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ABNORMAL NEURONAL MORPHOLOGY AND NEUROCHEMISTRY IN THE AUDITORY BRAINSTEM OF FMR1 KNOCKOUT RATS

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Abstract—Fragile X syndrome (FXS) is an inherited neurodevelopmental disorder affecting nearly one in 5000 newborn males and is a leading genetic cause of autism spectrum disorder. In addition to developmental delays and intellectual impairment, FXS is characterized by seizures, attention deficit, and hypersensitivity to visual, tactile and auditory stimuli. The Fmr1 gene encodes Fragile X mental retardation protein (FMRP), which is abundant in neurons, binds select mRNAs and functions as a negative regulator of mRNA translation. A deficiency in FMRP, as in FXS and Fmr1 knockout (KO) animals, results in neuronal dysmorphology and altered synaptic function. Additionally, there is evidence for disruption of GABAergic circuits in subjects lacking FMRP. Our previous studies demonstrated widespread expression of FMRP in human auditory brainstem neurons. Given this observation, we hypothesized that FMRP is highly expressed in rat auditory brainstem neurons and that the auditory hypersensitivity characteristic of FXS results from dysfunction of brainstem networks secondary to decreased expression of FMRP. In our investigation of postnatal day 50 (P50) control rats, we found that FMRP was widely expressed in neurons of the superior olivary complex (SOC). In P50 Fmr1 KO rats, many SOC neurons had a smaller soma when compared to controls, indicative of abnormal neuronal morphology. Additionally, neurons in the medial superior olive (MSO) were more round in Fmr1 KO rats. There was also reduced expression of glutamic acid decarboxylase (GAD67) in neurons of the superior paraolivary nucleus (SPON) and a reduction in the number of calretinin-immunoreactive terminals associated with neurons of the medial nucleus of the trapezoid body (MNTB). Together, these findings support the conclusion that the auditory dysfunction characteristic of FXS arises, at least in part, from defective brainstem networks.

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Abbreviations: ABR, auditory brainstem response; CI, confidence interval; CR, Calretinin; Fmr1, Fragile X mental retardation 1 gene; FMRP, Fragile X mental retardation protein; FXS, Fragile X syndrome; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; GBC, globular bushy cell; IC, inferior colliculus; IR, immunoreactive; KO, knockout; LSO, lateral superior olive; MNTB, medial nucleus of the trapezoid body; MSO, medial superior olive; NDS, normal donkey serum; PB, phosphate buffer; PFA, paraformaldehyde; SOC, superior olivary complex; SPON, superior paraolivary nucleus.

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INTRODUCTION

Fragile X syndrome (FXS), a leading genetic cause of autism spectrum disorder, is a sex-linked disorder associated with mild to severe intellectual disability, behavioral and learning difficulties, attention deficit and hypersensitivity to visual, tactile, olfactory and auditory stimuli (Miller et al., 1999; Eliez et al., 2001; Berry-Kravis, 2002; Hagerman et al., 2009; Pfeiffer and Huber, 2009). FXS is the most common heritable cause of intellectual impairment and due to linkage with the X chromosome, affected males (1:3600) develop a more severe phenotype compared to affected females (1:8000; O'Donnell and Warren, 2002; Pfeiffer and Huber, 2009). FXS results from an expansion of a CGG repeat in the Fragile X mental retardation gene on the X chromosome (Fmr1; Verkerk et al., 1991). The number of CGG repeats ranges from 230 to over 1000 and a higher number of repeats are associated with more severe FXS phenotypes (Verkerk et al., 1991; Warren and Nelson, 1994; Till, 2010; Bagni et al., 2012). In FXS, there is reduced or silenced expression of the Fmr1 gene and therefore very little or no expression of Fragile X mental retardation protein (FMRP; Verkerk et al., 1991; De Boulle et al., 1993; Warren and Nelson, 1994; McLennan et al., 2011). Additionally, FXS is associated with a number of gross brain abnormalities such as reduced volume of the cerebellar vermis, enlargement of the 4th ventricle (Mostofsky et al., 1998; Hoeft et al., 2010) and hypertrophy of the hippocampus (Kates et al., 1997), caudate nucleus and thalamus (Reiss et al., 1995; Eliez et al., 2001; Hoeft et al., 2010).

FMRP is expressed at high levels in both the brain and testis and widespread expression is also seen among other peripheral organs such as the placenta, liver, kidney, and skeletal muscle (Hinds et al., 1993; Feng et al., 1997; Wang et al., 2004, 2014; Jacobs et al., 2012; Beebe et al., 2014). Intense neuronal expression of FMRP is localized to cholinergic neurons in the nucleus basalis, pyramidal cells of the hippocampus and the granular layers of the cerebellum (Hinds et al., 1993; Eberhart et al., 1996). However, FMRP expression is not ubiquitous in neurons as only about 10% of neurons in the human inferior olive express FMRP (Beebe et al., 2014). FMRP appears to function as a translational

repressor and thereby regulates activity-dependent translation of a number of mRNAs (Brown et al., 2001; Akins et al., 2009, 2012; Strumbos et al., 2010; Zhang et al., 2012). Additionally, FMRP is believed to selectively bind approximately 4% of all mRNAs in the mammalian brain, many of which are known to code for proteins involved in neuronal maturation and synaptic plasticity (Brown et al., 2001; Till, 2010; Bagni et al., 2012).

The absence of FMRP (in humans and Fmr1 KO animals) results in dysregulated translation of select mRNAs, abnormal synaptic function, dendritic dysmorphology characteristic of immature neurons, and a loss of protein synthesis-dependent plasticity leading to disordered expression of normally regulated proteins (Rudelli et al., 1985; Hinton et al., 1991; Comery et al., 1997; Irwin et al., 2000; Nimchinsky et al., 2001; Galvez et al., 2003; Pfeiffer and Huber, 2009; Zhang et al., 2009; Strumbos et al., 2010; Deng et al., 2011). Such issues are believed to contribute to both the cognitive and functional impairments associated with FXS (Fiala et al., 1998; Irwin et al., 2000; Bassell and Warren, 2008). Furthermore, FMRP is normally expressed in GABAergic neuronal populations (Olmos-Serrano et al., 2010) and numerous studies have provided evidence for alterations in GABAergic circuits in FXS. Specifically, there is reduced GABA receptor expression and diminished glutamic acid decarboxylase (GAD67)-immunostaining in the amygdala (GAD; El Idrissi et al., 2005; D'Hulst et al., 2006, 2009; Adusei et al., 2010; Olmos-Serrano et al., 2010), although elevated levels of GAD65/67 have been reported in the Fragile X mouse (El Idrissi et al., 2005). Finally, there is evidence that pharmaceutical therapies directed at GABA receptors reduce FXS symptoms (Tervonen et al., 2009; Olmos-Serrano et al., 2010, 2011; Heulens et al., 2012).

Subjects with FXS are hypersensitive to both auditory and visual stimuli (Van der Molen et al., 2012b). Furthermore, FXS subjects display numerous auditory processing abnormalities (Hanson et al., 1986; St Clair et al., 1987; Rojas et al., 2001; Castrén et al., 2003; Brady et al., 2006; Hall et al., 2009; Van der Molen et al., 2012a,b) despite normal auditory thresholds (Arinami et al., 1988; Roberts et al., 2005). Auditory processing difficulties are also evident in animal models of FXS (Fmr1 knockout (KO) rats and mice). Specifically, Fmr1 KO animals display elevated acoustic startle responses (Chen and Toth, 2001; Nielsen et al., 2002; Yun et al., 2006) and *in vivo* recordings from the auditory cortex revealed more action potentials in response to pure tone stimuli, larger variability in first spike latency and broader frequency tuning (Rotschafer and Razak, 2013). Furthermore, Fmr1 KO mice have elevated auditory brainstem response thresholds (Rotschafer et al., 2015) and are susceptible to audiogenic seizures, which can be fatal (Musumeci et al., 2000; Chen and Toth, 2001; Musumeci et al., 2007; Dansie et al., 2013).

We have previously studied the brainstem of a 32-year-old autistic male with FXS (Kulesza and Mangunay, 2008). In this subject, we found neurons in the medial superior olive (MSO) to be significantly smaller and more round compared to an age-matched control. We interpreted such dysmorphology in the MSO to be indicative of immaturity and dysfunction of this nucleus. Furthermore, we have recently demonstrated that coincidence detector neurons in the MSO (gerbil and human) and nucleus laminaris (alligator and chicken) express FMRP (Wang et al., 2014). Additionally, we have shown that FMRP is widely expressed in the human cochlear nucleus and superior olivary complex (SOC) (Beebe et al., 2014), both of which are important brainstem centers involved in the processing of auditory information.

The rat auditory brainstem is well characterized (see Malmierca, 2014) and therefore will serve as an excellent model to study the distribution of FMRP and the impact that loss of this protein might have on auditory brainstem circuits. Based on aforementioned observations, we hypothesize that FMRP is widely expressed in neurons of the rat SOC and that loss of FMRP expression will be accompanied by significant dysmorphology and fewer GABAergic neurons in the rat SOC. Herein, we have utilized immunofluorescence to map the expression of FMRP in the control rat SOC and have examined neuronal morphology, the expression of GAD and the organization of calretinin-immunoreactive (CR-IR) calyx terminals in the medial nucleus of the trapezoid body (MNTB) in both control and Fmr1 KO rats.

EXPERIMENTAL PROCEDURES

Experimental animals

This investigation is based on the study of 12 control male albino rats (age = postnatal day 50 [P50]) and 10 Fmr1 KO rats (P50, male albino; obtained from SAGE Research Labs). The Fmr1 KO rats were hemizygous males with a deletion of the Fmr1 gene and exhibited complete loss of FMRP expression as demonstrated by Western blot analysis (SAGE Research Labs). Only male animals were used since FXS affects predominantly males. Animals were housed (1 per cage after weaning) under a 12-h light/dark cycle with *ad libitum* access to food and water. Animals were only included in the study if their external auditory canals were clear of infection and debris and their middle ears were without effusion. All animal handling was approved by the Institutional Animal Care and Use Committee.

Perfusion and tissue processing

Animals were anesthetized with an intraperitoneal injection of pentobarbital (80 mg/kg). When the animals were unresponsive to toe pinch, they were perfused through the ascending aorta with a rinse of normal saline followed by 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer, (PB; pH 7.2). Brains were dissected from the skull, the right side of the brainstem was marked with a register pin and the brain was post-fixed in 4% PFA–PB at least overnight. At least 24 h before tissue sectioning, brainstems were trimmed and cryoprotected in a solution of 30% sucrose in 4% PFA–PB at 4 °C. Tissue blocks were sectioned, in the coronal plane, on a freezing microtome at a thickness of 40 μm and collected into three separate wells in