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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.

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1. 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, Hagerman, Chamberlain, & Hagerman, 2000; Tassone et al., 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, Kim, Willemsen, & Berman, 2012; Hunsaker, Wenzel, Willemsen, & Berman, 2009) and impaired visuomotor processing (Diep et al., 2012; Hunsaker, von Leden et al., 2011). 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

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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, 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.

2. Materials and methods

2.1. 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.

2.2. 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.

2.3. Skilled forelimb reaching apparatus

The apparatus for the skilled forelimb reaching task (Fig. 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).

3. Experimental methods

3.1. Skilled forelimb reaching task

3.1.1. 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

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