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Unaltered hormonal response to stress in a mouse model of fragile X syndrome

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Summary

Reports in the clinical literature and studies of *fmr1* knockout mice have led to the hypothesis that, in addition to mental retardation, fragile X syndrome is characterized by a dysregulation of hypothalamic–pituitary–adrenal axis function. We have systematically examined this hypothesis by studying the effects of stress on adrenocorticotrophic hormone and corticosterone levels in adult, male *fmr1* knockout mice. Initially we determined the circadian rhythms of the plasma hormone levels in both wild-type and *fmr1* knockout mice and established the optimal time to impose the stress. We found no genotypic differences in the circadian rhythms of either hormone. We studied two types of stressors, immobilization and spatial novelty; spatial novelty was 5 min in an elevated plus-maze. We varied the duration of immobilization and followed the time course of recovery of hormones to their pre-stress levels. Despite the lower anxiety exhibited by *fmr1* knockout mice in the elevated plus-maze, hormonal responses to and recovery from this spatial novelty were similar in both genotypes. Further, we found no genotypic differences in hormonal responses to immobilization stress. The results of our study indicate that, in FVB/NJ mice, the hormonal response to and recovery from acute stress is unaltered by the lack of fragile X mental retardation protein.

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1. Introduction

Fragile X syndrome (FraX), the most common inherited form of mental retardation, is caused by silencing of a single gene, *FMR1*, resulting in the absence of the gene product, fragile X mental retardation protein (FMRP). FraX phenotype includes cognitive impairments (Rousseau et al., 1994); behavioral dysfunction such as hyperactivity, social anxiety, attention problems and autistic-like behavior (Miller et al.,

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1999); and some physical abnormalities including macroorchidism in males (Hagerman, 2002). It has also been suggested that boys with FraX have dysregulation of hypothalamic–pituitary–adrenal (HPA) axis function. Two clinical reports show higher salivary cortisol concentrations in boys with FraX under both normal and stressful conditions (Wisbeck et al., 2000; Hessler et al., 2002), and some data suggest that higher levels of cortisol may be associated with greater severity of behavioral problems (Hessler et al., 2002).

In the *fmr1* knockout (KO) mouse model of FraX (Bakker et al., 1994), effects on the HPA axis have also been reported. Immobilization stress affected changes in *c-fos* expression that varied with duration of stressor, brain region, and genotype (Lauterborn, 2004). In paraventricular nucleus (PVN) *c-fos* expression was increased similarly in both WT and KO immediately following 30 min of immobilization. After 2 h of immobilization, *c-fos* was close to pre-stress levels in WT, but remained elevated in KO mice. Serum corticosterone (CORT) levels were elevated after either 30 min or 2 h of immobilization in both genotypes, but effects were greater in KOs after 2 h of stress. Following immobilization stress, serum CORT concentrations recovered more slowly in KO mice (Markham et al., 2006). In addition, one of the mRNAs bound by FMRP is a glucocorticoid receptor mRNA (Miyashiro et al., 2003). Although total glucocorticoid receptor levels appear to be normal in KOs, there is a reduced immunoreactivity in stratum radiatum of the hippocampus (Miyashiro et al., 2003). Normally, circulating glucocorticoids acting on glucocorticoid receptors suppress HPA axis responses through a negative feedback loop. The hippocampus, rich in glucocorticoid receptors, likely plays a major role in the negative feedback. Taken together with results of the clinical studies, these findings suggested a dysregulation of the HPA axis in FraX.

Our previous finding that *in vivo* rates of regional cerebral protein synthesis are increased in selective brain regions in adult *fmr1* KO mice (Qin et al., 2005) sparked our interest in a possible dysregulation of HPA axis function. Affected regions in our study included the PVN of the hypothalamus, hippocampus, and the basolateral amygdala, all regions known to be involved in mediating the response to stress. The present study was undertaken to further examine possible dysregulation of HPA axis function in FraX. We have studied the effects of two types of stressors (immobilization and spatial novelty) on stress hormone levels in adult, male *fmr1* KO mice. We have varied the duration of the immobilization and followed the time course of recovery of hormones to their pre-stress levels.

2. Material and methods

2.1. Animals

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. FVB/NJ-Fmr1^{tm1Cgr} breeding pairs (heterozygous females and hemizygous males) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). 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 (7 am–7 pm) and darkness (7 pm–7 am). Mice were housed 2–4/cage. Food (NIH-31 rodent chow) and water were provided *ad libitum*. Breeding pairs of heterozygous female and WT males provided offspring in two experimental groups: hemizygous and WT males. Mature mice at 100 ± 10 days of age were studied.

2.2. 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 DNA, a polymerase chain reaction (PCR) buffer, and *Taq* DNA polymerase (AmpliTag Gold, Applied Biosystems, Foster City, CA, USA) were combined and subjected to 35 cycles at 95, 62, and 72 °C. After amplification, the products were separated by electrophoresis on a 2.0% agarose gel at 100 V for 15 min. The PCR product at ≈ 800 bp indicated the presence of the null allele.

2.3. Hormone assays

At the outset of the study we determined the optimum conditions for collection of blood for hormone assays. We measured cort levels in blood samples collected from tail or trunk under different conditions which included with or without light halothane anesthesia, guillotine decapitation versus decapitation with scissors and tapered plastic film tube restraint (DecapiCone, Disposable rodent restrainers, Braintree Scientific INC., MA). We found that rapid decapitation using momentary tapered plastic film tube restraint without any anesthesia is the best way to sample blood (data not shown). This method subjected animals to minimal stress and provided stable cort measurements.

Following rapid decapitation, core blood was collected into heparinized tubes containing EDTA. Blood was centrifuged to separate the plasma, and plasma samples were stored at –70 °C until assayed. Concentrations of ACTH (200 μl) and cort (5 μl) in plasma samples were determined by radioimmunoassay (CORT ¹²⁵I RIA kit and hACTH ¹²⁵I RIA kit, MP Biomedicals, LLC, Orangeburg, NY, USA). Samples were counted in a Wallac Wizard Gamma Counter 1480 (PerkinElmer, Waltham, MA, USA). 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%. For a given experiment, hormone levels were assayed in a single batch.

2.4. Circadian fluctuations in HPA hormones

Before embarking on studies in which we perturbed the HPA system, we analyzed the daily rhythms of these hormones in the *fmr1* KO and WT mice to test for differences between

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