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Reduced Activity-Dependent Protein Levels in a Mouse Model of the Fragile X Premutation

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Abstract

Environmental enrichment results in increased levels of Fmrp in brain and increased dendritic complexity. The present experiment evaluated activity-dependent increases in Fmrp levels in the motor cortex in response to training on a skilled forelimb reaching task in the CGG KI mouse model of the fragile X premutation. Fmrp, Arc, and c-Fos protein levels were quantified by Western blot in the contralateral motor cortex of mice following training to reach for sucrose pellets with a non-preferred paw and compared to levels in the ipsilateral motor cortex. After training, all mice showed increases in Fmrp, Arc, and c-Fos protein levels in the contralateral compared to the ipsilateral hemisphere; however, the increase in CGG KI mice was less than wildtype mice. Increases in Fmrp and Arc proteins scaled with learning, whereas this relationship was not observed with the c-Fos levels. These data suggest the possibility that reduced levels of activity-dependent proteins associated with synaptic plasticity such as Fmrp and Arc may contribute to the neurocognitive phenotype reported in the CGG KI mice and the fragile X premutation.

Keywords

Fragile X Premutation; CGG KI Mouse; Fmrp; Arc; c-Fos; Western Blot; Endophenotype

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Introduction

The fragile X mental retardation gene (*FMR1*), a gene that codes for fragile X mental retardation protein (FMRP), is polymorphic for the length of a CGG trinucleotide repeat in the 5' untranslated region. Individuals in the general population have 6–45 CGG repeats in the *FMR1* gene. Carriers of the full mutation underlying fragile X syndrome (FXS) carry greater than 200 CGG repeats, which transcriptionally silences the *FMR1* gene transcription and FMRP translation (Hagerman & Hagerman, 2004). In the fragile X premutation there are between 55 and 200 CGG repeats in the *FMR1* gene leading to increased transcription of *FMR1* mRNA (Garcia-Arocena & Hagerman, 2010) and decreased FMRP levels (Tassone & Hagerman, 2003; Tassone et al., 2000; Tassone, Hagerman, Chamberlain, & Hagerman, 2000). The premutation is associated with a late onset neurodegenerative disorder known as Fragile X-Associated Tremor/Ataxia Syndrome, (FXTAS), which results in cognitive and behavioral deficits characterized by motor ataxia and intention tremor (Hagerman et al., 2001).

To investigate the consequences of the fragile X premutation, a transgenic CGG knock-in (KI) mouse was developed in which the native mouse CGG repeat was replaced with an expanded CGG_{98} repeat of human origin by homologous recombination (Willemsen et al., 2003). Behavioral analyses of these CGG KI mice have shown deficits in spatiotemporal processing (Borthwell, Hunsaker, Willemsen, & Berman, 2012; Hunsaker, Goodrich-Hunsaker, Willemsen, & Berman, 2010; Hunsaker, Wenzel, Willemsen, & Berman, 2009; Hunsaker, Kim, Willemsen, & Berman, 2012b) and impaired visuomotor processing (Diep et al., 2012; Hunsaker et al., 2011b). Furthermore, female CGG KI mice were delayed in acquiring a skilled reaching task compared to wildtype (wildtype) mice, suggesting impairments in visuomotor learning (Diep et al., 2012). The molecular and cellular processes underlying these impairments are unknown, but they are thought to be related to the increased *Fmr1* mRNA and decreased Fmrp levels in the brain of CGG KI mice.

It is known that Fmrp associates with translating polyribosomes and mRNA and is believed to act as a negative regulator of protein synthesis (*cf.*, Huber, Gallagher, Warren, & Bear, 2002). Fmrp is translated *in vitro* in response to neurotransmitter activation (Weiler et al., 1997), and Irwin and colleagues reported an increase in the Fmrp/Actin ratio in Western blot analysis of the hippocampus and visual cortex of rats after exposure to a complex environment, suggesting that levels of Fmrp can be altered by increased neuronal activity and activity-dependent plasticity (Irwin et al., 2000; Irwin et al., 2005; Weiler et al., 1997). As it has been shown that there is an increase in dendritic complexity in the motor cortex of mice after training on a skilled forelimb reaching task (Greenough, Larson, & Withers, 1985; Xu et al., 2009), it is possible that elevations in Fmrp levels in response to neuronal activity result in, or are the result of increased synaptic density in the cortex.

The present experiment was designed to examine Fmrp levels in response to training on a skilled forelimb reaching task in mice, based on work by Whishaw and colleagues (Diep et al., 2012; Farr & Whishaw, 2002) to determine whether there is an activity-dependent increase in Fmrp levels in the motor cortex of mice. As skilled reaching is the product of several discrete movements, neuronal plasticity may link areas responsible for learning the fluid motion necessary for successful reaching (Whishaw, Whishaw, & Gorny, 2008). We also used the task to evaluate levels of activity-dependent translation of Arc (also called Arg3.1) and c-Fos, proteins whose levels of expression have been shown to be correlated with learning and general neuronal activity, respectively (Bramham, Worley, Moore, & Guzowski, 2008; Park et al., 2008; Vazdarjanova, McNaughton, Barnes, Worley, & Guzowski, 2002). *Arc* mRNA has been shown to associate with Fmrp at the polyribosome and is translated when Fmrp is phosphorylated after group I mGluR (mGluR1/5) activation

(Chowdhury et al., 2006; Pfeiffer & Huber, 2006). This is important as *Arc* mRNA has been shown to be elevated after performance on learning and memory tasks (Vazdarjanova et al., 2002), whereas *c-Fos* mRNA levels have not been shown to be related to learning *per se*, but rather show a relationship with cellular activation in itself (Dragunow & Faull, 1989).

To evaluate any alterations in activity dependent plasticity in CGG KI mice relative to wildtype littermate mice, CGG KI mice were trained to reach for a 20 mg sucrose pellet with a non-preferred paw. Levels of Fmrp, Arc, and c-Fos proteins in the contralateral somatosensory/motor cortex (in relation to the trained hand) were quantified by Western blot and compared to levels in the ipsilateral somatosensory/motor cortex, using Gapdh as a loading control. The difference in Fmrp levels between the two hemispheres was used as a measure of activity-dependent increase in protein levels.

After training in the task, both CGG KI and wildtype littermate mice showed activity-dependent increases in Fmrp, Arc, and c-Fos protein levels in the contralateral motor cortex as compared to the ipsilateral motor cortex. Notably, the increase in all three proteins in the CGG KI mice was of lesser magnitude than the increase seen in wildtype mice. These results suggest that reduced levels of activity-dependent proteins associated with synaptic plasticity may contribute to the neurocognitive deficits seen in CGG KI mice with the fragile X premutation.

Materials and Methods

Mice

Nine male CGG KI mice heterozygous for the fragile X premutation at 6 months of age and 9 male wildtype mice at the same age were used as subjects for this task. All wildtype mice were littermates with CGG KI mice included in the study. All CGG KI mice were bred onto a congenic C57BL/6J background over greater than 12 generations from founder mice on a mixed FVB/N x C57BL/6J background (Willemsen et al., 2003). Mice were housed in same sex, mixed genotype groups with three or four mice per cage in a temperature and humidity controlled vivarium on a 12 h light-dark cycle. Mice had *ad libitum* access to water and were maintained at 90–95% their free feeding weight throughout experimentation. Mouse weights did not differ among genotypes during experimentation. All experiments were conducted during the light phase of the diurnal cycle. Experimental protocols conformed to University of California, Davis approved IACUC protocols.

Genotyping

DNA was extracted from mouse tails by incubating with 10 mg/mL Proteinase K (Roche Diagnostics; Mannheim, Germany) in 300 μL lysis buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% SDS overnight at 55°C. One hundred μL saturated NaCl was then added and the suspension was centrifuged. One volume of 100% ethanol was added, gently mixed, and the DNA was pelleted by centrifugation and the supernatant discarded. The DNA was washed and centrifuged in 500 μL 70% ethanol. The DNA was then dissolved in 100 μL milliQ-H_2O. CGG repeat lengths were determined by PCR using the Expanded High Fidelity Plus PCR System (Roche Diagnostics). Briefly, approximately 500–700 ng of DNA was added to 50 μL of PCR mixture containing 2.0 μM/L of each primer, 250 μM/L of each dNTP (Invitrogen; Tigart, OR), 2 % dimethyl sulfoxide (Sigma-Aldrich; St. Louis, MO), 2.5 M Betaine (Sigma-Aldrich), 5 U Expand HF buffer with Mg (7.5 μM/L). The forward primer was 5’-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3’ and the reverse primer was 5’-AGCCCCGCACTTCCACCACCAGCTCCTCCA-3’. PCR steps were 10 min denaturation at 95°C, followed by 34 cycles of 1 min denaturation at 95°C, annealing for 1 min at 65°C, and elongation for 5 min at 75°C to end each cycle. PCR

ended with a final elongation step of 10 min at 75°C. DNA CGG repeat band sizes were determined by running DNA samples on a 2.5% agarose gel and staining DNA with ethidium bromide. Genotyping was performed twice on each mouse, once using tail snips taken at weaning and again on tail snips collected at sacrifice. In all cases the genotypes matched.

Skilled Forelimb Reaching Apparatus

The apparatus for the skilled forelimb reaching task (Figure 1) was a transparent Plexiglas box 19.5 cm long, 8 cm wide, and 20 cm tall. A 1 cm wide vertical window ran up the front of the box centered along the front wall. A 0.2 cm thick plastic shelf (8.3 cm long and 3.8 cm wide) was mounted 1.1 cm from the floor on the front of the box. Single 20 mg banana-flavored sucrose pellets (Bioserve Inc.; Frenchtown, NJ) were placed in indentations spaced 1 cm away from the window. The pellets were placed in the indentations on the right or left edge of the shelf, depending on which paw (right or left) was being trained such that the mouse could only reach the pellets with one paw and could not reach them with their tongue (Diep et al., 2012; Farr & Whishaw, 2002).

Experimental Methods

Skilled Forelimb Reaching Task

Pretraining: Mice were food deprived to 90–95% free feeding weight and given access to 20 mg banana flavored sucrose pellets in their home cage to habituate to the food reward for 2 days. Thirty minutes after each training day mice were provided sufficient food to maintain 95% free feeding weight. All training was carried out following the protocols described in Diep et al. (2012) by a single experimenter blinded to the genotype of the individual mice.

Six wildtype and six CGG KI littermates were chosen to receive skilled reaching task training and three CGG KI and three wildtype littermates were chosen to receive daily exposures to the testing apparatus and sucrose pellet rewards but not receive skilled reaching training. These untrained mice served as controls for any influences of exposure to the testing apparatus or sucrose pellet rewards on protein expression

Training: On days 3–5, mice were placed in the apparatus with sucrose pellets on the floor and at the base of the open window within reach of the mouse's tongue for 30 min and allowed to consume sucrose pellets. On days 6–10, mice were placed in the apparatus with sucrose pellets available immediately outside the open window for 15 min, allowing the mice to use their tongues to obtain the reward pellet. When mice freely ate rewards, they moved on to task acquisition.

Acquisition: Prior to the first day of acquisition, mice were placed in the apparatus with sucrose pellets available in the indentations on both sides of the ledge outside of the window. The mice were allowed to reach and obtain as many rewards as possible for 15 min. The paw preference of each mouse was determined as the paw used during the majority of individual reaches (*i.e.*, > 50%). Starting the next day, all mice were trained against their individual paw preference. This protocol was chosen since training the animals to reach with their opposite paw created a more complex task that required learning relative to reaching with a preferred paw.

Mice were placed in the apparatus for 15 min with one sucrose pellet placed on the side of the open window such that the mouse could only obtain it using the non-preferred paw. Each time the mouse reached, an experimenter blinded to mouse genotype recorded whether the reach was successful or whether or not errors occurred, and immediately replaced the

sucrose reward pellet when displaced. A successful reach was defined as the mouse obtaining and consuming the food pellet. If the mouse knocked the pellet away or dropped it prior to eating it an error was recorded. This acquisition phase was continued for 11 days.

The untrained group of mice (n=6; 3 wildtype and 3 CGG KI mice) were placed in the apparatus daily with access to sucrose reward pellets but were never trained to reach. This served as a control for any nonspecific effects on brain protein levels associated with exposure to the reaching task apparatus or sucrose reward.

Western Blotting: On Day 11, mice were given one final session in the reaching task and then returned to their home cage for 60 min. After 60 min, the mice were sacrificed by cervical dislocation and the somatosensory/motor cortices were dissected and flash frozen in liquid nitrogen by experimenters blinded to the mouse genotype and stored at −80°C until further processing. The rest of the brain was separated into hippocampus, brainstem, cortex, midbrain, and subcortical forebrain (*i.e.*, basal ganglia, thalamus, septal nuclei), similarly frozen, and stored at −80°C. For the present experiment, western blotting was performed on somatosensory/motor cortices.

The somatosensory/motor cortex tissue was homogenized (1000 µl buffer × 0.01 g of tissue) in HEPES buffer (10 mM HEPES, 300 mM KCL, 3 mM $MgCl_2$, 100 µM $CaCl_2$, 0.45% Triton X-100, and 0.05% Tween 20, pH 7.6) with a protease inhibitor cocktail (Roche Diagnostics) in a sterilized vial, and centrifuged for 30 min at 12,000 rpm at 4°C. The supernatant was collected and assayed for total protein concentration using a micro Bradford assay (Bradford, 1976).

Sixty micrograms (µm) of protein were diluted with Laemmli sample buffer (BioRad, Hercules, CA) and were heated for 5 min at 98°C. Samples were loaded onto an acrylamide gel (4% stacking gel and 8% separating gel). A subset of gels were evaluated for total protein using Coomassie blue staining prior to transfer to a nitrocellulose filter paper at 4°C. Efficient protein transfer was confirmed using Ponceau red staining. The blot was placed in a blocking solution for 120 min in a rotating 50 mL conical vial in Odyssey blocking buffer (BioRad). After blocking, the tissue was incubated in primary antibodies diluted in blocking buffer with 0.1% Tween-20 overnight at 4°C with gentle agitation. Primary antibodies included a polyclonal antibody targeting the C-terminus of Fmrp raised in chicken (1:15,000; *cf.*, Hunsaker et al., 2011a), a rabbit polyclonal antibody targeting Arc protein (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), a polyclonal antibody targeting the c-Fos protein (1:1000; Cell Signaling Technology, Danvers, MA), and a monoclonal antibody targeted to Gapdh (Abcam, Inc; 1:5,000). After incubation in the appropriate primary antibodies, the blots were incubated in secondary antibodies (1:10,000) optimized for the Odyssey imaging system (Li-Cor, Lincoln, NE) for 60 min at room temperature and rinsed. Levels of Fmrp, Arc, and c-Fos proteins were quantified using the Odyssey system and normalized to Gapdh as a loading control. The methods reported by Brouwer et al. (2008) were employed to quantify Fmrp levels, involving pooling signal from the 4 prominently visible Fmrp bands. This method was adapted to similarly quantify the multiple bands present on blots of c-Fos.

Dependent Measures and Statistical Analysis

Behavior: The number of times that the mouse successfully reached and obtained a sucrose pellet reward was collected as the dependent variable. If the mouse reached and missed/displaced the pellet during a reach or dropped the pellet before consuming it, an error was recorded and the pellet was immediately replaced by the experimenter. Qualitative observations concerning the behavior/strategy of each mouse were also recorded by the

observer. For analysis, the percentage of reaches that were successful was calculated for each day.

To determine whether parametric analyses of variance (ANOVA) were appropriate for the data, tests of normality, homoscedasticity, and sphericity were performed. Once it was determined that parametric statistics were appropriate for the data, the data were plotted and placed into groups as follows: the mice in the wildtype group all had between 8–12 CGG repeats (mean 10 +/− 0.2 SEM; n=6), and the mice included in the CGG KI group ranged between 138–185 CGG repeats (mean 168 +/− 19; n=6). A 3 (CGG repeat group) × 11 (Day) mixed design repeated measures analysis of variance (ANOVA) was used to determine differences among the groups for acquisition of the skilled reaching task while taking into account the total number of times each mouse reached during each session. ANOVA were used to confirm that the total number of reaches did not differ among genotypes.

To specifically determine differences in the day the mice learned the task to asymptotic performance levels, the data for each mouse was evaluated for the point at which the learning curved changed from being linear to curvilinear and confirmed using a frequentist change point algorithm reported by Gallistel, Fairhurst, and Balsam (2004). The original change point algorithm was translated into R from the original MATLAB code; *cf.*, Diep et al., 2012 and freely available for download at http://github.com/mrhunsaker/Change_Point). The first change point in the returned change point array corresponded to the point at which the learning curve significantly changed shape (discrimination threshold was set at logit = 3: odds against = 1,000:1 or $p < .001$). This point was chosen as the index of learning for each mouse and was then used as a dependent variable to compare the learning index across groups using a oneway ANOVA, replicating the analytical methods used by Diep et al. (2012). Analysis using a Bayesian change point algorithm gave similar results to the frequentist data that are presented here.

Subsequent analyses were performed to further characterize all main effects, and Tukey-Kramer post hoc pairwise comparison tests were used to characterize all significant main effects and interactions among factors. All analyses were considered significant at $p_{(adj)} < .05$. Statistical analyses were performed using the R 3.0 statistical language and environment (R Development Core Team, 2013).

Western Blotting: Western blotting data for Fmrp, Arc, and c-Fos proteins were first normalized to Gapdh. The values for the brain hemisphere contralateral to the trained hand were then normalized to the protein values in the hemisphere ipsilateral to the trained hand using the following formula (in units of percentage):

(Contralateral protein levels / Ipsilateral protein levels) × 100

This normalized value reflects activity-dependent percent change in protein levels in the trained (contralateral) versus untrained (ipsilateral) cortex and was used as the unit of measure for statistical analysis. For each experiment, every Western Blot was stripped and re-probed with antibodies for each protein by a second experimenter blinded to the results of the first experiment to confirm each finding. Reported data are from the first probe of each blot for each antibody (multiple antibodies were probed on each blot as well as during each re-probe on a given blot).

Once it was determined that parametric statistics were appropriate for the data, the data were plotted and placed into the same groups as for behavior, with the addition of a group of mice that were exposed to reward and the reaching task apparatus, but were never trained to reach (n=3 wildtype mice, n=3 CGG KI mice with CGG repeats between 138–185 repeats; mean

168 +/− 19). A 1 way (CGG repeat group) ANOVA was used to determine differences among the groups for the baseline amount of Fmrp, Arc, and c-Fos protein levels in the untrained hemisphere. A 1 way (CGG repeat group) ANOVA was used to determine differences among the groups for the amount of activity-dependent increases in of Fmrp, Arc, and c-Fos protein levels. To control for potential differences in overall protein levels among groups, baseline protein levels in the CGG KI mice were adjusted to wildtype levels and the statistical analyses were performed again (using a mean correction across all animals). Similar ANOVA were used to confirm that the total number of reaches did not differ among genotypes.

To characterize any possible relationship of performance on the skilled forelimb reaching task with increasing protein levels, a Pearson's correlation coefficient was calculated comparing performance on day 11 and Fmrp, Arc, and c-Fos protein levels. To control for the false discovery rate (FDR) given the number of analyses performed on the data, all p values have been FDR adjusted as outlined by Benjamini, Drai, Elmer, Kafkafi, and Golani, (2001).

Results

Behavioral Performance

For all mice, data were grouped by CGG repeat length (wildtype, CGG KI) and analyzed across days of training using mixed-design Repeated Measures ANOVA with percent successful reaches as the dependent variable, CGG repeat group as the grouping factor, and day of training as a repeated within-subjects factor with total number of attempted reaches during each session as a covariate. There was a main effect of group ($F(1,66) = 20.02$, $p_{(adj)} < .001$), an effect for training day ($F(10,64) = 83.48$, $p_{(adj)} < .0001$), and there was a significant interaction between group and day ($F(10,66) = 2.87$, $p_{(adj)} < .005$), suggesting that the performance trajectory across training days differed among CGG repeat groups (Figure 2). Total number of reaches per session did not significantly contribute to skilled forelimb reaching task acquisition ($F(10,66) = 1.30$, $p_{(adj)} = .21$), and did not differ among CGG repeat groups ($F(1,66) = 1.45$, $p_{(adj)} = .23$; wildtype mean 68 +/− 11 (SEM) reaches per session; CGG KI group 57 +/− 9 reaches per session).

To further characterize the significant interaction, a Tukey-Kramer post hoc pairwise comparisons test demonstrated that the two groups did not differ in performance during days 1–6 of training (all $p_{(adj)} > .25$), whereas on days 7–11 the wildtype group showed a greater percentage of successful reaches than the CGG KI mouse group (all $p_{(adj)} < .001$).

Based on the results of the paired comparisons, it appears that the two trained groups show differential time-courses for reaching asymptotic performance on the skilled forelimb-reaching task (Figure 2). A confirmatory analysis of differences in the learning curve among CGG groups was performed using a change point algorithm described by Gallistel, Fairhurst, and Balsam (2004). The first change point in the data (corresponding to the first alteration to the learning curve at a $p < .001$ threshold) returned by the algorithm was selected for each mouse and compared across groups: There was a significant difference between the wildtype and CGG KI group for this first change point ($F(1,11) = 9.3$, $p_{(adj)} = .011$). The wildtype mice showed a significant change in the slope of the learning curve on day 7 (group mean 6.7 +/− 0.15 SEM), whereas the CGG repeat group showed a change in slope on day 9 (group mean 8.8 +/− 0.28).

Western Blotting

Overall Protein Levels in Ipsilateral (untrained) Cortex—For all mice, data were grouped (wildtype, CGG KI, untrained mice) for analysis. For Fmrp, there was a significant difference for levels of Fmrp protein levels between the wildtype and CGG KI mice in the somatosensory/motor cortex of untrained hemispheres. Specifically, CGG KI mice showed a 19.3% +/− 2.11 standard error (sem) reduced level of Fmrp ($F(1,11) = 4.93$, $p_{(adj)} = .043$), but no evidence for significant differences in Arc ($F(1,11) = 2.13$, $p_{(adj)} = .18$) or c-Fos ($F(1,11) = 1.13$, $p_{(adj)} = .31$) proteins. Including the untrained CGG KI and wildtype mice into the analysis did not alter these results (Fmrp ($F(1,17) = 4.57$, $p_{(adj)} = .047$); Arc ($F(1,17) = 1.47$, $p_{(adj)} = .24$); c-Fos ($F(1,17) = .92$, $p_{(adj)} = .35$)). These data suggest that despite the presence of 19% baseline reductions in Fmrp expression levels in untrained CGG KI compared to untrained control mice, there were not significant increases or decreases in Arc or c-Fos baseline protein expression levels.

Activity-Dependent Expression of Fmrp protein—As shown in Figure 3, the activity-dependent change in levels of Fmrp between hemispheres (*i.e.*, trained versus untrained) differed significantly between the three experimental groups ($F(2,15) = 37.35$, $p_{(adj)} < .0001$), with CGG KI mice showing lower Fmrp levels compared to wildtype littermate mice ($p_{(adj)} = .006$). Both wildtype and CGG KI trained mice showed significantly greater levels of Fmrp protein compared to mice that did not receive skilled reaching training ($p_{(adj)} < .0001$, $p_{(adj)} < .006$, respectively). When these analyses were performed on protein levels adjusted for changes in baseline protein expression levels, results of the statistical analysis did not change.

Activity-Dependent Expression of Arc protein—There was a significant difference between groups in activity-dependent Arc levels ($F(2,15) = 37.22$, $p_{(adj)} < .0001$), with CGG KI mice showing lower levels of Arc compared to wildtype littermate mice ($p_{(adj)} < .002$; Figure 3). Both wildtype and CGG KI mice showed significantly greater activity-dependent Arc protein levels compared to mice that did not receive skilled reaching training ($p_{(adj)} < .0001$, $p_{(adj)} < .002$, respectively). When these analyses were performed on protein levels adjusted for changes in baseline protein expression levels, results of the statistical analysis did not change.

Activity-Dependent Expression of c-Fos protein—There was a significant difference between groups for levels of c-Fos protein ($F(2,15) = 25.70$, $p_{(adj)} < .0001$), with CGG KI mice showing lower activity-dependent protein levels compared to wildtype littermate mice ($p_{(adj)} = .004$; Figure 3). Both wildtype and CGG KI mice showed significantly greater activity-dependent c-Fos protein levels compared to mice that did not receive skilled reaching training ($p_{(adj)} < .0001$, $p_{(adj)} = .011$, respectively). When these analyses were performed on protein levels adjusted for changes in baseline protein expression levels, results of the statistical analysis did not change.

Relationship Between Skilled Reaching Performance and Protein Levels—To characterize any possible relationship between performance on the skilled forelimb reaching task and activity-dependent increases in protein levels, Pearson's correlation coefficients were calculated between the elevation of activity dependent protein levels and performance on day 11 for CGG KI mice (Figure 4). A tentative positive correlation was observed between the asymptotic level of skilled reaching performance on training day 11 and Fmrp protein levels in the wildtype and CGG KI mice (wildtype corr = .79, $p_{(adj)} = .011$; $R_{(adj)}^2 = .62$; CGG KI corr = −.72, $p_{(adj)} = .025$; $R_{(adj)}^2 = .51$). A positive association was also observed between the asymptotic level of skilled reaching performance on day 11 and Arc protein levels in the wildtype and CGG KI mice (wildtype corr = .83, $p_{(adj)} = .031$; $R_{(adj)}^2$

= .69; CGG KI corr = .82, $p_{(adj)}$ = .028; $R_{(adj)}^2$ = .67). However, there was no association between the asymptotic level of skilled reaching performance on day 11 and c-Fos protein levels in the wildtype and CGG KI mice (wildtype corr = .25, $p_{(adj)}$ = .37; $R_{(adj)}^2$ = .06; CGG KI corr = .15, $p_{(adj)}$ = .48; $R_{(adj)}^2$ = .02). It must be noted that although these correlations appear robust, there were not a sufficient number of data points in in each group to draw any definitive conclusions.

Discussion

Our results demonstrate that wildtype mice perform more successfully at the skilled reaching task than CGG KI mice. CGG KI mice required a greater number of sessions to learn the task, and never achieved the same number of successful reaches as the wildtype mice. Although Fmrp protein levels increased in the contralateral motor cortex in both groups of mice after learning, the effect was less pronounced in CGG KI than wildtype mice. This finding, in light of the observed increased latency to acquire the skilled reaching task and reduced asymptotic performance levels seen in CGG KI mice, supports our hypothesis that Fmrp plays a role in experience dependent plasticity. Additionally, our findings correlate with the documented neurocognitive deficits seen in previous studies of CGG KI mice (Diep et al., 2012; Hunsaker et al., 2009; Hunsaker et al., 2010; Hunsaker et al., 2011b; Hunsaker, 2012; Hunsaker, Arque, Berman, Willemsen, & Hukema, 2012a; Hunsaker et al., 2012b).

The results of this experiment indicate an activity-dependent increase in protein levels of Fmrp, Arc, and c-Fos proteins in somatosensory/motor cortex contralateral to the trained limb when compared to protein levels in the ipsilateral untrained cortex. There was also a difference in both reaching performance and protein levels between CGG KI and wildtype mice. These findings support previous work demonstrating that levels of Fmrp in the brain are influenced by activity and environmental enrichment (Irwin et al., 2005; Weiler et al., 1997). This is consistent with the previous finding that similar training in mice results in increased numbers and stabilization of synapses in the contralateral motor cortex following training in a similar forelimb reaching task (Xu, et al., 2009).

Knock-out mouse models of fragile X syndrome (*Fmr1* KO mice) have been used previously to show that Fmrp is involved in synaptic plasticity (Huber et al., 2002). Protein levels increase in response to stimulation with the mGluR5 agonist DHPG as a part of development as well as in learning and memory (Antar, Dictenberg, Plociniak, Afroz, & Bassell, 2005; Auerbach & Bear, 2010). Our results reflect this response through increased levels of Fmrp after training and further elevated levels in mice that effectively performed the skilled reaching task.

The Arc protein has been used as a marker for activity and activity-dependent plasticity (Lyford et al., 1995). Due to its translation at activated synaptic sites, it is believed to play a role in learning and memory, including LTP and AMPA receptor trafficking associated with LTD (Bramham et al., 2010; McIntyre et al., 2005). We therefore examined Arc protein levels after mice learned the skilled reaching task to verify that learning-dependent changes in somatosensory/motor cortex had occurred. Accordingly, we observed increased levels of Arc protein in the contralateral motor cortex of both wildtype and CGG KI mice after training in comparison with untrained mice. Additionally, these learning-dependent increases in Arc protein were markedly higher in wildtype than CGG KI mice, supporting our argument that the mutation in the CGG KI mice results in impeded plasticity leading to lower levels of activity-dependent changes after training, and potentially impaired learning.

The increase in Arc protein levels is consistent with an earlier report that similar skilled forelimb motor training increased the number of Arc immunostained neurons in the trained contralateral sensory/motor cortex compared to the untrained ipsilateral hemisphere (Hanlon, et al., 2009). Interestingly, in that study the asymmetry in increased Arc was seen in awake animals at the time of sacrifice, but not in animals that underwent non-REM sleep after training. Evidence that the increase in Arc protein levels may be due to increased translation resulting from training comes from a recent study, also using the skilled reaching task, showing increased numbers of *Arc* mRNA positive neurons in motor cortex (*i.e.*, M1) quantified by in situ hybridization immunochemistry (Hosp, et al., 2013). Similar to the present findings, the number of *Arc* mRNA positive neurons in the contralateral, but not ipsilateral motor cortex, was significantly correlated with the magnitude of improvement in reaching success after training.

Expression of the c-Fos protein is used as a measure of overall neuronal activity, but not as a marker of activity-dependent changes underlying synaptic plasticity. c-Fos is expressed at neuronal sites when action potentials are fired, leading to their use as a confirmation of activity in the cell (Dragunow & Faull, 1989). Accordingly, although we saw increased levels of c-Fos protein in the trained versus untrained hemispheres of brain, we did not find a relationship with overall performance on the task. This result suggests that c-Fos protein expression was increased by neuronal firing in motor cortex related to success on the reaching task (*e.g.*, number of successful reaches). Interestingly, levels of the c-Fos protein remained lower in CGG KI mice, consistent with the results for Fmrp and Arc proteins, further supporting the possibility that premutation length CGG repeats on the *Fmr1* gene may lead to decreases in protein translation and cellular activity that are associated with learning. These data also support the findings of Hunsaker et al. (2012b), who demonstrated reduced synaptic plasticity in the CGG KI mouse relative to wild type littermates in acute hippocampal slices.

It is important to note that there exists an alternative explanation for these data. We observed that overall levels of Fmrp and Arc proteins are lower in CGG mice after training than wildtype mice. However, the relationship between learning and protein expression levels is evident, suggesting that learning mechanisms related to the expression of these proteins is in fact intact in the CGG KI mice. As such, it may be overly speculative to attribute the decreases in learning observed in these mice to absolute lower levels of Fmrp and Arc protein. Perhaps the reduction in Fmrp levels resulted in synaptic changes that were not observable at the resolution of the Western Blot.

The prevailing model is that Fmrp is represses translation of the Arc protein, so that more Fmrp should lead to reduced Arc translation in a more or less linear fashion. Interestingly, in the present study both Arc and Fmrp protein levels increase, contradicting this simple model. Furthermore, less Fmrp expression in CGG KI mice would predict higher Arc protein levels, but this was also not found in this experiment, again contradicting the prevailing model. However, the prevailing model is based on studies in wildtype mice with normal Fmrp expression levels and *Fmr1* KO mice with zero Fmrp expression. This limited dataset makes interpreting our present data difficult using this simple model since the CGG KI mouse has reduced, but not zero, Fmrp expression levels.

Indeed, *Fmr1* KO mice exhibit increased Arc protein basal expression, but decreased mGluR5-dependent Arc induction (*cf.*, Niere et al., 2012). What may be most relevant in the Niere et al. (2012) report is that Arc expression differs in dendrites compared the soma, and this greatly complicates interpretation of total protein levels. It is entirely possible that any differences in Fmrp and Arc protein expression in the soma relative to the dendrites may underlie the seemingly contradictory results in the present study, particularly since it has

been shown that there is increased dendritic complexity/arborization after skilled forelimb reaching training (Greenough, Larson, & Withers, 1985; Xu et al., 2009). Additionally, it is also likely that there are fundamental differences between the present reductions in Fmrp levels in the CGG KI mouse and the total lack of expression of Fmrp in the *Fmr1* KO mouse. The present report of 19% reduced (*i.e.*, 81% of normal), but still nonzero Fmrp levels is consistent with what has been reported in other CGG KI mouse models (*cf.*, Iliff et al., 2013; Qin et al., 2011). At present, the relationship between reductions in Fmrp levels and Arc protein expression are at best unclear. Further research using mice with parametrically altered Fmrp expression levels will be needed to elucidate any relationships.

The similar pattern of activity-dependent increases in protein levels between Fmrp and Arc may be attributed to the proposed role of Fmrp as a translational repressor for a number of proteins involved with long term depression, including Arc. It has been proposed that Arc is rapidly translated in response to group I mGluR (mGluR1/5) activation and is then involved in the rapid internalization of AMPA receptors at the synapse through endocytotic mechanisms (Pfeiffer & Huber, 2006). c-Fos proteins, on the other hand, has not been shown to interact with Fmrp, thus it is not surprising that Fmrp and c-Fos protein levels do not show the same relationship with behavior. This hypothesis is supported by the present data as plotted in Figure 4.

What the present experiment was unable to determine was the mechanism whereby the general activity-dependent increase in protein levels were reduced in CGG KI mice relative to their wildtype littermates. Future studies are necessary to determine if these results were mediated by changes to protein turnover, protein synthesis, or reductions in the activity-dependent increases in dendritic complexity seen in wildtype mice which result in general increases in protein levels. Additionally, similar motor tasks using more acute training protocols would be necessary to determine if *Fmr1*, *Arc*, and *c-Fos* mRNA immediate early gene expression show a similar pattern.

In summary, CGG KI mice acquired the forelimb reaching task more slowly than wildtype mice and did not reach the same level of performance as wildtype by the end of training. These results are similar to our earlier findings of learning and performance deficits in CGG KI mice (Diep, et al., 2012). They also demonstrate an additional dimension to the already documented motor impairments associated with the fragile X premutation and FXTAS, in both human carriers and in animal models. Increased levels of Fmrp, Arc, and c-Fos proteins were found in somatosensory/motor cortex contralateral to the forelimb trained in the reaching task when compared to the homologous cortex in the ipsilateral hemisphere. However, only Fmrp and Arc proteins were significantly correlated with performance of the task at the end of training. This suggests that increased neural activity associated with training on the reaching task may have induced expression of Fmrp and Arc proteins as part of an activity dependent plasticity underlying or contributing to forelimb motor skill learning. Such induction of these IEG's could contribute to the cellular mechanisms underlying rapid formation and stabilization of synapses in motor cortex during forelimb motor training (Xu, et al., 2009). Training related increases in Fmrp and Arc proteins in CGG KI mice were of lower magnitude than found in wildtype mice. Impaired activity-dependent synaptic translation of Fmrp has been reported in CGG KI mice (Iliff, et al., 2013). Specifically, induction of Fmrp by the Group 1 mGluR agonist DHPG was reduced or absence in cortical synaptoneurosome and in cultured hippocampal neurons (Iliff, et al., 2013). Combined with the present in vivo evidence for reduced activity dependent induction of Fmrp in CGG KI mice, the results suggest that some cognitive deficits associated with the Fragile X premutation may be due to impaired activity-dependent Fmrp translation. As described earlier, evidence for increases in Arc levels has been reported in a variety of learning tasks (Bramham, et al., 2010), including the forelimb reaching task used in this

study (Hanlon, et al., 2009, Hosp, et al., 2013). Because Fmrp is a regulator of mRNA translation for a variety of proteins including Arc (Darnell, et al., 2011), it is interesting to consider the possibility that activity dependent induction of Arc and other proteins may be influenced by alterations on activity dependent induction of Fmrp, and future studies should examine this possibility.

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Highlights

- The CGG KI mouse shows reduced levels of Fmrp relative to wildtype littermates
- The CGG KI Mouse shows lower levels of activity dependent proteins Arc, Fmrp, and c-Fos relative to wildtype littermates
- Smaller increases in Fmrp and Arc after motor training are associated with impaired performance

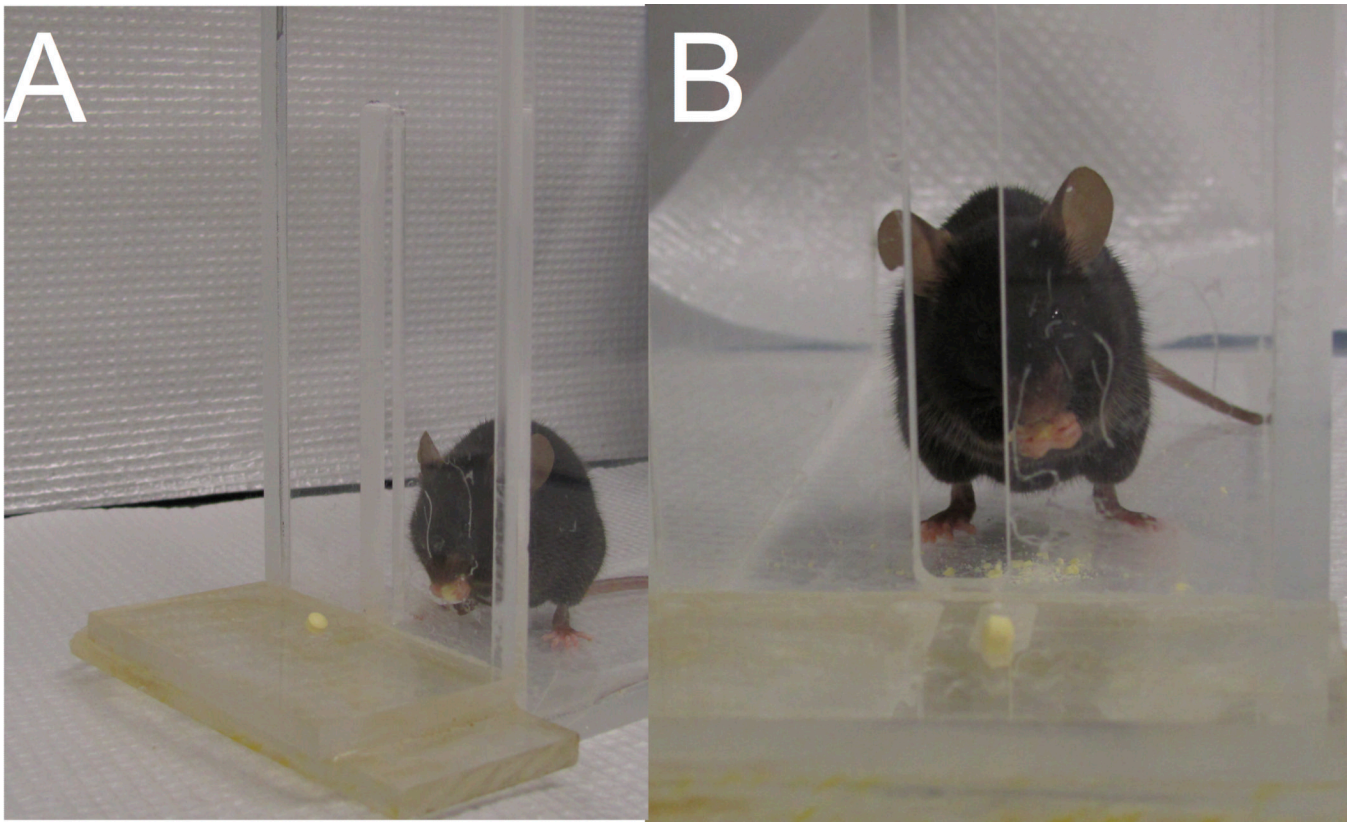

Figure 1. Skilled reaching task apparatus

A. Side view of apparatus with a mouse inside. **B.** Front view of the apparatus with same mouse.

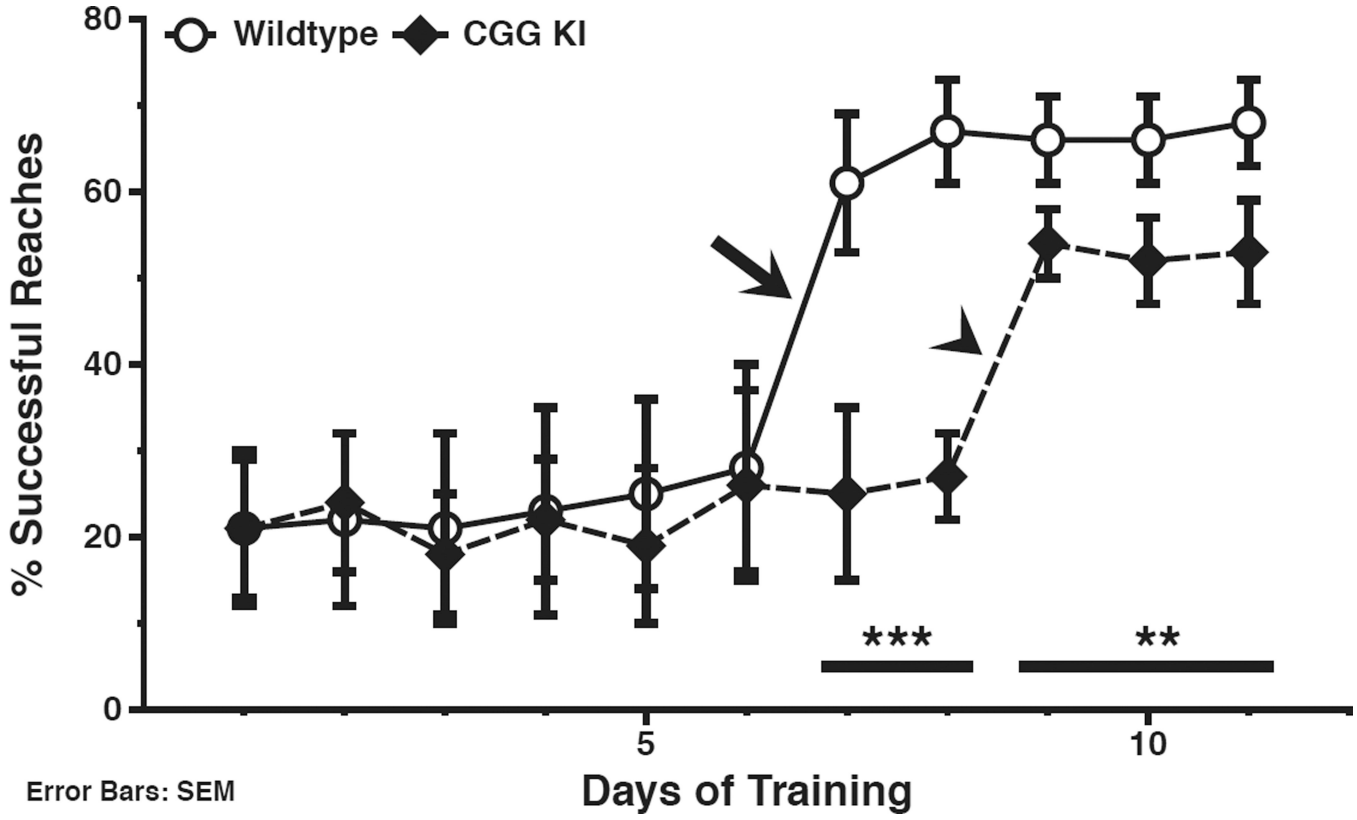

Figure 2. wildtype perform better at skilled reaching than CGG KI mice

Arrow signals first change point for wildtype mice at day 7. Arrowhead signals the first change point for CGG KI mice at day 9. ** p < .01 ***p < .001 Day × Group interaction based on Tukey's-HSD post hoc paired comparison.

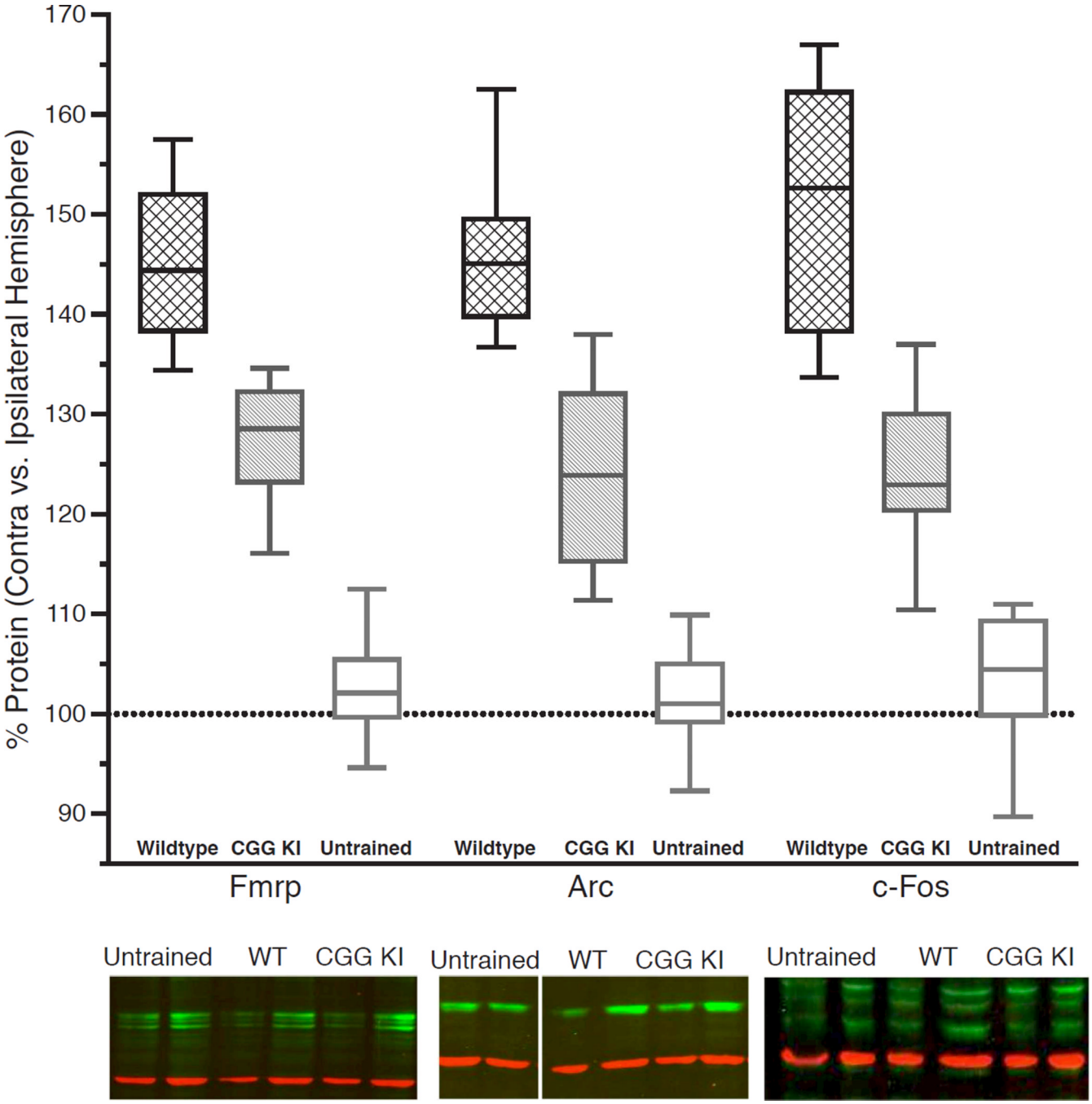

Figure 3. Activity dependent plasticity after skilled reaching

A. Wildtype have higher protein levels in the trained hemisphere (contra) vs untrained (ipsilateral) hemisphere compared to CGG KI mice. All trained mice have higher activity-dependent protein levels than untrained mice. **B.** Example Western blots for Fmrp, Arc, and c-Fos proteins. Each blot contains data from the ipsilateral and contralateral hemispheres of untrained wildtype, trained CGG KI, and trained wildtype mice alternating across each blot. The Fmrp bands were centered at 75 kD, Arc bands were at 45 kD, and c-Fos bands at 40 kD. The blots presented are at different magnifications to emphasize hemispheric differences.

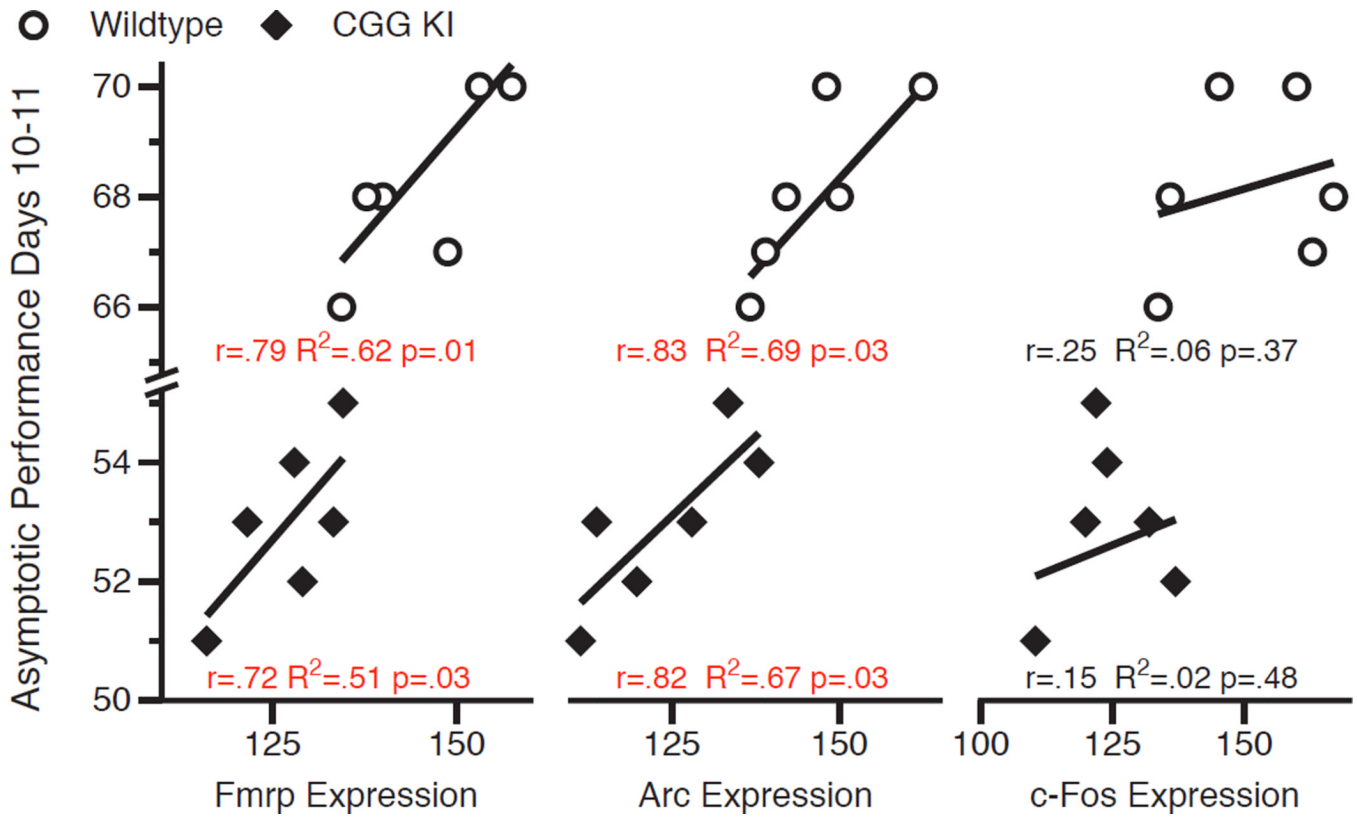

Figure 4. Associations between skilled reaching performance and protein expression

Asymptotic performance on day 11 associated with protein expression levels within wildtype and CGG KI mice for Fmrp and Arc proteins, both involved with learning/plasticity; but not c-Fos protein, which reflects cell activity, rather than learning. Expression = percent increase in protein in the contra versus ipsilateral hemisphere.