may be diminished in these animals. Immobilization stress resulted in enhanced changes in *c-fos* expression in the paraventricular nucleus (PVN) (Lauterborn, 2004) and a more prolonged recovery of serum corticosterone (CORT) levels to baseline (Markham et al., 2006). In our previous study, we found no difference between *Fmr1* KO and wild type (WT) mice in CORT and ACTH responses to and recovery from acute (30 or 120 min) restraint stress (Qin and Smith, 2008). We also found no difference between genotypes in CORT and ACTH responses to spatial novelty in the elevated plus maze (EPM) despite the fact that *Fmr1* KO mice showed less anxiety-like behavior than WT. We now extend these studies to examine the response to chronic stress (CS). In normal rats, CS increases activation of the amygdala, induces dendritic hypertrophy in BLA, and increased anxiety behavior (Vyas et al., 2003; 2006). The alterations in dendrite and dendritic spine morphology in amygdala and anxiety-like behavior reflect experience-dependent forms of plasticity. The purpose of the present study was to investigate experience-dependent plasticity in *Fmr1* KO mice.

EXPERIMENTAL PROCEDURES

Animals

Male WT and *Fmr1* KO offspring were generated by FVB/NJ-*Fmr1*^{tm1Cgr} breeding pairs (heterozygous females and WT males). We studied male WT and *Fmr1* KO mice at 96±1 days of age. Mice with no previous exposure to these or any other stressful conditions were singly housed beginning one week before the study. Four groups of mice were studied: WT-unstressed control (WT-US) (n=24), *Fmr1* KO-unstressed control (KO-US) (n=24), WT-chronic stress (WT-CS) (n=19), *Fmr1* KO-chronic stress (KO-CS) (n=20). All mice were housed in a central facility and maintained under controlled conditions of normal humidity and temperature with standard alternating 12-h periods of light and darkness. Food (NIH-31 rodent chow) and water were provided *ad libitum*. All procedures were carried out in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and an animal study protocol approved by the National Institute of Mental Health Animal Care and Use Committee.

Genotyping

At the time of weaning, we analyzed genomic DNA extracted (Puregene, Gentra Systems, Inc, Minneapolis, MN, USA) from a small section of tail to test for the presence or absence of the KO allele as previously described (Qin et al., 2002). Primers to screen for the presence or absence of the mutant allele were 5′-ATCTAGTCATGCTATGGATATCAGC-3′ and 5′-GTGGGCTCTATGGCTTCTGAGG-3′. The PCR product at ≈800 bp indicated the presence of the null allele.

Chronic immobilization stress

Mice were subjected to CS by restraint in a plastic cylinder (Model 500M, Braintree Scientific, Inc, Braintree, MA) 120 min once a day from 8 am to 10 am for 10 days.

Elevated plus maze

On the 11th day, mice were tested in an EPM for 5 min. The EPM test took place in the same room as the chronic restraint and at the same time of day. Mice had had no previous exposure to the EPM. The apparatus consisted of two dark arms (30 × 5 × 15 cm) and two open arms elevated 50 cm from the floor. Initially animals were placed at the center facing an open arm. The times spent in the dark and open arms were recorded.

Body weight and adrenal weight change

The body weight of each mouse was measured each day. At the end of the EPM test, mice were decapitated in a separate procedure room and trunk blood was collected for hormone measures as previously described (Qin and Smith, 2008). Adrenal glands were carefully dissected and weighed.

Hormone assays

Blood was collected into tubes containing EDTA (BD Biosciences, Franklin Lakes, NJ), centrifuged 7000 × ***g*** for 5 min at 4°C to separate the plasma, and plasma samples were stored at −70°C until assayed. Concentrations of ACTH and CORT in plasma samples were determined by radioimmunoassay (Corticosterone ^{125}I RIA kit and *h*ACTH ^{125}I RIA kit, MP Biomedicals, LLC, Orangeburg, NY). Samples were counted in a Wallac Wizard Gamma Counter 1480 (PerkinElmer, Waltham, MA). Inter- and intra-assay variability was monitored by the use of a standard. For the CORT assay the coefficient of variation within assays ranged from 4% to 8% and between assays was 11%. For the ACTH assay the coefficient of variation within assays ranged from 1% to 10% and between assays was 15%.

Golgi staining and morphological analysis of dendrites and dendritic spines in the basal amygdala (BLA)

After blood sampling, brains were quickly removed and impregnated with the Rapid GolgiStain™ Kit according to the manufacturer's protocol (FD NeuroTechnologies, Ellicott City, MD). Coronal sections 100 μm in thickness were prepared with a Leica CM1850 cryostat, (Leica Microsystems Inc, Bannockburn, IL). Spiny pyramidal-like neurons from BLA were selected for analysis on the basis of the following criteria: (i) presence of untruncated dendrites, (ii) consistent and dark impregnation along entire extent of all dendrites, and (iii) relative isolation from neighboring impregnated neurons (Mitra et al., 2005; Vyas et al., 2002). BLA neurons analyzed in the present study were located between bregma −1.46mm and 1.82mm. Several aspects of dendritic morphology were analyzed with NIH ImageJ software. The numbers of dendritic branch intersections were determined by means of a Sholl Analysis with 25 μm concentric spheres and total dendrite lengths were measured in 40 neurons per group (8 mice/group) under light objective (16X, 0.63 numerical aperture, Leitz Wetzlar). Length and number of dendritic spines on 50 μm segments of primary basal dendrites starting 25 μm from the soma and on secondary apical dendrites originating 25 μm from the apical trunk were quantified. For the spine measurements we used an oil objective (100 X, 0.63 numerical aperture, Leitz Wetzlar). A total of 30 segments per experimental group in 6 mice per group were analyzed.

Statistical analysis

Data are expressed as mean ± SEM. Effects of CS on weight of adrenal glands, behavior in the EPM, hormone concentrations, dendritic length, and spine density were assessed by means of two-way analysis of variance (ANOVA) with condition and genotype as factors and Bonferroni *post-hoc t*-tests to assess differences between conditions for each genotype. Body weight data were analyzed by means of repeated measures (RM) 3-way ANOVA with genotype and stress condition as between subjects factors and day of stress as a within

subjects factor. Dendritic intersection data were analyzed with a RM 3-way ANOVA with genotype and stress condition as between subjects factors and distance from the soma as a within subjects factor. Spine length distributions were compared by two-way Kruskal-Wallis tests followed by Kolmogorov-Smirnov tests. The criterion for statistical significance was $P \leq 0.05$. We used SPSS and Partek Express programs for statistical analyses.

RESULTS

Effects of CS on body weight

We monitored body weight during the 10 days of CS (Fig.1A). Body weight tended to decrease on Day 3 of CS in both genotypes and continued to decrease in *Fmr1* KO mice while leveling off in WT. The genotype × condition × day of stress interaction was statistically significant ($F_{(4.1,316.8)} = 2.401$, P=0.048). At each time point we tested for differences between four pairs of groups by means of *post hoc t*-tests. Results indicate that in unstressed mice, body weights were higher in *Fmr1* KO mice compared with age-matched WT (Day 1, t(41)=2.882, P=0.005; Day 2, t(41)=2.497, P=0.015; Day 3, t(41)=2.765, P=0.007; Day 4, t(41)=2.649, P=0.010; Day 5, t(41)=2.916, P=0.005; Day 6, t(41)=2.929, P=0.004; Day 7, t(41)=3.150, P=0.002; Day 8, t(41)=3.119, P=0.003; Day 9, t(41)=3.095, P=0.003; Day 10, t(41)=3.169, P=0.002). Similarly in mice subjected to chronic stress, body weights were higher in *Fmr1* KO mice compared with WT (Day 1, t(36)=2.977, P=0.004; Day 2, t(36)=3.249, P=0.002; Day 3, t(36)=3.335, P=0.001; Day 4, t(36)=3.168, P=0.002; Day 5, t(36)=2.831, P=0.006; Day 6, t(36)=2.320, P=0.023; Day 7, t(36)=2.662, P=0.009; Day 8, t(36)=2.617, P=0.010; Day 9, t(36)=2.256, P=0.027; Day 10, t(36)=2.343, P=0.022). Differences between CS and US weights of *Fmr1* KO mice were statistically significant from Day 7–Day 10 (Day 7, t(39)=2.128, P=0.037; Day 8, t(39)=2.174, P=0.033; Day 9, t(39)=2.514, P=0.014; Day 10, t(39)=2.047, P=0.044). In WT mice, body weight was not significantly affected by CS at any of the time points.

Effects of CS on adrenal weights

Adrenal glands were removed and weighed on Day 11 following the EPM test (Fig. 1B). The genotype × condition interaction was statistically significant ($F_{(1,75)} = 5.083$, P=0.027), so we probed for specific differences between groups by means of Bonferroni *t*-tests. In unstressed mice, adrenal gland weight was higher (18%) in *Fmr1* KO mice compared with age-matched WT (t(39)=3.101, P=0.0027). Adrenal glands showed the expected hypertrophy following CS in both genotypes. In WT mice, adrenal glands increased in weight by 36% after CS (t(38)=5.735, P<0.0001), whereas in *Fmr1* KO mice the increase was smaller, c. 13% of KO-US (t(37)=2.498, P=0.015).

Effects of CS on anxiety and hormonal responses to a novel environment

On Day 11, the day following the last day of restraint stress, we subjected mice to the EPM as a test of anxiety (Fig. 2A). The genotype × condition interaction was statistically significant ($F_{(1,82)} = 7.753$, P=0.007), so we probed for specific differences between groups by means of *t*-tests. In unstressed mice, we confirmed our previous finding (Qin and Smith, 2008, Liu and Smith, 2009) that *Fmr1* KO mice have reduced time in the dark arms suggesting less anxiety than WT (t(46)=2.742, P=0.007). In animals subjected to CS, we found that time spent in the dark arms increased by 28% in WT (t(38)=4.527, P<0.0001), whereas in *Fmr1* KO mice there was very little effect. Following EPM we collected trunk blood for measurement of the hormonal response to this novel spatial environment (Fig. 2B & C). We also included a control group of each genotype that was not exposed to either CS or EPM for comparison. Plasma ACTH and CORT concentrations immediately after exposure to the spatial novelty of the EPM were increased in both genotypes regardless of exposure to chronic stress. For both hormones interactions between genotype and condition

were not significant. The main effect of genotype regardless of condition was statistically significant for CORT ($F_{(1,51)}$=5.069, P=0.029) but not for ACTH, indicating that levels of CORT were generally higher in *Fmr1* KO mice. Main effects of condition were statistically significant for both ACTH ($F_{(2,51)}$=23.515, P<0.0001) and CORT ($F_{(2,51)}$=17.626, P<0.0001). Exposure to the spatial novelty of the EPM increased levels of ACTH 2–5-fold and CORT 2–3-fold in both genotypes.

Dendritic analyses in BLA

Tracings of Golgi-impregnated pyramidal-like neurons of BLA from each experimental group are shown in Fig. 3A. Branch intersections on dendrites were analyzed at 25 μm concentric intervals from the soma (Fig. 3B). The curves for the *Fmr1* KO mice (both US and CS) are below those for the WT mice indicating reduced complexity of the dendritic arbor in fragile X mice. There appears to be little, if any, effect of CS on dendritic arbors in the *Fmr1* KO mice as US-KO and CS-KO curves are nearly superimposable. In contrast, in WT mice there is a clear hypertrophy of the arbor proximal to the soma following CS. The genotype × condition × distance from the soma interaction approached statistical significance ($F_{(4.6,718)}$ =1.803, P=0.116), so we compared the groups at each 25 μm interval with *t*-tests. In the US groups the number of branch intersections were statistically significantly higher in WT compared with KO between and including 75 and 125 μm (75 μm, t(78)=3.533, p=0.0005; 100 μm, t(78)=3.122, p=0.002; 125 μm, t(78)=2.330, p=0.021) and at 200 μm (t(78)=2.235, p=0.027) from the soma. In the CS groups the number of branch intersections were statistically significantly higher in WT compared with *Fmr1* KO between and including 25 and 150 μm (25 μm, t(78)=2.011, p=0.046; 50 μm, t(78)=3.865, p=0.0002; 75 μm, t(78)=3.600, p=0.0004; 100 μm, t(78)=2.379, p=0.019; 125 μm, t(78)=2.451, p=0.015; 150 μm, t(78)=2.595, p=0.010) and at 200 μm (t(78)=2.484, p=0.014) from the soma. In WT mice, the numbers of branch intersections were statistically significantly higher in CS compared with US at 25 (t(78)=1.974, p=0.050) and 50 μm (t(78)=2.803, p=0.006) from the soma. In *Fmr1* KO mice, the number of branch intersections was statistically significantly lower in CS compared with US at 175 μm from the soma (t(78)=2.528, p=0.012). We also assessed total dendritic length in these cells (Fig. 3C). This measure also indicates that dendritic arbors in *Fmr1* KO mice are less complex (KO-US, 17% lower compared with WT-US; t(78)=3.698, p=0.0003), and that CS results in increased total dendrite length in WT (11%, t(78)=2.320, p=0.022) but has no effect in *Fmr1* KO mice.

Dendritic spine analyses in BLA

Following CS, dendritic spine densities on BLA apical and basal dendrites were affected in both genotypes, but the direction of the effects differed in WT and *Fmr1* KO mice. Representative Golgi-impregnated dendrite segments from all four groups are shown in Fig. 4A. In WT mice, spine densities (Fig. 4B & C) increased (18%, apical (t(58)= 2.726, p=0.007); 23%, basal (t(58)=3.692, p=0.0003)) with CS, whereas in *Fmr1* KO mice they decreased (−16%, apical (t(58)=3.065, p=0.003); −11%, basal (t(58)=2.302, p=0.023)). Genotype × stress interactions were statistically significant for both apical ($F_{(1,116)}$ =16.77, P<0.0001) and basal ($F_{(1,116)}$ =17.96, P<0.0001) dendrites. Spine densities were higher in US-KO mice compared with US-WT by 31% on both apical (t(58)=4.657, p<0.0001) and basal (t(58)=4.968, p<0.0001) dendrites. Cumulative frequency distributions of spine lengths on both apical and basal dendrites (Fig. 4C) indicate that spines were longer in KO-US mice compared with WT-US (P≤0.05, apical; P≤0.01 basal; Kolmogorov-Smirnov tests), but in mice subjected to CS spines were longer in WT (P≤0.01, apical; P≤0.05 basal; Kolmogorov-Smirnov tests). Chronic stress significantly increased median spine lengths of both apical and basal dendrites 13% and 16%, respectively, in WT mice, but in KO mice CS had no effect.

DISCUSSION

The central finding of this study is that stress-induced remodeling of dendritic arbors in murine amygdala is altered in the absence of FMRP. Moreover, *Fmr1* KO mice fail to show the increased anxiety induced by chronic stress in rodents. Our findings indicate that these differences in response to CS between WT and *Fmr1* KO mice are not the result of a deficiency at the level of circulating stress hormones, because hormone responses are intact in the *Fmr1* KO mouse. Results of our study indicate that long term adaptive responses to stress in amygdala are altered in adult fragile X mice.

To our knowledge, this is the first study to demonstrate a deficiency in adaptive response of the amygdala in the mouse model of FXS. The amygdala is involved in storage of memories of fearful and stressful experiences (LeDoux, 2003). It is also thought to be involved in social behavior (reviewed by Kling and Brothers, 1992). Both clinical and animal studies provide evidence for amygdala dysfunction in FXS. Subjects with FXS have reduced amygdala volume (Gothelf et al, 2008; reviewed by Schneider et al, 2009) and aberrant processing of direct gaze stimuli (Watson et al, 2008). *Fmr1* KO mice exhibit abnormalities in social behavior (Spencer et al, 2008; McNaughton et al, 2008; Mineur et al, 2006; Liu and Smith, 2009) and decreased freezing responses to both contextual and cued conditioning (Paradee et al, 1999; Zhao et al, 2005). Fear conditioning, like social interaction, is thought to be an amygdala-based function. Consistent with deficits in fear conditioning response is the finding that long-term potentiation (LTP) is reduced in lateral amygdala (LA) in *Fmr1* KO mice (Zhao et al, 2005). More recent studies report deficits in mGluR-dependent LTP and surface expression of AMPA receptor subunit GluR1 in LA in *Fmr1* KO mice (Suvrathan et al, 2010). In addition, Suvranthan et al (2010) presented evidence that presynaptic transmitter release was also decreased at synapses of thalamic inputs to principal neurons of the LA. Another study found dramatic reductions in measures of GABAergic neurotransmission in BLA in *Fmr1* KO mice (Olmos-Serrano et al., 2010). We have shown that in *Fmr1* KO mice, rates of energy metabolism and rates of protein synthesis measured *in vivo* are increased in BLA (Qin et al, 2002; 2005). In the present study, we demonstrate less complex dendritic arbors and increased spine density and spine length on pyramidal-like cells of BLA in US *Fmr1* KO mice. We have also reported similar changes in BLA in a study of a mouse model of the fragile X premutation in which FMRP levels are reduced to 10–15% of control (Qin et al., 2011). Taken together these findings indicate that structure and function of the amygdala is clearly affected by reductions in FMRP.

We used restraint for two hours per day for 10 days to induce a state of CS. This approach has been used in many other studies of normal rodents designed to examine the effects of CS on behavior and brain structure (Rao *et al.*, 2009). Rats subjected to this form of CS have increased anxiety-like behavior as demonstrated by their behavior in the elevated plus maze (Vyas *et al.*, 2004). They also have structural changes in amygdala, prefrontal cortex and hippocampus. These changes include increased dendritic arborization on both stellate and pyramidal cells and increased spine density on spiny neurons in BLA (Mitra *et al.*, 2005). CS also results in decreased dendritic arborization in dorsal hippocampus CA3 pyramidal cells (Vyas *et al.*, 2002) and in prefrontal cortex (Liston *et al.*, 2006).

The difference between WT and *Fmr1* KO mice in the effects of repeated restraint stress on body weight may reflect a difference between the genotypes in adaptability. We saw an initial tendency (on Day 3) for body weight to decline in both WT and *Fmr1* KO mice, but only in the *Fmr1* KO mice did the decline in weight continue through Day 9. Repeated daily 3 h of restraint stress in rats produced a pattern of weight loss similar to that observed in our WT mice (Harris et al, 2002). In the rat study, plasma CORT levels were elevated approximately 10-fold on Day 1 of restraint and declined gradually to about 4–5-fold on

Day 9 of restraint (Harris et al, 2002). These results are consistent with an adaptation to the stress in the normal animals. It is possible that *Fmr1* KO mice do not adapt or take longer to adapt to the stressor.

We tested for anxiety-like behavior with the EPM. The EPM is a standard test of anxiety in rodents. It is based on the conflict between an inclination to explore a novel environment and the aversive attributes of brightly lit and open spaces. In previous studies, we demonstrated reduced anxiety-like behavior in *Fmr1* KO mice by their behavior in the open field, EPM, and the elevated zero maze (EZM) (Qin et al, 2002; Liu and Smith, 2009). In the open field, *Fmr1* KO mice spent a greater percentage of time in the center of the field and less time close to the walls of the apparatus compared to WT. In the EZM, *Fmr1* KO mice spent a greater percentage time in the open quadrants of the maze compared with WT. All of these behaviors indicate reduced generalized anxiety levels in the absence of FMRP. In contrast we found evidence of *increased* social anxiety in these animals. Behavior of *Fmr1* KO mice in a three-chambered test of social interaction indicated reduced social approach and less preference for social novelty suggesting behavior akin to social anxiety (Liu and Smith, 2009). Reduced generalized anxiety and increased social anxiety were all partially normalized by chronic treatment with lithium carbonate (Liu et al, 2010). In the present study CS in WT mice resulted in increased time in the dark enclosed arms of the EPM. *Fmr1* KO mice did not have this response. The fact that lithium treatment can normalize behavior on these tests of anxiety in *Fmr1* KO mice indicates that differences from WT mice were not due to sensory or physical defects and that *Fmr1* KO mice do have the capacity to express generalized anxiety in the EPM.

In our study we measured plasma levels of ACTH and CORT following the 5 min test in the EPM and 24 h after the last administration of restraint stress. Comparison with hormone levels measured after 5 min in the EPM with no prior stress show that both ACTH and CORT levels were similar in both groups. This suggests that hormone levels reflect the response to the spatial novelty of the EPM rather than basal hormone levels at the end of 10 days of CS. Levels of both hormones were much lower than those determined after two hours of acute restraint in which CORT increased 25–30 fold over control (Qin and Smith, 2008). With acute restraint stress we had shown previously that both hormones return to normal after 2 h of recovery (Qin and Smith, 2008). We did not measure hormone levels in groups of mice at various time points during the 10 days of CS, but in a similar study in rats in which animals were subjected to 3 h daily sessions of restraint CORT levels were measured every other day during restraint (Harris et al, 2002). In the rat study CORT levels were highest on Day 1 (c. 20 times control) but progressively decreased over time so that by Day 9 CORT levels were about 4–5 times control. These results indicate some adaptation of the rats to the recurring stress. We don’t know to what extent adaptation occurred in our mice and whether it was similar in both genotypes. These results and comparisons with our previous results on the effects of acute stress suggest that prior chronic restraint had negligible effects on the stress hormonal response to 5 min in the EPM 24 h after the last exposure to restraint.

In the present study, we confirm in WT mice the findings that CS effects structural changes in dendritic arbors and spines in BLA and that these changes are accompanied by increases in anxiety. We also expand on previous studies of the effects of CS in rodents with our finding that spine lengths are significantly increased in BLA. Elongated spines may represent an unstable state of spines during the dynamic plasticity response. Strikingly, none of these effects occurred in *Fmr1* KO mice. In *Fmr1* KO mice, dendritic arbors and spine length were unaffected by CS. Spine density was *decreased* in *Fmr1* KO mice, an effect in the opposite direction to that found in WT suggesting a maladaptive response in the KO. In keeping with the correlation between morphological changes in BLA and increases in

anxiety, *Fmr1* KO mice did not show the effects on anxiety-like behavior. Our study does not establish a direct relationship between the morphological and behavioral changes. The evidence for a relationship between the structural remodeling and increased anxiety comes from the finding in rodents that chronic restraint stress, but not chronic unpredictable stress, results in both increased anxiety and dendritic changes in BLA principal neurons (Vyas et al, 2002). Moreover, with recovery from the chronic restraint stress dendritic and the behavioral changes follow similar time courses (Vyas et al, 2004). It is likely that other parts of the amygdala may also participate in the response to CS. In the medial nucleus (MeA), spine loss is induced in spiny stellate neurons by chronic restraint stress, and both spine loss in MeA and the accompanying increased anxiety-like behavior depend on the upregulation of serine protease tissue-plasminogen activator (tPA) (Bennur et al, 2007; Pawlak et al, 2003). Expression of tPA is not seen in BLA (Pawlak et al, 2003).

We propose that the results of our study of CS reveal a deficiency in experience-driven plasticity in amygdala in the absence of FMRP. Disruptions in experience-driven plasticity have been demonstrated in neocortex, hippocampus and cerebellum in the mouse model of FXS. The critical period with respect to thalamus-to-barrel cortex synapses is delayed in perinatal *Fmr1* KO mice (Harlow et al, 2010), and the response in visual cortex to chronic monocular deprivation occurs more rapidly in adolescent *Fmr1* KO mice compared to WT (Dölen et al, 2007). In adult *Fmr1* KO mice, the classical eye blink conditioning response is attenuated (Koekkoek et al, 2005), and this response is dependent on the function of cerebellar Purkinje cells. In our study, *Fmr1* KO mice failed to undergo both the behavioral changes and the morphological alterations in BLA seen in WT mice in response to CS.

The differences between the two genotypes in response to stress occurred despite the similar stress hormone response. This suggests that the problem is not at the level of the stress hormones. Rather, our results suggest that there is a defect further down the pathway, e.g., at the level of the glucocorticoid receptor. Glucocorticoid receptor mRNA is one of the cargoes found to associate with FMRP, and in *Fmr1* KO mice, immunostaining for glucocorticoid receptor has been shown to be decreased in hippocampal dendrites (*stratum radiatum*) (Miyashiro et al, 2003). How the glucocorticoid receptor is affected in amygdala is not known, but a reduction in the receptor or an effect on the signaling pathway could be involved in the altered response to stress. Another possibility is that stress could have a differential effect on a pathway modulating the hormonal response. For example, activation of the noradrenergic system in BLA is required for enhanced memory consolidation in response to an emotionally arousing experience (reviewed by Roozendaal et al, 2009). This activation may occur via brain stem-to-BLA circuits stimulated by systemic adrenaline released in response to stress (Clayton and Williams, 2000). Stress has also been shown to result in release of corticotrophin releasing factor (CRF) in the amygdala which interacts with glucocorticoids and β-adrenoreceptors to affect memory consolidation (Roozendaal et al., 2008). Endocannabinoids are also released in BLA (Marsicano et al., 2002) in response to stress and endocannabinoid receptor activation in BLA can enhance memory consolidation (Campolongo et al, 2009). GABA (γ-aminobutyric acid) receptor antagonists infused in BLA also enhance memory consolidation (Brioni et al, 1989) and it has been proposed that endocannobinoids and glucocorticoids may enhance memory consolidation by inhibiting GABAergic activity in BLA (Roozendaal et al, 2009). The deficient response to stress in *Fmr1* KO mice could be mediated via an effect on any of these factors.

The fact that GABAergic transmission is already markedly reduced in BLA in *Fmr1* KO mice (Olmos-Serrano et al, 2010) suggests to us that there may be insufficient latitude in the system in BLA to allow for its modulation. Further inhibition via endocannaboids, glucocorticoids, or some other mechanism may be inadequate to enhance memory consolidation. This is consistent with the model proposed by Rao et al (2009) in which the

disinhibition caused by stress permits glutamatergic synapses to undergo plastic changes. With repeated bouts of stress, inputs are strengthened over time by means of spinogenesis and increased dentritic arborization. We propose that in *Fmr1* KO mice in which inhibition is reduced in the unstressed state, the stress response does little to further disinhibit and, consequently, cannot induce the adaptive changes.

Conclusions

Long term adaptive changes are essential for optimal function of the nervous system. They endow the nervous system with the ability to respond to the environment and make adjustments important for survival. In WT mice such changes occur in response to chronic stress and are manifest as increased anxiety-like behavior and structural remodeling in the amygdala. *Fmr1* KO mice appear to lack this capacity to respond to CS in BLA at least in the time frame that we have studied. Our findings indicate that this fundamental property is deficient in the BLA in the absence of FMRP and suggests that FMRP is essential for the normal expression of experience-driven plasticity in BLA.

Bullet Points

- Chronic stress (CS) on anxiety and amygdala dendrites in WT and *Fmr1* KO mice
- Control *Fmr1* KO mice had reduced dendritic arbors and elevated spine densities
- ACTH and CORT increased in both genotypes in response to spatial novelty after CS
- CS increased anxiety and dendritic branching in WT but not in *Fmr1* KO mice
- Following CS, spine density increased in WT but decreased in *Fmr1* KO mice

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Abbreviations

FXS	fragile X syndrome
FMRP	fragile X mental retardation protein
***Fmr1* gene**	fragile X mental retardation-1
WT	wild-type
KO	knockout
US	unstressed
CS	chronic stress
HPA	hypothalamic-pituitary-adrenal
CORT	corticosterone
ACTH	adrenocorticotrophic hormone
EPM	elevated plus maze

LTP	long term potentiation
LA	lateral amygdala
BLA	basal lateral amygdala
RM	repeated measure
ANOVA	analysis of variance
GABA	γ-aminobutyric acid

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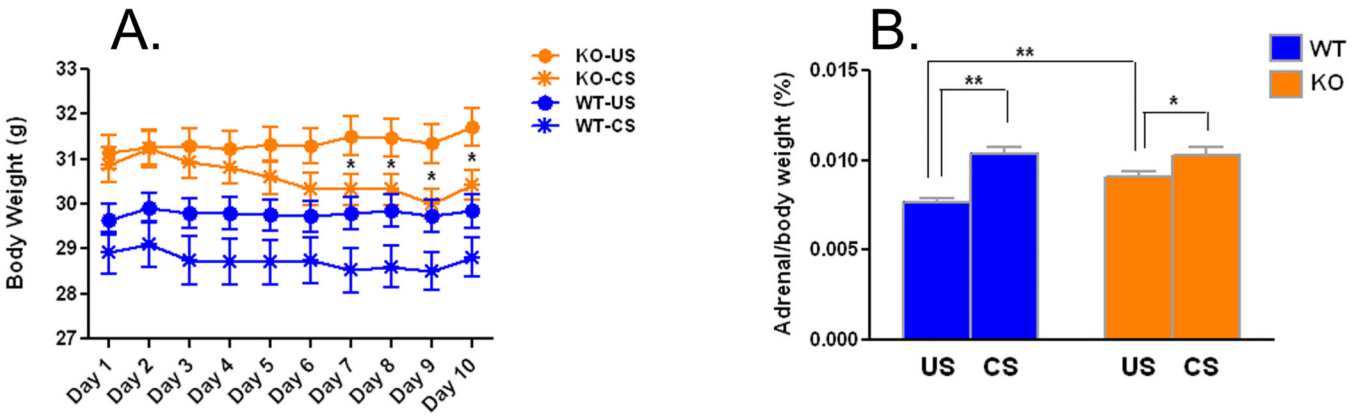

Figure 1.

A. Effects of chronic restraint stress on body weight in WT (US, n=22; CS, n=18) and *Fmr1* KO (US, n=21; CS, n=20) mice. Each point represents the mean ± SEM for each day of stress. Data were analyzed by means of RM ANOVA with genotype (WT, KO), treatment (US, CS) and day of stress as factors with RM on day of stress. The genotype × treatment × day of stress interaction was statistically significant ($F_{(4.1,316.8)} = 2.401$, p=0.048). Specific differences between groups at each time point were assessed for statistical significance by means of Bonferroni *post-hoc t*-tests. Differences between WT-US and KO-US and differences between WT-CS and KO-CS were statistically significant ($p<0.05$) at all time points. There were no significant differences between WT-US and WT-CS at any time point. Differences between KO-US and KO-CS were statistically significant as indicated on the graph (*, $p<0.05$).

B. Effects of chronic restraint stress on adrenal gland weight (as a percent of body weight) in WT (US, n=21; CS, n=19) and *Fmr1* KO (US, n=20; CS, n=19) mice. Bars are the means ± SEMs. Data were analyzed by means of ANOVA with genotype (WT, KO), treatment (US, CS) as factors. The genotype × treatment interaction was statistically significant ($F_{(1,75)} = 5.083$, p=0.027). Specific differences between groups were assessed for statistical significance by means of *post-hoc* Bonferroni *t*-tests (*, $p<0.05$; **, $p<0.01$).

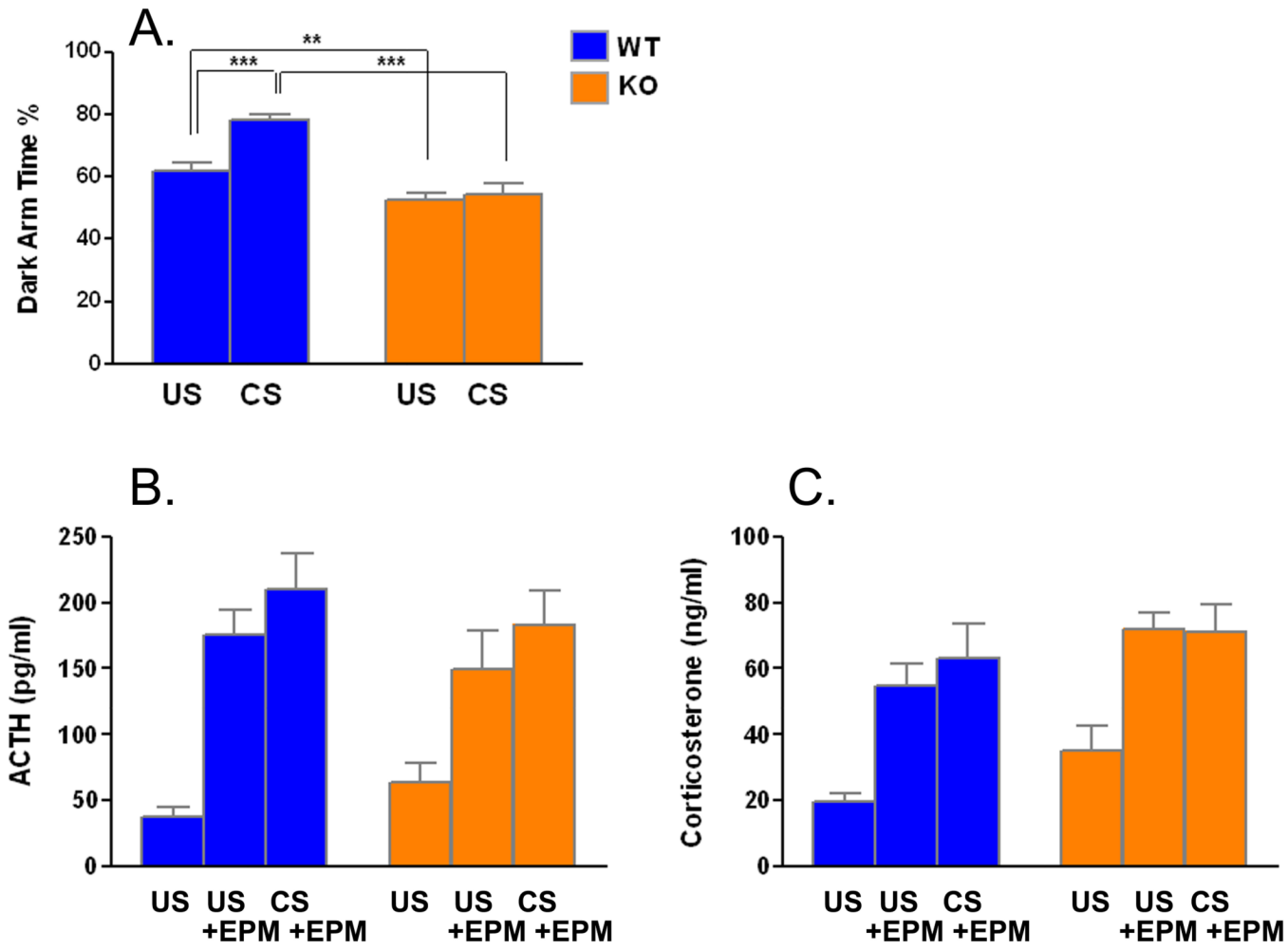

Figure 2.

A. Effects of chronic restraint stress on percent time spent in closed arms of the elevated plus maze in WT (US, n=24; CS, n=19) and *Fmr1* KO (US, n=24; CS, n=19) mice. Bars are the means ± SEMs. Data were analyzed by means of ANOVA with genotype (WT, KO), treatment (US, CS) as factors. The genotype × treatment interaction ($F_{(1, 82)} = 7.753$, p = 0.007) was statistically significant. Specific differences between groups were assessed for statistical significance by means of *post-hoc* Bonferroni *t*-tests (**, p<0.01; ***, p<0.001). Plasma concentrations of ACTH (**B.**) and corticosterone (**C.**) in WT (US, n=9; US+EPM, n=9, CS+EPM, n=10) and *Fmr1* KO (US, n=10; US+EPM, n=9, CS+EPM, n=10) mice measured after five min in the EPM. Bars are the means ± SEMs. Data were analyzed by means of ANOVA with genotype (WT, KO), treatment (US, US+EPM, CS+EPM) as factors. B. ACTH. The genotype × treatment interaction ($F_{(2, 51)} = 0.921$, NS) and the main effect of genotype ($F_{(1, 51)} = 0.229$, NS) were not statistically significant, but the main effect of condition ($F_{(2, 51)} = 23.515$, P<0.0001) was. C. Corticosterone. The genotype × treatment interaction ($F_{(2, 51)} = 0.203$, NS) was not statistically significant, but the main effects of both genotype ($F_{(1, 51)} = 5.069$, P=0.029) and condition ($F_{(2, 51)} = 17.626$, P<0.0001) were.

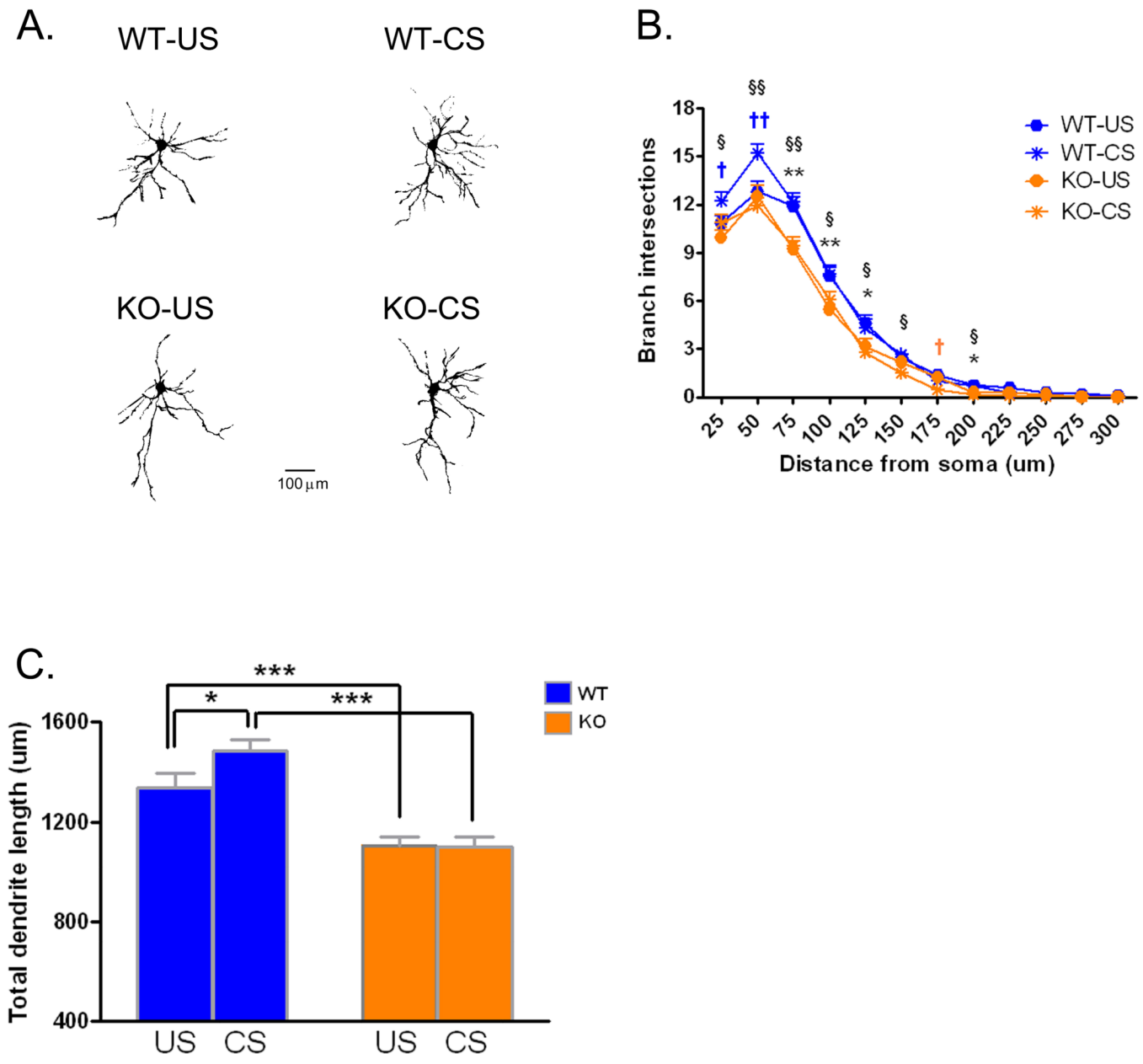

Figure 3.

Dendritic branching on BLA pyramidal-like neurons. **A.** Tracings of Golgi-impregnated neurons in BLA from each experimental group. B. Results of Sholl analysis of dendritic branching in WT (US, n=8; CS, n=8) and *Fmr1* KO (US, n=8; CS, n=8) mice. Each point represents the mean ± SEM branches on 40 dendrites (5 per animal) of each genotype. Results were analyzed by RM ANOVA with genotype (WT, KO), treatment (US, CS) and distance from the soma as factors and repeated measures on distance from the soma. Both the genotype × condition × distance from the soma ($F_{(4.6,718)}$=1.803, P=0.116) interaction and the condition × distance from the soma ($F_{(4.6,718)}$=2.255, P=0.053) interaction approached statistical significance. The genotype × distance from the soma ($F_{(4.6,718)}$=6.593, P<0.0001) interaction was statistically significant. Because the three-way interaction approached statistical significance we probed for specific group differences at each distance point by means of *post-hoc* Bonferroni *t*-tests. Results are shown in the figure as follows:

*, p<0.05, **, p<0.01; statistically significant difference between WT-US and KO-US

§, p<0.05, §§, p<0.01; statistically significant difference between WT-US and KO-US

†, p<0.05, ††, p<0.01; statistically significant difference between WT-US and WT-CS

†, p<0.05; statistically significant difference between KO-US and KO-CS

C. Total dendrite length of pyramidal-like BLA cells in WT (US, n=8; CS, n=8) and *Fmr1* KO (US, n=8; CS, n=8) mice. Each point represents the mean ± SEM branches on 40 dendrites (5 per animal) of each genotype. Data were analyzed by means of ANOVA with genotype (WT, KO) and treatment (US, CS) as factors. The genotype × treatment interaction ($F_{(1, 156)} = 2.935$, $p = 0.089$) and the main effect of condition ($F_{(1, 156)} = 2.458$, $p = 0.119$) approached statistical significance. The main effect of genotype ($F_{(1, 156)} = 48.209$, $p<0.001$) was statistically significant. Pairwise comparisons indicate statistically significant differences between genotypes under either US or CS conditions (***, $p\leq0.001$) and between CS and US conditions in WT mice (*, $p\leq0.05$).

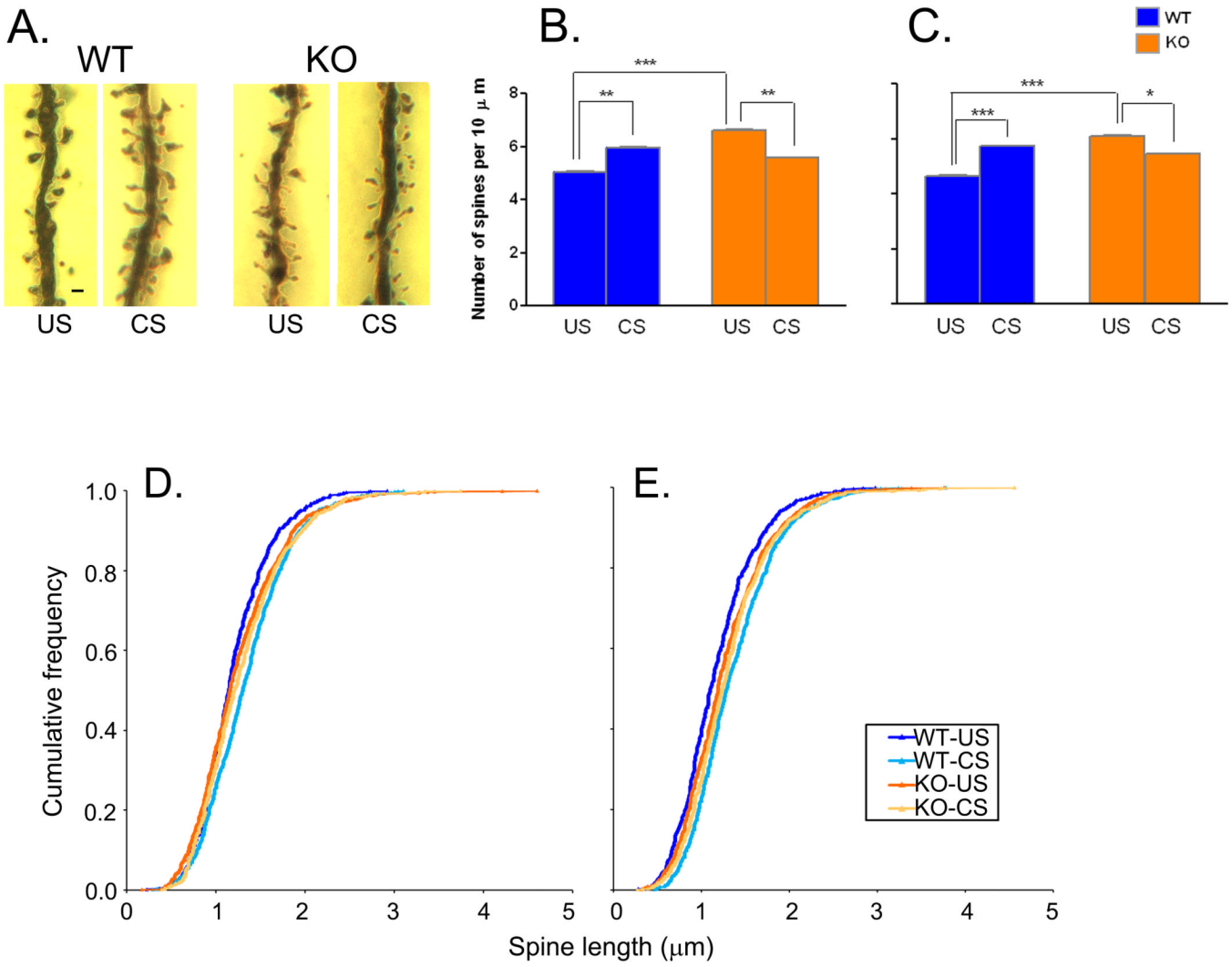

Figure 4.

Effects of chronic restraint stress on dendritic spines. **A.** Examples of spine morphology in each experimental group. Scale bar in WT-US segment represents 2 μm. Spine density on secondary apical (**B**) and primary basal (**C**) dendrites of pyramidal-like BLA cells in WT (US, n=8; CS, n=9) and *Fmr1* KO (US, n=8; CS, n=7) mice. Each point represents the mean ± SEM density of 30 dendrite segments (1–7 per animal) of each genotype. Measurements were made on primary basal dendrites 25 μm from the soma and on secondary apical dendrites 25 μm from the apical trunk. Data were analyzed by means of ANOVA with genotype (WT, KO) and treatment (US, CS) as factors. B. Secondary apical dendrites. The genotype × condition interaction ($F_{(1, 116)}$=16.769, p<0.0001) was statistically significant. C. Primary basal dendrites. The genotype × condition interaction ($F_{(1, 116)}$=17.961, p<0.0001) was statistically significant. We probed for specific group differences by means of *post-hoc* Bonferroni *t*-tests. Results are shown in the figure as follows: *, p<0.05; **, p<0.01; ***, p<0.001.

Cumulative frequency distributions of spine lengths in WT (US, n=8; CS, n=9) and *Fmr1* KO (US, n=8; CS, n=7) mice. (D) Apical dendrites. Lengths were measured in 758, 896, 993, and 836 spines in WT-US, WT-CS, KO-US, KO-CS, respectively. (E) Basal dendrites. Lengths were measured in 699, 861, 917, and 817 spines in WT-US, WT-CS, KO-US, KO-CS, respectively. Statistical analysis of all four cumulative frequency distributions of spine

length on both apical and basal dendrites indicate statistically significant differences for both apical and basal dendrites (2- way Kruskal-Wallis test; apical: $H = 45.89$, $p \leq 0.001$; basal: $H = 76.03$, $p \leq 0.001$). Pairs of cumulative frequencies were further probed by means of Kolmogorov-Smirnov Tests; results indicate that for apical dendrites differences between WT-US and WT-CS ($p \leq 0.001$), WT-US and KO-US ($p \leq 0.05$), and WT-CS and KO-CS ($p \leq 0.01$) were statistically significant. For basal dendrites differences between WT-US and WT-CS ($p \leq 0.001$), WT-US and KO-US ($p \leq 0.01$), and WT-CS and KO-CS, $p \leq 0.05$ were statistically significant.