Effects of Regulating Rac1 in a Mouse Model of Fragile X Syndrome

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A Dissertation Presented to

The Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of

The Requirements for the Degree

Doctor of Philosophy

By

Luis Alberto Martinez

August, 2016

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ABSTRACT

Autism is a complex neurodevelopmental disorder characterized by social deficits, communication and language impairments, and restricted or stereotyped patterns of behavior. Intellectual disability (ID), reported in nearly 70% of those with autism, is a pervasive co-morbidity that exacerbates cognitive functions and impedes behavioral development. The most effective treatment for autism and ID are behavior-based interventions such as Applied Behavior Analysis (ABA) that require rigorous training methodologies. Furthermore, treatment must be started early and must be continuous and consistent for optimal efficiency. The high cost of behavior therapies and demanding schedule often precludes a successful outcome.

Over the years, studies have revealed structural and functional synaptic impairments in autism and ID in areas important for cognitive functions such as learning and memory formation. The observation in these learning disabilities that repeated training can overcome cognitive deficiencies suggests that mechanisms of learning and memory are not entirely defective. Autism and ID have been found to share dysregulation of molecular signaling cascades involved in synaptogenesis, spinogenesis and synaptic plasticity. The structural integrity of synapses and dendritic spines within those synapses relies on the underlying actin cytoskeleton. Examination of post-mortem brain tissue of autistic individuals reveals not just an unusually high number of dendritic spines but a

high density of immature spines. Post-mortem brain tissue also exhibits high levels of the small Rho GTPase Rac1, a well-recognized regulator of actin dynamics at the synapse. The integral role of Rac1 in dendritic spine development, synaptic plasticity, and learning and memory has been extensively studied. Together these studies present Rac1 as an intriguing target in the treatment of cognitive deficits associated with autism and ID.

Herein, we studied whether regulation of Rac1 might represent a promising treatment for cognitive impairment in autism, using Fragile X syndrome (FXS) as a model. FXS is the leading single gene cause of autism and ID. Neurons express a high density of underdeveloped dendritic spines in FXS humans and animal models. Synaptic plasticity deficits are prevalent throughout the brains of FXS mouse models including the cortex and hippocampus, areas critical for various forms of learning and memory. Moderate to severe learning deficiencies are also characteristic in FXS patients and is paralleled in mouse models. Therefore, FXS is an ideal model in the clinical and laboratory setting to investigate therapies aimed at autism and ID. In FXS mouse models, hyperactive Rac1 has been demonstrated in hippocampus and cortex where dendritic spine abnormalities are a common feature.

Our results show that in the Fmr1 KO mice (an animal model of FXS) deficits in memory and synaptic plasticity are associated with the presence and localization of Rac1.

Furthermore, treatment of Fmr1 KO mice with a specific Rac1 inhibitor improves memory and increases hippocampal LTP. Taken together these observations show that Rac1 may contribute to FXS related learning and memory impairments in humans. Importantly, this study suggests that targeting Rac1 in FXS may rescue cognitive impairments. Such a therapy may be translated into broader applications in autism and ID.

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1. INTRODUCTION AND STATEMENT OF THE PROBLEM

1.1 Introduction

Autism is a neurodevelopmental disorder with a complex behavioral phenotype comprised of three core symptoms: social deficits, communication impairments and repetitive patterns of behavior (Park et al, 2016). The most recent CDC report on the prevalence of autism diagnosis, based primarily on medical records, is 1 in 68 in children ages 8-14 (CDC, 2016). The National Center for Health Statistics conducted an independent parental survey which estimates the prevalence at 1 in 45 (Zablotsky et al, 2015). In 2015, the collective economic burden of autism in the U.S. was estimated at $268 Billion in 2015 and is predicted to climb to $461 Billion by 2025 (Buescher et al, 2014). Medical and behavioral complications that are often associated with autism include seizures, attention deficit hyperactivity disorder, sleep disorders, depression, anxiety and aggression (Volkmar and Nelson, 1990; Simonoff et al, 2008; Devnani and Hegde, 2015), which can be treated with medications (Doyle and McDougle, 2012; Ji and Findling 2015; Ji and Findling, 2016). Currently there are no pharmacological treatment options for core symptoms or cognitive impairments in autism, which pose the primary developmental barriers. The most effective treatments for learning

disabilities associated with autism are one-on-one (individualized) behavior-based intervention programs such as Applied Behavior Analysis (ABA) that utilize rigorous task repetition and classical associative learning paradigms (McEachin et al, 1993; Smith et al, 1997; Granpeesheh et al, 2009; Estes et al, 2015). The age of the child and symptom severity dictates intensity of training required and determines program efficiency (Howlin et al, 2009; MacDonald et al, 2014). Although gains in cognitive function accumulate slowly after structured teaching programs, improvements ranging from mild to full recovery are reported within different learning domains such as experiential, social and academic learning (Howard et al, 2005; Eikeseth et al, 2007). Progress, however great or small, indicates that mechanisms of learning and memory are not entirely defective. Therefore, it is important to investigate signaling pathways possibly associated with learning and memory that may be modulated pharmacologically, to complement behavioral therapies.

Episodic memories are autobiographical facts and experiences (Tulving, 2002; Dickerson and Eichenbaum, 2010) that inform and guide new learning (Schacter and Wagner 1999). The hippocampus is a limbic structure within the medial temporal lobe important for learning and the formation of new memories, including episodic memories (Burgess et al, 2002). Functional MRI studies in autistic patients undergoing learning tasks have shown disruptions in sub-regions of the hippocampus engaged when processing

episodic memories (Goh and Peterson, 2012; Gaig et al 2015). The structural impairments of the hippocampus in autism have been explored (Delong, 1992) and studies show enlarged hippocampal volume (Groen et al, 2010), and hippocampal sub-region area CA1 presenting smaller neurons and reduced dendritic branching (Raymond et al, 1996; Saitoh et al 2001). Neurons receive excitatory neurotransmission primarily within the dendritic tree, specifically on spiny protrusions known as dendritic spines (Megias et al, 2002). These dendritic spines are actin- and receptor-rich structures (Nimchinsky et al, 2002) that undergo proliferation in normal early development (Petanjek et al, 2011). During adolescence the number of spines are reduced drastically in an experience-driven pruning process (Purves and Lichtman, 1980; Tropea et al, 2010) believed to help refine synaptic connections between individual neurons and neuronal networks. Stable spines have been associated with learning (Roberts et al, 2010) and long-term memories (Yang et al, 2009), leading to a proposed role in information processing and storage. Post-mortem brain tissue from individuals with autism reveals not only increased dendritic spine density in temporal lobe neurons (Hutsler and Zhang, 2010), but a failure of spines to undergo age-specific pruning (Tang et al, 2014). A lack of spine elimination has been proposed to maintain hyper-connectivity, which has been shown to weaken synaptic strength during early postnatal development (Testa-Silva et al, 2012). It is not known what factors or mechanisms maintain an overabundance of dendritic spines in individuals with autism.

About 60 to 70% of individuals with autism are also diagnosed with intellectual disability (ID; Fombonne, 2006; Charman et al, 2011), which further confounds existing core symptoms and impedes cognitive development (Ben et al, 2008). ID, formerly referred to as mental retardation, is defined as an IQ of below 70 accompanied by deficits in adaptive behaviors (AAIDD, 2010) and negatively impacts cognitive, motor and language skills in early development (WHO, 1992). A consistent neuronal feature in ID is a high density of structurally malformed dendritic spines (Table 1 LITERATURE REVIEW, Purpura, 1974; Kaufmann and Moser, 2000). Singe gene X-linked intellectual disabilities have guided the generation of animal models that recapitulate the human behavioral and synaptic abnormalities (Branchi et al, 2003).

A leading cause of autism and ID is Fragile X Syndrome (FXS), a neurodevelopmental disorder that has become an ideal model in the clinical setting to study these co-occurring conditions (Budimirovic and Kaufmann, 2011). In FXS patients, an unstable CGG triplet codon expansion (>200 repeats) in the promoter region of the Fragile X Mental Retardation 1 gene (Fmr1) has led to hypermethylation and transcriptional silencing of the gene. Loss of the Fmr1 gene product, Fmr1 protein (FMRP), a negative regulator of mRNA translation at the synapse, has been strongly associated with a series of behavioral disturbances including hyperactivity, social and cognitive deficits as well as seizures (Freund et al 1991; Roberts, et al, 2005; Kidd, et al, 2014; Boyle and Kaufmann,

2010; MacLeod et al, 2010). In the laboratory setting, a mouse model of FXS with a null Fmr1 gene recapitulates many of the behavioral abnormalities seen in Fragile X patients, including autistic features and a significant learning deficit (Dutch Belgian Consortium, 1994; Musumeci et al 2000; Mineur, et al 2002; Yan et al, 2004; Spencer et al, 2005; Kazdoba et al 2014; Kazdoba, et al 2016). Interestingly, both FXS patients and mouse models exhibit a high density of immature dendritic spines throughout the brain but most notably in the hippocampus, cortex and amygdala (Comery et al, 1997; Irwin et al, 2001). These areas play critical roles in the integration of sensory and emotional stimuli and are important for learning and memory formation (Richter-Levin, 2004; Rodrigues et al 2004; Maren, 2011). The Fmr1 KO mouse central nervous system also exhibits impaired synaptic plasticity (Li et al 2002; Zhao, et al 2005; Zhang, et al 2009; Xu, et al 2012; Strumbos et al 2010; Yang, et al 2014; Rotschafer, et al 2015). Therefore, the abnormal dendritic spine and synapse development with accompanying impaired synaptic plasticity might be implicated in perturbing cognitive function in FXS. These observations in FXS provide an ideal foundational model for determining pathophysiology of autism and ID.

As mentioned, dendritic spines are rich in actin filaments that form a dynamic cytoskeleton (Nimchinsky et al, 2002). Spines develop from filopodia into long thin immature spines that are then converted to mature mushroom-shaped spines

(Hotulainen and Hoogenrad, 2010). Once mature, spines can shrink, grow or be eliminated (Cingolani and Goda, 2008; Bhatt et al, 2009). The actin cytoskeleton must be remodeled during morphological changes that require de-polymerization, re-polymerization and branching of existing actin filaments (Cingolani and Goda, 2008; Spence and Soderling, 2015). For example, studies have demonstrated a synaptic activity-dependent, short-lived reduction in actin filaments (Ouyang et al, 2005) prior to the stable, long-lived enhancement of actin filament content in dendritic spines (Fukazawa et al, 2003). Preventing such changes may diminish the plastic properties of synapses that underlie learning and memory formation processes.

Actin cytoskeleton dynamics factor critically into the morphological and functional changes of dendritic spines resulting from synaptic activity (Cingolani and Goda, 2008) suggesting regulation of actin remodeling must be strict to match synaptic activity magnitude. Providing tight control of actin cytoskeleton remodeling at the synapse are members of the Rho subfamily of small GTPases (Luo, 2000; Nakayama et al, 2000; Tashiro et al, 2000; Dillon and Goda, 2005). Small Rho GTPases cycle between inactive (GDP-bound) and active (GTP-bound) states and promote actin assembly (Rac1) or disassembly (RhoA) (Cingolani and Goda, 2008). Impaired molecular signaling, which has been reported in both autism and ID, converges on small Rho GTPases (Newey et al 2005; Ba, et al 2013). The small Rho GTPase Rac1 has recently been reported to be

upregulated in cortex and cerebellum in post-mortem tissue from individuals with autism (Fatemi et al, 2013). Furthermore, several rare X-linked intellectual disabilities are known to impact Rac1 signaling directly (Faucherre et al, 2005; Bacon et al, 2013; Ellery, et al 2014). Studies show that enhancing Rac1 activity increases while reducing Rac1 activity lowers dendritic spine number (Nakayama et al, 2000; Tashiro, et al, 2000), emphasizing its role in spine dynamics (Impey et al, 2010; De Rubeis, et al 2013; Wiens et al. 2005). Perturbing Rac1 activity also impairs various forms of synaptic plasticity such as long term potentiation (LTP) and long term depression (LTD), widely regarded neuronal substrates of learning (Bongmba, et al. 2011; Martinez and Tejada-Simon, 2011). At the behavioral level, Rac1 is also determined to be significant in addiction (Dietz et al 2012), mood disorders (Golden, et al, 2013), and learning and memory (Martinez et al, 2007; Bongmba, et al. 2011; Wu et al 2014; Gao, et al 2015). Interestingly, increased levels of active-state Rac1 (Rac1-GTP) have been found in whole brain homogenates of Fmr1 KO mice (de Diego-Otero et al, 2009). Additionally, hyperactive-state Rac1 levels were detected in Fmr1 KO specifically in hippocampus and cortex by our laboratory (Bongmba et al, 2011). These observations in the Fmr1 KO mouse model of FXS present parallel findings in upregulated Rac1 in autism.

A deficiency in theta-burst stimulation-LTP (TBS-LTP) has been reported in Fmr1 KO hippocampus under low-intensity stimulation conditions (Lauterborn, et al, 2007). Theta

burst stimulation is patterned after the physiological theta frequency detected in rodents exploring novel environments (King et al., 1998; Penley, et al, 2013) and which has also been shown to be important in spatial and fear learning (Winson, 1978; Ognjanovski et al, 2014). A major step in Rac1 activation is translocation to the plasma membrane where Guanine Nucleotide Exchange Factors (GEFS) load Rac1 with GTP (Bustelo et al, 2012). Previous studies have demonstrated that in mice, Rac1 translocates to the plasma membrane after exposure to novel environments (Zhou et al, 2008), long-term exposure to enriched environments (Yuan et al 2015) and fear conditioning (Martinez et al, 2007), behaviors that have been associated with changes in dendritic spine morphology (Leuner and Shors, 2004). Rac1 controls actin dynamics through p21-activated kinases (PAKs) by inhibiting the actin filament severing protein ADF/cofilin (DesMarais et al, 2005). Additionally, increased levels of active ADF/cofilin have been detected in fibroblasts from Fmr1 KO mice (Castets et al, 2005). Therefore, hyperactive Rac1 may prevent ADF/cofilin activation required for synaptic activity-dependent functional changes at the synapse (Gu et al, 2010). Other studies have revealed that in Fmr1 KO mice, pharmacological and genetic inhibition of PAK leads to rescue of synaptic plasticity and learning deficiencies (Hayashi, et al, 2007; Dolan et al, 2013) and that a reduction in Rac1 activity results in improvements in fear learning and memory (de Diego-Otero et al, 2009).

Herein, we confirmed that Rac1 is upregulated in Fmr1 KO mice. Moreover, Fmr1 KO mice exhibit abnormal Rac1 membrane localization at three critical developmental stages of the hippocampus (neonate, juvenile, and adult). In the hippocampus, Fmr1 KO mice exhibit an LTP deficit that can be overcome by increasing stimulus intensity. Fmr1 KO mice displayed significant learning and memory deficits during hippocampus-dependent associative learning tasks, which were normalized after increasing training trials. Fmr1 KO mice that underwent a higher number of training trials exhibited normalized hippocampal LTP magnitude that was associated with a decrease in membrane bound Rac1. Additionally, treatment of hippocampus slices from Fmr1 KO mice with a specific Rac1 inhibitor corrected this LTP deficit. Furthermore, Fmr1 KO mice treated with the Rac1 inhibitor displayed significant learning and memory recovery even with reduced training trials. Taken together these observations show that Rac1 may be contributing to dendritic spine abnormities, underlying pathological behavior in FXS. Rac1 may also be a suitable modulatory target in correcting cognitive deficiencies in FXS that could translate into broader applications in autism and ID.

1.2 Statement of the problem

In the past couple of decades, the prevalence of autism has been rapidly climbing (Hansen et al, 2015). According to the CDC, currently 1 in 68 school-aged children are diagnosed with an autism spectrum disorder (CDC, 2016). This increase has been partially explained by a wider awareness of the condition, improved diagnostic tools, and a broadening of the diagnostic criteria (Hansen et al, 2015). The remaining cases are believed to result from nascent environmental factors interacting with a large array of unidentified susceptible genes as indicated by the wide variability of symptom severity (Chaste et al, 2012). Up to 70% of those diagnosed with autism also exhibit intellectual disability (ID), which is defined as having an IQ below 70 (average 100) and accompanied by deficits in adaptive behaviors. ID impedes behavioral and occupational development, and obligates lifelong assistance. Furthermore, autism co-occurring with ID puts much heavier financial burdens on families and the economy (Buescher et al 2014; Ouyang, et al, 2014; Leigh and Du 2015). Medications exist to treat common comorbidities such as seizures (anti-epileptics), aggression (sedatives, anti-psychotics), sleep disturbances (sedative hypnotics), depression (anti-depressants) and attention deficit hyperactivity disorder (CNS stimulants). However, there are no pharmaceutical options to treat core symptoms or the cognitive deficiencies, which pose the primary developmental impairments. Behavioral therapies have proven effective in attenuating core symptom

severity and diminishing cognitive deficits (Ben and Zachor, 2007). However, the effectiveness of these costly behavioral interventions depends on stringent regimens and consistent schedules and oftentimes hinges on the age of the child and severity of symptoms (Howlin et al, 2009; MacDonald et al, 2014). Many states now require insurance companies to cover Applied Behavioral Analysis (ABA), which is considered to be the "gold standard" in the treatment of autism. Despite these efforts, a large percentage of people with autism undergo truncated treatment and therefore experience a diminished outcome. Those with autism also diagnosed with ID benefit the least after behavior interventions (Sigman and McGovern, 2005; Itzchak and Zachor, 2007). They will likely require continued care after age 21, after which ABA becomes less effective and is no longer covered by insurance.

Despite the undefined etiology of autism, a recurring theme in autism and ID is problems with synapse development, dynamics and maintenance (Purpura, 1974; Kaufmann and Moser, 2000; Hutsler and Zhang, 2010). Behavioral intervention programs can therefore benefit synergistically from pharmaceutical therapies aimed at correcting synaptic dysfunctions, believed to pose an impediment during the learning process.

1.3 Significance of the study

This study supports the idea that cognitive functions in intellectual disabilities might improve by utilizing mechanisms unique to the disability. Behavior intervention programs prove to be efficient with long term commitment. In preclinical studies, the spotlight is invariably focused on the deficiency. However, animal models of intellectual disability often exhibit a decrement in face validity when a learning impairment phenotype is not detected or is mildly different than the control. The mechanism of memory recovery uncovered here can be only one of many more that are engaged during the recovery process. Based on these findings in a mouse model of Fragile X Syndrome, Rac1 dysregulation appears to be an important contributor of impaired synaptic plasticity and learning and memory. Furthermore, these investigations are highly suggestive of a self-regulating mechanism induced under conditions favorable to the learning process. These conditions potentially invoke Rac1 inhibition that is conducive to learning, an observation that introduces one of perhaps many therapeutic targets in the treatment of FXS. The pathophysiological state, which by definition is responding differently to the environment, will induce compensatory mechanisms uniquely different from the healthy state. Therefore, it is equally valuable to study self-correcting pathways that function beyond the deficit. Many more animal models of autism have been developed that consistently display similar impairments in synaptic

structure and function to those observed in the FXS mouse model. Therefore, this study points to the need to investigate how recovery is achieved.

2. Review of Literature

Autism was initially defined in 1943 by Leo Kanner when he wrote about 11 boys who would not make eye contact and "who were oblivious to everything around them" (Kanner, 1943). This internalization, or "mindblindness" (Lombardo and Cohen, 2011) is an overarching behavioral feature of autism that prevents emotional reciprocity. Severity of the core symptoms vary independently from each other over a wide spectrum from mild to severe. Social interaction deficits can be reliably observed and used as diagnostic indicators in 2 year olds who demonstrate an apathy towards the sound of their name and exhibit eye-gaze avoidance (Jones et al, 2008). Indeed, eye-gaze avoidance is becoming such an important hallmark of autism that it is being investigated in the diagnosis of patients younger than 1-year-old to begin behavior interventions sooner (Jones and Klin, 2013; Frazier et al, 2016). Medical and behavioral complications that are frequently associated with autism include seizures, attention deficit hyperactivity disorder, sleep disorders, depression, anxiety and aggression (Volkmar and Nelson, 1990; Simonoff et al, 2008; Devnani and Hegde, 2015). These

comorbidities often can be treated with available drugs (Doyle and McDougle, 2012; Ji and Findling 2015; Ji and Findling, 2016). A single cause of autism is unlikely, given that several hundred genetic risk factors have been associated with autism (Neale et al, 2012; Sanders et al, 2012) and many environmental factors have also been linked to high incidences of autism (Grabrucker, 2013). Interactions of genetic risks with changing environmental factors have also been implicated (Chaste et al, 2012). The rapid increase in prevalence in the past couple of decades suggests a change in genetic risk factors or environment, however studies have revealed improved and broadened diagnostic practices largely explain the dramatic increase in autism prevalence (Hansen et al, 2015). The cause notwithstanding, autism currently takes a monumental economic toll at the societal (Buescher et al, 2014; Leigh and Du 2015) and household level (Ouyang, et al, 2014). The recorded increase in prevalence coupled with the high cost of special education has prompted federal intervention. Laws have been passed requiring insurance companies to cover behavioral intervention programs. The “gold standard” in the treatment of autism is Applied Behavior Analysis (ABA), which utilizes classical associative learning with positive reinforcement. ABA curricula aimed at improving cognitive and social deficits are intensive and can be effective when started early, which stresses the importance of early diagnosis.

Fragile X syndrome (FXS) is the leading known single gene cause of autism and ID. FXS, also referred to as Martin-Bell syndrome, affects 1:4000 boys and 1:8000 girls with boys

exhibiting more severe cognitive impairment (Garber et al, 2008). FXS is caused by the loss of the Fragile X Mental Retardation Protein (FMRP), an mRNA translation regulator that is highly expressed in the brain. In people without FXS, the Fmr1 gene, which codes for FMRP, contains a series of CGG codon repeats numbering less than 35 in the promoter region. A pre-mutation results when the repeats expand to between 35 and 200 CGG repeats. The permutation is unstable and highly prone to further replication. In FXS, the CGG codon series expands to greater than 200 repeats that attract methyl-transferases (Jin and Warren, 2000). Subsequent hyper-methylation of the promoter leads to chromatin condensation and silencing of the gene. The irregular chromatin condensation in the region of the Fmr1 gene locus during metaphase of cell division produces a kink (or fragile site) in the chromosome that gives the condition its name (Jin and Warren, 2000). The explanation for the repeat expansion remains unknown, but this feature groups FXS into a set of conditions resulting from unstable repeat expansions (Gatchel and Zoghbi, 2005). Examination of post-mortem tissue from FXS patients revealed an over-abundance of long, thin immature dendritic spines (Irwin *et al*. 2001; Irwin *et al.* 2002), which are the major sites of excitatory input of neurons (Nimchinsky et al, 2002). The generation of a mouse model of FXS (Dutch Belgian Consortium, 1994) expanded and greatly accelerated the unraveling of the function of FMRP as a negative regulator of basal (Brown et al 2001) and synaptic activity-dependent (Todd, et al, 2003; Niere, et al, 2012) protein synthesis. Many mRNA targets

of FMRP have been identified to code for proteins involved in synaptic plasticity (Darnell et al 2011). Neurons from FXS mouse models also exhibited an abundance of deformed dendritic spines (Comery et al, 1997), a key feature of ID (Kaufmann and Moser, 2000). Various forms of synaptic plasticity have been discovered to be impaired throughout the brain of Fmr1 KO mice including the hippocampus (Li et al 2002; Zhao, et al 2005; Zhang, et al 2009; Xu, et al 2012; Strumbos et al 2010; Yang, et al 2014; Rotschafer, et al 2015). Research into the function of metabotropic glutamate receptors (mGluRs) in control of mRNA translation converged on investigations into long-term depression (LTD), a regulated weakening of synaptic efficacy. Hippocampal slices from Fmr1 KO mice were found to exhibit exaggerated mGluR-dependent LTD (Huber et al, 2002), resulting from excessive removal of AMPA receptors from the post-synaptic membrane after mGluR5 activation (Nakamoto et al, 2007). These studies provided the underpinnings for the metabotropic glutamate receptor (mGluR) theory of FXS which states that the clinical manifestations of Fragile X syndrome resulting from functional loss of FMRP are initiated and propagated at overstimulated group 5 mGluRs (Bear, et al 2004). mGluR-mediated exaggerated LTD coupled with an excess of immature dendritic spines offered a well-grounded explanation for cognitive deficiencies seen in FXS. The mGluR theory was further bolstered when it was demonstrated that FmR1 KO mice crossed with mGluR5 subunit haplo-insufficient mice corrected the enhanced mGluR-mediated protein synthesis (Dolen et al, 2007) and corrected abnormal behaviors (Thomas et al 2011).

Further excitement grew when treatment of Fmr1 KO mice with selective mGluR5 inhibitors also corrected abnormal behaviors (Yan et al, 2005; Thomas et al, 2012) as well as rescuing dendritic spine deformities (Su et al, 2011). These promising preclinical studies sparked clinical trials using mGluR5 antagonists. Although initially hopeful in animal models, tests of the mGluR theory in various clinical trials failed to improve primary outcome measures such as sociability and cognitive function (Jacquemont et al 2014; Berry-Kravis et al, 2016). FXS has often been utilized to screen treatments in autism (Wang et al, 2010). However, the challenges to the treatment of FXS has stimulated interest in the identification of novel targets (Hagerman et al, 2009). Many of these targets have been revealed in the hippocampus.

The importance of the hippocampus to human memory was made clear in 1953 after Henry Molaison underwent brain surgery to treat epileptic seizures (Dossani et al, 2015). Ablation studies in experimental animals further supported the role of the hippocampus in learning and memory (Ross et al 1984; Chen et al, 1996). Earlier investigations using rabbits had revealed that synapses of the hippocampus undergo long lasting increases in synaptic efficacy after applying artificial high frequency synaptic activity, a phenomenon termed long-term potentiation (LTP) (Bliss and Lomo, 1973). LTP would later be observed in more experimental species from slugs and flies to rats and mice. Naturally, LTP fit the requirements for the Hebbian learning rule, which described the selective strengthening of synapses relevant to learned behavior (Hebb, 1949).

Modern techniques have better supported a functional link between LTP in the hippocampus and the formation of memories (Gruart et al, 2006; Nabavi et al, 2014). Specific patterns of synaptic stimulation have been shown to induce different forms of LTP (Buonomano, 1999) suggesting an inherent flexibility of hippocampal synapses required to process environmental stimuli of differing saliency or relevance. Pyramidal neurons throughout the brain are covered in receptor-rich spiny protrusions that oppose the neurotransmitter-rich presynaptic membrane. Spines develop from filopodia into long thin immature spines, which are then converted to mature mushroom-shaped spines (**Figure 1**).

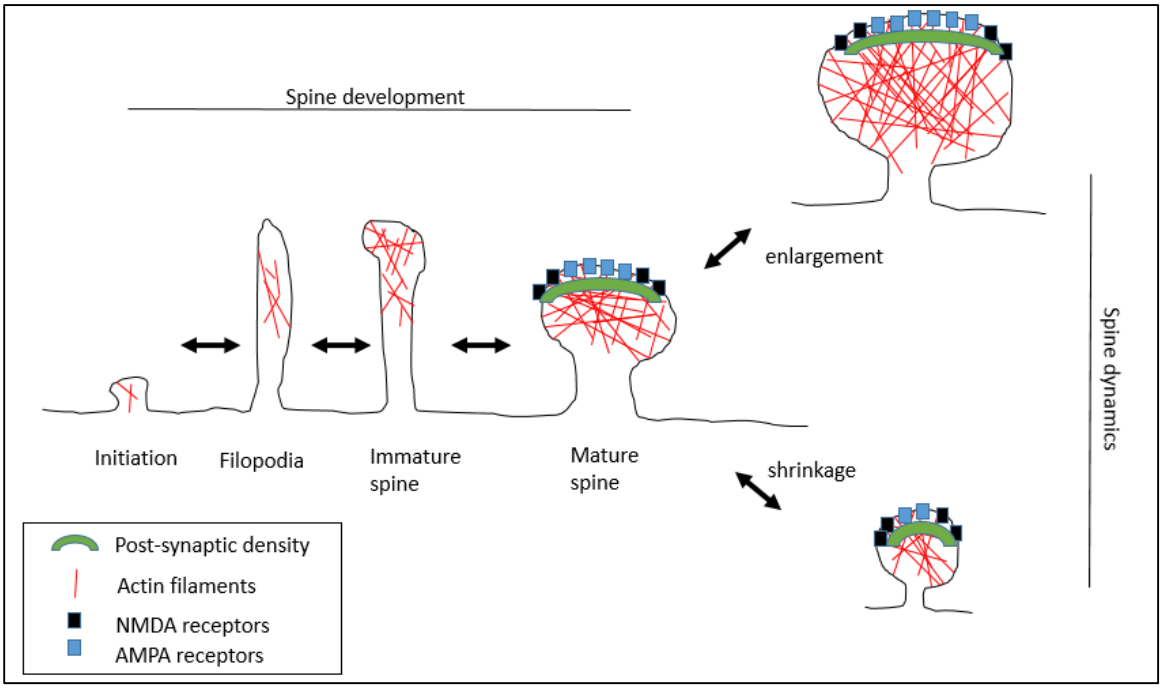

Figure 1. Spine development and spine dynamics.

Although the dendritic shaft is composed primarily of microtubules, dendritic spines are enriched with a dynamic actin cytoskeleton (Korobova and Svitkina, 2010) linked to a dense scaffolding protein complex that clusters receptors at the tip (Okabe, 2007). The majority of excitatory synapses in hippocampal area CA1 innervate neurons on dendritic spines (Megias et al, 2001). Furthermore, induction of LTP leads to activity-dependent changes in spine morphology (Maletic-Savatic et al, 1999) along with increases in actin filament content within dendritic spines (Fukazawa et al, 2003; Cingolani and Goda, 2008). The process of actin remodeling within dendritic spines is strongly implicated in subserving hippocampus-mediated learning and synaptic plasticity (**Figure 2**).

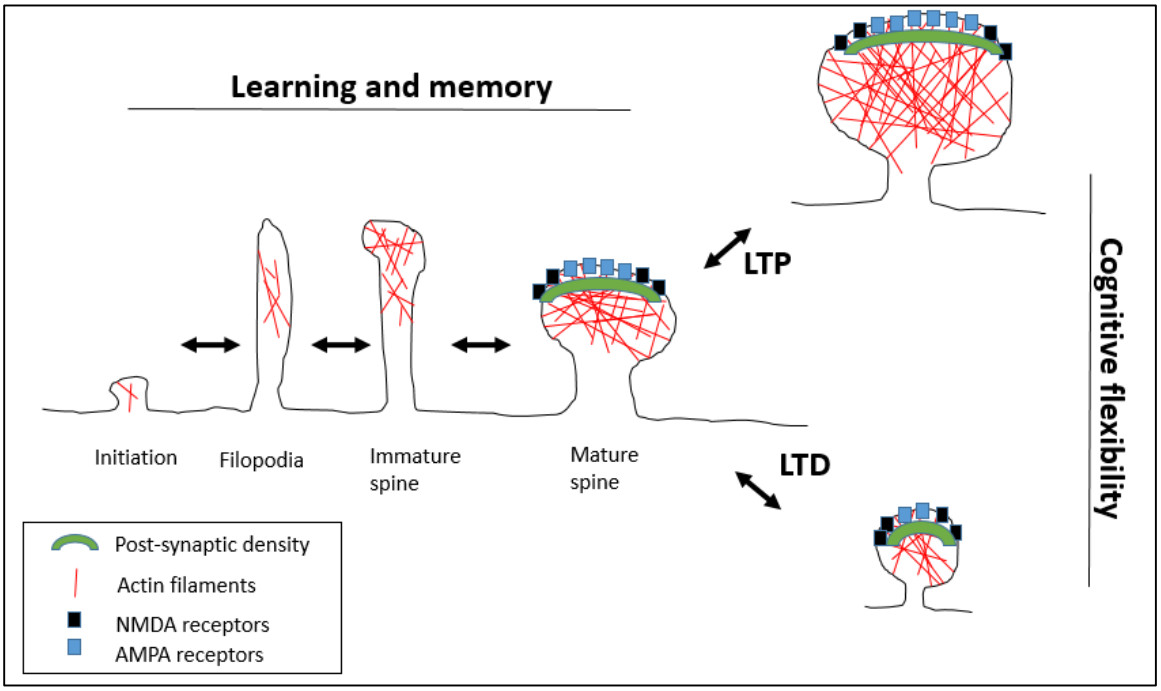

Figure 2. Spine development and dynamics are associated with synaptic plasticity and cognitive functions.

The neurotransmitter glutamate plays a critical role at the synaptic cleft of hippocampal area CA1. Induction of LTP is dependent on glutamatergic AMPA and NMDA receptors (Bashir et al, 1993). In the hippocampus, high frequency stimulation of area CA3 Schaffer-collateral pathway depolarizes the post-synaptic membrane of area CA1 synapses through high quantal release of glutamate. NMDA receptors, which conduct a fast, high calcium current when open, bind glutamate as well, but do not open due to occlusion by magnesium (Blanke and VanDongen, 2009). Post-synaptic membrane depolarization, mediated by AMPA receptors, pushes out the magnesium and allows the secondary messenger calcium to enter the dendrite through the NMDA receptor. Calcium entry activates alpha calcium calmodulin kinase II (αCamKII), which is the first significant intracellular step that initiates LTP, and the morphological and functional changes to the dendritic spine (Lisman et al, 2012). A functional consequence following LTP induction is an increased AMPA receptor current due to additional AMPA receptors added to the post-synaptic density (Makino and Malinow, 2009). This scenario, in which depolarization and glutamate binding together but not individually can induce LTP, provides synaptic cooperativity and selectivity through the NMDA receptor that would satisfy aspects of Hebbian learning. A presumed function of the structural changes to dendritic spines is to support a larger post-synaptic density and greater number of

AMPA receptors. To support such changes to dendritic spine morphology would require remodeling of the underlying actin cytoskeleton.

Studies scrutinizing regulators of actin cytoskeleton dynamics have provided crucial insight into their role in brain development and function. Molecular switches that provide tight regulation of actin filament elongation, severing, and branching at the synapse are members of the subfamily of small Rho GTPases (**Figure 3**). Small Rho GTPases cycle between active (GTP-bound) and inactive (GDP-bound) states. At synapses, Rho GTPases are known to regulate actin cytoskeleton dynamics, a process that is integral to neuronal development, survival and synaptic plasticity (Luo, 2000). Rho GTPase activity therefore must be tightly regulated within spines to ensure that actin remodeling matches synaptic activity. Several factors determine the activity state of Rho GTPases. Guanine Nucleotide Exchange Factors (GEFs) catalyze exchange of GDP for GTP, thus activating Rho GTPases (Rossman et al, 2005). Binding of GTP to Rho GTPases induces conformational changes that permit interaction and activation of downstream effectors, particularly actin binding proteins (ABPs) (Cherfils and Zeghouf, 2013). Intrinsic GTPase activity is much too slow to provide adequate temporal inhibition of Rho proteins. Therefore, GTPase Activating Proteins (GAPs), accelerate GTPase activity that returns Rho GTPases to the inactive, GDP-bound state (Cherfils and Zeghouf, 2013). Small Rho GTPases are also post-translationally modified with a lipid tail

that is added to the cysteine of the C-terminal CAAX motif (Roberts et al, 2008). The lipid tail allows Rho GTPases to incorporate into the plasma membrane. Rho GDP-dissociation inhibitors (Rho GDIs) bind to the lipid tail and prevents membrane binding (Garcia-Mata et al, 2011). Control of membrane binding localizes Rho GTPase activity within specific membrane domains and focuses actin cytoskeleton remodeling. This action is important for polarization of neurons during development of axons and dendrites as well as synaptogenesis (Luo, 2000; Gonzalez-Billault et al, 2012).

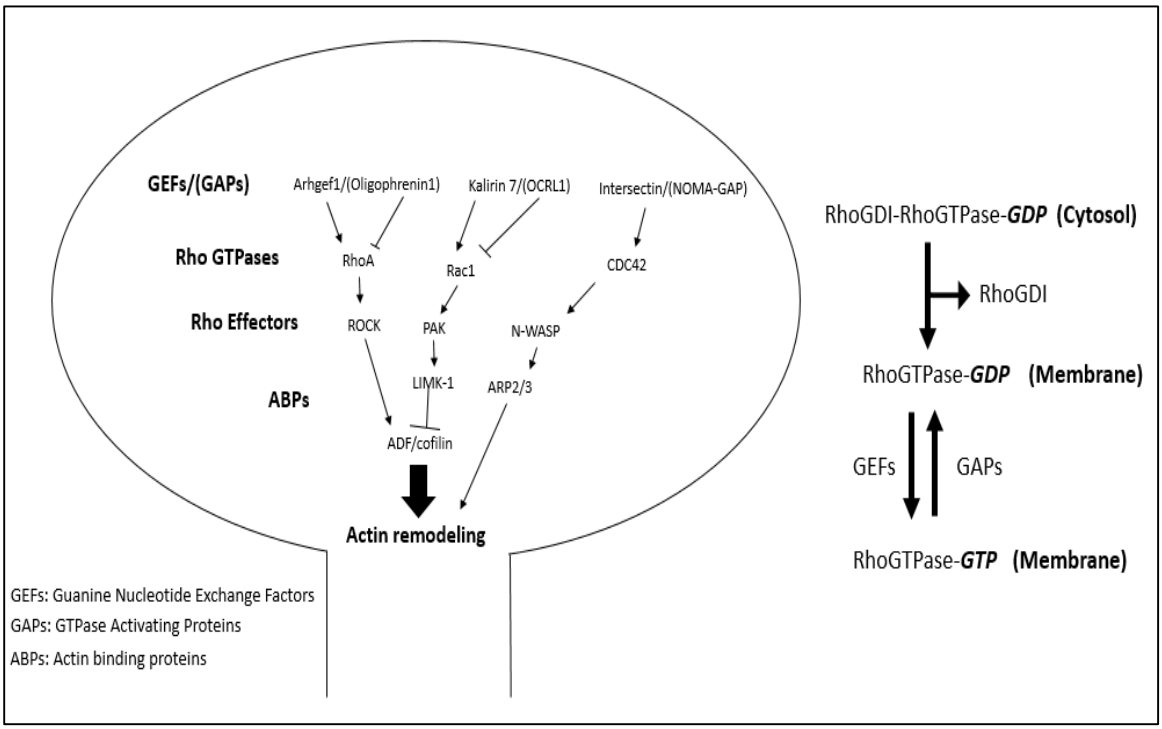

Figure 3. Actin remodeling is regulated by small Rho GTPases at the synapse.

Morphological studies performed on post mortem brain tissue of individuals with ID have consistently revealed impairments in dendritic spine shape and number (Kaufmann and Moser, 2000). Furthermore, detailed examination of genes associated with ID continue to return the focus on regulators of the actin cytoskeleton (**Table 1**; Tassabehji et al, 1996; Billuart et al, 1998; Faucherre et al, 2005; Margolis et al, 2010; Bacon et al, 2013; Ellery, et al 2014). In addition to abnormal neuronal size in the hippocampus, dendritic spine pathology has recently been emerging as a pathophysiological hallmark in autism (Hutsler and Zhang, 2010; Tang et al, 2014). Only a small percentage of autism cases have an identified etiology (Bhat et al, 2014). However, a significant observation in behavioral therapies that aim to improve cognitive function is that deficits can be overcome. Given the role of the actin cytoskeleton in dendritic spine structure and regulators of actin dynamics in synaptic plasticity, absent a suitable cause, these factors are becoming attractive targets in the treatment of autism spectrum disorders.

Table 1. Intellectual disabilities consistently reveal direct or indirect links to regulators of the actin cytoskeleton.

Actin Regulator	Functional link to actin regulator	Reference
LIM Kinase	(downstream effector of Rac1/CDC42) promotes actin polymerization. Mutated or missing in **Williams syndrome** leading to global cognitive impairment (mean IQ 55)	Tassabehji et al, 1996
Oligophrenin1	(GAP for RhoA) mutated in **X linked mental retardation**	Billuart et al 1998
PAK3	(downstream effector of Rac1/CDC42) promotes actin polymerization. Mutated in **X-linked mental retardation**	Allen et al, 1998
ARHGef6	(also known as αPIX, is a GEF for Rac1 and CDC42) gene mutated in **X-linked mental retardation**	Kutsche et al, 2000
MEGAP	(GAP for Rac1) gene mutated after translocation breakpoint in **3p- syndrome**	Endris et al, 2002
FMRP	(in **Fragile X syndrome**) along with Rac1/CYFIP complex mediates actin nucleation	Schenck et al, 2003
OCRL1	(GAP for Rac1) mutated in **Lowe syndrome**, X-linked mental retardation	Faucherre et al, 2005
CDKL5	Cyclin-dependent kinase-like 5. Gene mutated in **Rett syndrome**; forms complex with Rac1 in developing neurons	Chen et al, 2010
Ube3a	(ubiquitin ligase; mutated in **Angelman syndrome**; severe mental retardation) controls degradation of Ephexin1 (RhoA GEF)	Margolis et al, 2010
Interleukin-1-receptor accessory protein like 1 (IL1RAPL1)	Localizes RhoGAP2, a Rac1 GAP, to post-synaptic density. mutated in **X-linked mental retardation**	Valnegri et al, 2012

3. MATERIALS AND METHODS

3.1 Animals

Mice were handled in compliance with the principles and procedures of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Guide Revised version 2011). Protocols for experimental tests were approved by the Institutional Animal Care and Use committee (IACUC) at the University of Houston. Mice used in these studies were at postnatal day 5, 5 weeks and 20 weeks of age and on the FVB background, *Fmr1* WT (wild type; stock No. 4828) and *Fmr1* KO (knockout; stock No. 4624) from The Jackson Laboratory (Bar Harbor, Me., USA). Previous studies have found that both male and female Fmr1 KO mice exhibit similar behavioral impairments (Baker et al, 2010; Ding et al, 2014; Nguy and Tejada-Simon, 2015). In this study, the extent of phenotypic impairment did not differ between male and female. Therefore, data from male and female mice were pooled. Mice were housed in groups of 2-5 per cage and kept in a 12:12 light:dark cycle with food and water *ad libitum*. Behavior experiments were carried out at the same time each day. For behavior experiments with inhibitor treatment, different cohorts were used. A single dose of freshly prepared N^6-[2-[[4-(Diethylamino)-1-methylbutyl]amino]-6-methyl-4-pyrimidinyl]-2-methyl-4,6-quinolinediamine trihydrochloride (NSC23766 Tocris, Ellisville, MO) (5mg/kg b.w. in

50µL 0.9% normal saline) was given by intraperitoneal route 2 hours prior to start of experiment. Controls were given 50µl 0.9% normal saline.

3.2 *Western blotting*

Whole hippocampus from Fmr1 WT and Fmr1 KO mice was collected for protein analysis by western blot. Hippocampi from mice of postnatal day 5, 5 weeks and 20 weeks of age were collected and washed with ice-cold PBS and transferred to ice-cold homogenizing buffer complete (HBC; 200 mM HEPES, 2.5 mM NaCl, 100 mM EDTA, 100 mM EGTA, 200 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM sodium orthovanadate, 10ug/ml leupeptin, 2ug/ml aprotinin, 1 µM microcystin-LR, and 200 nM calyculin A). Samples were then lysed by sonication (Branson Digital Sonifier, Danbury, CT) with 10x 0.1sec pulses set at lowest amplitude (10%). Protein concentration was determined by the Bradford method (Bradford, 1976) using a spectrophotometer (BioRad Laboratories). Equal amounts of protein were then loaded onto sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE, 12%) followed by electrophoretic blotting to a polyvinylidene difluoride (PVDF) membrane (0.45µm pore size), and incubated in Tris-buffered saline with Tween 20 (TTBS, 50 mM Tris-HCl [pH 7.5–8.0], 150 mM NaCl, and 0.1% Tween 20) containing 5% non-fat milk for 1 hour at room temperature. Blots were incubated with primary

antibodies overnight at 4°C in 5% non-fat milk (Rac1, 1:10.0000 from Millipore; total PAK1 and phosphorylated PAK1 1:1000; Na^+/K^+ ATPase 1:1000 from Cell Signaling). Blots were washed three times for 15 min in TTBS and then treated with corresponding secondary antibodies for 2 hours at room temperature (horseradish peroxidase-conjugated goat anti-mouse IgG [1:5,000] from Promega, Madison, WI for Rac1; anti-rabbit for total PAK1 and phosphorylated PAK1). This was followed by washing three times in 1 X TTBS and visualized using enhanced chemiluminescence (ImmunoCruz, Santa Cruz Biotechnology Inc, Santa Cruz, CA) and then exposed to autoradiography film for ~ 15 s to 1 min. Densitometric analysis of all samples was carried out using NIH ImageJ software.

3.3 Fractionation

A 200µg portion of each homogenate was centrifuged at 100,000*g* for 45 min using an air driven ultracentrifuge (Beckman Coulter, Palo Alto, CA). The supernatant (cytosolic fraction) was collected and the pellet (membrane fraction) was resuspended and sonicated (3x 0.1sec pulses set at lowest amplitude) in HBC. Protein concentration was determined by Bradford method for each fraction. Equal amounts of protein from each fraction were run on a 12% SDS-PAGE gel, followed by western blotting to determine

quantities of Rac1 in fractions relative to total homogenates. The purity of fractions was determined by probing for the integral membrane protein Na^+/K^+ ATPase.

3.4. Golgi-Cox staining

To determine neuronal morphology and dendritic spine density, whole brains were removed from 5 week old mice and immediately process by FD Rapid Golgi stain kit according to the manufacturers manual (FD Neurotechnologies, Columbia, MD). Mice were sacrificed and whole brains were carefully removed, briefly washed by ice cold milli-Q water and immersed into mixed impregnation solution (mixed 24 hours ahead of use) for 2 weeks stored in the dark at room temperature. Impregnation solution (Solutions A and B with milli-Q water; 1:1:2) was replaced twenty-four hours after initial immersion. Brains were then immersed in Solution C for one week at 4°C. Brains were then snap frozen in dry ice-cooled isopentane and stored at -80°C until ready for sectioning (less than 3 days). Brains were transferred to cryostat and allowed to acclimate to -25°C before sectioning. Brains were embedded in Tissue Tec Optimal Cutting Temperature (O.C.T.; Sakura Finiteck U.S.A, Inc Torrance, CA). Sections of 50-100µm were obtained by cryostat (Leica CM 1850, Leica Microsystems Inc., Bannockburn, IL) at -25°C and mounted set on gelatin coated slides. A drop of Solution C was added to each slice and allowed to air dry. Mounted slices were then washed briefly with milli-Q water followed by immersion in freshly prepared staining solution for ten

minutes. Staining was followed by milli-Q water 2x 4 minutes. Sections were them dehydrated in graded ethanols (50%, 75%, 95% 4 minutes each) followed by clearing in xylene 3x 4 minutes. Finally, slices were cover slipped in Permount. To image and quantify cell morphology, confocal microscopy was used. High-resolution confocal images were obtained using an Olympus BX51WI Microscope with Spinning Disk Confocal (DSU) and Hamamatsu ORCA-ER Deep Cooled Camera and images processed with StereoInvestigator and Neurolucida software (MBF, Williston, Vermont). Spines were examined from apical dendrites at stratum radiatum layer on pyramidal neurons from hippocampal CA1. Dendrites for quantification were randomly chosen for analysis. Oil lenses (60x [1.35 NA] and 100x [1.4 NA] were used to image fine structures.

3.5 Electrophysiology

3.5.1 Hippocampal slice preparation

Mice were sacrificed by cervical dislocation and brains immediately immersed in ice cold, carb-oxygenated (95%O_2, 5%CO_2) cutting solution (CS: 5 mM glucose, 110 mM sucrose, 60 mM NaCl, 28 mM $NaHCO_3$, 3 mM KCl, 1.25 mM NaH_2PO_4, 7 mM $MgCl_2$, and 0.5 mM $CaCl_2$, 0.6 mM ascorbate). Saggital slices (400um) of hippocampus were obtained by a 1000Plus Vibratome sectioning system (Vibratome Co., St. Louis, Missouri). Slices were then transferred to room temperature carb-oxygenated 1:1

CS:ACSF for 30 minutes (ACSF: 25mM glucose, 125mM NaCl, 2.5mM KCl, 1.25mM NaH2PO4, 25 NaHCO3, 1mM MgCl2, and 2mM CaCl2). Slices were then transferred to room temperature carb-oxygenated ACSF for at least one hour before transfer to a submersion chamber (Harvard Apparatus, Holliston, MS) for recording of field excitatory post-synaptic potentials (fEPSPs).

3.5.2 Baseline measurement and Input-Output curve

ACSF was perfused through the recording chamber at 1-2mL/min. A stimulating bipolar platinum wire was placed on Schaffer-collateral pathway in area CA3 and a borosilicate recording electrode (1-5MΩ) filled with ACSF was placed at stratum radiatum in area CA1. For each slice, an input-output curve was produced by applying increasing stimulating intensities from 0.5mV to 10mV at 0.5mV increments. Stimulus intensity per slice used during baseline recordings was set to values evoking 40-50% the maximum fEPSP magnitude.

3.5.3 Theta burst long term potentiation (TBS-LTP)

After a stable baseline was obtained for at least 15 minutes, long term potentiation was induced by single theta burst stimulation (TBS; 5 bursts of 4 pulses given at 100Hz and interburst interval of 200ms). As previously reported by (Lauterborn et al, 2007) a TBS pattern composed of 5 bursts delineates an LTP deficit in hippocampus of Fmr1 KO

mice. Field-EPSPs were collected once per two minutes and the initial slope was measured and normalized to the 10-minute baseline mean prior to theta-burst. Short-term LTP was induced by applying a single series of 50 pulses given at 50Hz. High frequency stimulation-LTP (HFS-LTP) was induced by applying 4 100Hz HFS stimuli with 1- minute inter-stimulus intervals.

For drug treatments, slices were incubated in ACSF or ACSF + 100µM NSC23766 for at least 1 hour prior to the start of recording and subsequently perfused with ACSF only while in the recording chamber. Two to three slices per mouse were used per condition.

3.5.4 DHPG-long term depression

After a stable baseline was obtained for at least 15 minutes, metabotropic glutamate receptor dependent-long term depression (mGluR-LTD) was induced by bath application of 100µM *(S)*-3,5-dihydroxyphenylglycine, DHPG (Tocris, Ellisville, MO) for 5 minutes in the presence of 100µM ((2*R*)-amino-5-phosphonovaleric acid, AP-5 (Tocris, Ellisville, MO).

3.5.5 Paired-pulse long term depression

After a stable baseline was obtained for at least 15 minutes, metabotropic glutamate receptor dependent-long term depression (mGluR-LTD) was induced by application of

900 pulse-pairs (20ms interpulse interval) delivered at 1Hz for 15 minutes in the presence of 100μM APV.

3.6 Behavioral tests

3.6.1 Fear Conditioning

Fear learning and memory was examined in mice ages 5 weeks and 20 weeks using a conditioning chamber with background light (15lux) and noise (70dB) (Med Associates). Mice received pairs of conditioning stimuli (tone) and unconditioned stimuli (mild foot shock) during training, followed by examination twenty-four hours later of long-term contextual and tone (cue) memory. Freezing behavior exhibited by mice reflects acquisition and recall of the aversive experience and was monitored and collected by infrared camera by Med Associates Freeze software. Testing chambers were cleaned thoroughly with 70% ethanol between uses. Two separate fear conditioning protocols were carried as described below.

3.6.1.1 Delay-Fear Conditioning

To confirm an associative learning and memory deficit phenotype in the Fmr1 KO, mice underwent delay fear conditioning. In delay-FC, the tone co-terminates with the foot shock in a task which engages the hippocampus and amygdala. Mice were given 3 tone

shock pairs. Mice were put individually into a conditioning chamber and allowed to explore for 2 minutes after which tone-shock pairs were presented with a one-minute inter-pair interval. Duration of the tone was 30 seconds at 90dB, 5kHz and the shock (0.7mA) was delivered during the final 3 seconds of the tone. One minute of recovery followed the final tone-shock pair. Contextual memory was tested twenty-four hours later by returning mice to the conditioning chamber during which freezing behavior was monitored for seven minutes. Cue-dependent memory was tested 2 hours later by modifying the conditioning chamber wall, floor and odor (single application of 30% ascetic acid to wall) and measuring freezing behavior during a three-minute presentation of the tone.

To determine the extent of a learning impairment in Fmr1 KO mice after delay-fear conditioning, three levels of training intensities were conducted. Different cohorts received 1, 3 or 6 pairs of unconditioned stimuli (foot-shock) and conditioned stimuli (tone). Contextual memory was tested twenty-four hours later by returning mice to the conditioning chamber during which freezing behavior was monitored for seven minutes. Cue-dependent memory was tested 2 hours later by modifying the conditioning chamber wall, floor and odor (single application of 30% ascetic acid to wall) and measuring freezing behavior during a three-minute presentation of the tone.

To study changes in the hippocampus after delay-fear conditioning, mice underwent delay-fear conditioning with 3x or 6x tone-shock pairs as described above. Control mice were exposed to the chamber for seven minutes without tone or shock. One hour after training, mice were euthanized and whole hippocampus was collected and processed for western blot as described above.

For drug treatment studies to determine rescue of deficits observed, a single dose of NSC23766 (5mg/kg b.w. in 50µL 0.9% normal saline) was given by intraperitoneal route 2 hours prior to training. Controls were given 50µl 0.9% normal saline. Twenty-four hours later contextual and tone-memory were tested as described above.

To study changes associated to TBS-LTP after delay-fear conditioning, mice underwent delay-fear conditioning with 3x or 6x tone-shock pairs. One hour after training, mice were euthanized and hippocampi prepared for electrophysiology as previously described. TBS-LTP with 5 bursts was induced as described above. Control mice were exposed to the training chamber for 7 minutes then processed for TBS-LTP one hour later.

To study morphology changes in dendritic spines after delayed-fear conditioning, mice underwent delay-fear conditioning with 3x or 6x tone-shock pairs. One hour after training, mice were euthanized and 300µm hippocampi were isolated in ice-cold phosphate buffered saline and immediately processed with a slice Golgi kit (BioEnno

Tech, Santa Ana, CA). In 6-well culture plates slices were placed into fresh fixative overnight at room temperature and stored in the dark. Slices were then incubated in fresh impregnation solution for 3 days stored in the dark. After impregnation, slices were washed briefly with dH2O followed by 3x 5min washes with PBS-T. Slices were afterward transferred to staining solution C (5 minutes), a brief PBS-T wash, and post-staining solution D (3 minutes), concluding with 4x 5 minute washes in PBS-T. Slices were then mounted in pairs on gelatin-coated slides and allowed to air-dry. Dehydration was carried out by graded ethanols (25%, 50%, 75% and 100%) 5 minutes each. Slices were then cleared by xylene 3x 5 minutes. Each slice was individually cover slipped in Permount mounting medium.

3.6.1.2 Trace-Fear Conditioning

For trace-fear conditioning, experiments were carried out as described before (Zhao et al, 2005), however instead of 10 tone-shock pairs, mice received 6 tone-shock pairs presented with a 210 second inter-pair interval. A trace of 30 seconds separated the tone from its respective shock. Duration and intensity of tone and shock were the same as given during delay-fear conditioning.

For drug treatment studies to determine rescue of deficits observed, a single dose of NSC23766 (5mg/kg b.w. in 50µL 0.9% normal saline) was given by intraperitoneal route 2 hours prior to training. Controls were given 50µL 0.9% normal saline. Twenty-four hours later contextual and tone-memory were tested as described above

3.6.2 Hotplate analgesia test

Mice were individually placed onto a hotplate analgesia meter (Columbus Instruments, Ohio) set at 55°C while being closely monitored for response (paw licking or flinching). Latency to response was recorded manually and mice were removed immediately and returned to their home cage. The chamber was cleaned with 70% ethanol between mice. Pain is the aversive stimulus during fear conditioning tests that use a foot shock.

3.6.3 Hotplate analgesia test after Rac1 inhibitor treatment

To test pain sensitivity upon drug treatment, mice were injected with a single dose of 5mg/kg b.w. NSC23766 or saline (total volume 50 µL) by I.P. and placed two hours later onto the hotplate. Test was then performed as described above.

3.6.4 Acoustic startle and prepulse inhibition

Measurements of prepulse inhibition and acoustic startle response were carried out using the SR-Lab system (San Diego Instruments). The testing chamber consisted of a sound attenuated cabinet (28x28x30cm;) fitted with a speaker, house light (15 lux) and fan (65dB). Mice were placed into an acrylic cylinder connected to a motion sensor. Flinch reflexes were transmitted to a personal computer with SR-Lab software. To measure pre-pulse inhibition, after a 5-minute acclimation period mice were presented with five pre-pulse and pulse pairs in pseudo-random order. Pre-pulses were set at 74, 78, 82, 86 and 90dB; pulse level was set at 120dB. Responses from pulse-only sessions were averaged to determine acoustic startle response.

To test changes to prepulse inhibition/acoustic startle reflexes after drug treatment, mice were injected intraperitoneally with a single dose of NSC23766 (5mg/kg b.w.) or saline (total volume 50 µL). Test for PPI and acoustic startle response was carried out as described before.

3.6.5 Morris water maze

The Morris water maze was employed to assess spatial learning and memory. The pool was filled with water and allowed to reach room temperature before use. White tempera paint was added and mixed thoroughly to provide contrast for tracking by overhead camera. Swim patterns were tracked automatically by Noldus EthovisionXT 5.1 software (Noldus Information Technology). The pool was divided by tracking software into four quadrants, one of which contained the hidden escape platform 1cm below the water surface. Mice were transferred to the testing room and allowed to acclimate for at least 30 minutes. Mice were placed individually in holding cages with a paper towel. Each mouse was allowed 15 seconds for familiarization with the pool and platform and promptly returned to the holding cage. Training consisted of placing the mouse at one of four locations at the edge of the pool. Mice were given a maximum of one minute per trial to locate the platform. Large distal cues were place on each of the four walls surrounding the pool. If the mouse did not locate the pool at the end of the one-minute trial, the mouse was guided to the platform and held there for 10 seconds before transferring to holding cage. Mice that reached the platform under one minute were given 10 seconds on the platform before transferring to the holding cage. The latency to reach the platform was recorded to assess ongoing rate of learning of four days of training. Four trials were given on day 1, three trials on day 2 and two trials on

days 3 and 4 (inter-trial intervals of 30 minutes). On day 5, a probe test was conducted by removing the platform and allowing the mice to swim for one minute. The amount of time spent in each quadrant was measured to assess spatial memory performance.

3.7 Data Analysis

Spines were examined from apical dendrites at stratum radiatum layer on pyramidal neurons from hippocampal CA1. One dendrite per mouse was randomly chosen for spine analysis. A dendritic segment was magnified to 100x and calibrated for measurement. Total spines were quantified along a 30um dendrite segment. Protrusions were classified as spines if lengths were less than 7µm (Papa et al, 1995; Ballesteros-Yanes et al, 2006). Spines were classified as immature if length was greater than 1.5µm (Comery et al, 1997).

Immunoblots were incubated for one minute in enhanced chemiluminescent (ECL) reagent and then exposed to blue autoradiograph film. Desk top scanner was used to scan exposed film followed by analysis with NIH ImageJ software. Bands were normalized to loading control and averaged.

For analysis of behavior results, means were used to compare between wild type and Fmr1 KO during characterization. In treatment experiments, means were compared by one-way ANOVA with *post-hoc* Bonferroni correction when required.

For electrophysiology experiments, the four time points following LTP induction were averaged for each slice. Means of slices from each group were compared by Student's *t-test*. The final four time points after TBS-LTP were also compared between groups.

Data are presented as mean ±sem. Means were compared by Student's *t*-test to compare two means or one-way ANOVA followed by Bonferroni *post hoc* test when required. *p* values of less than 0.05 were designated as significant.

4. RESULTS

PHENOTYPIC CHARACTERIZATION OF FXS ANIMAL MODEL

4.1 Fmr1 KO mice express increased thin dendritic spine density in hippocampus area CA1

Previous reports have found an increase in immature dendritic spine density in Fmr1 KO mice (Grossman et al, 2006) with a specific impairment in pruning and maturation of spines (Cruz-Martin et al, 2010). To confirm the reported dendritic spine phenotype in the Fmr1 KO mouse hippocampus, brains were processed with the FD-Rapid Golgi staining kit as described in materials and methods section. In area CA1 of the hippocampus no differences in spine density between wild type and Fmr1 KO were observed (Figure 4b) which may lead to impaired synaptic transmission. This finding was similar to previous studies that did not detect abnormal spine density in hippocampus from young Fmr1 KO mice (Grossman et al, 2006). However, Fmr1 KO mice expressed an increased number of immature, thin dendritic spines on pyramidal neurons of hippocampal area CA1 (Figure 4b).

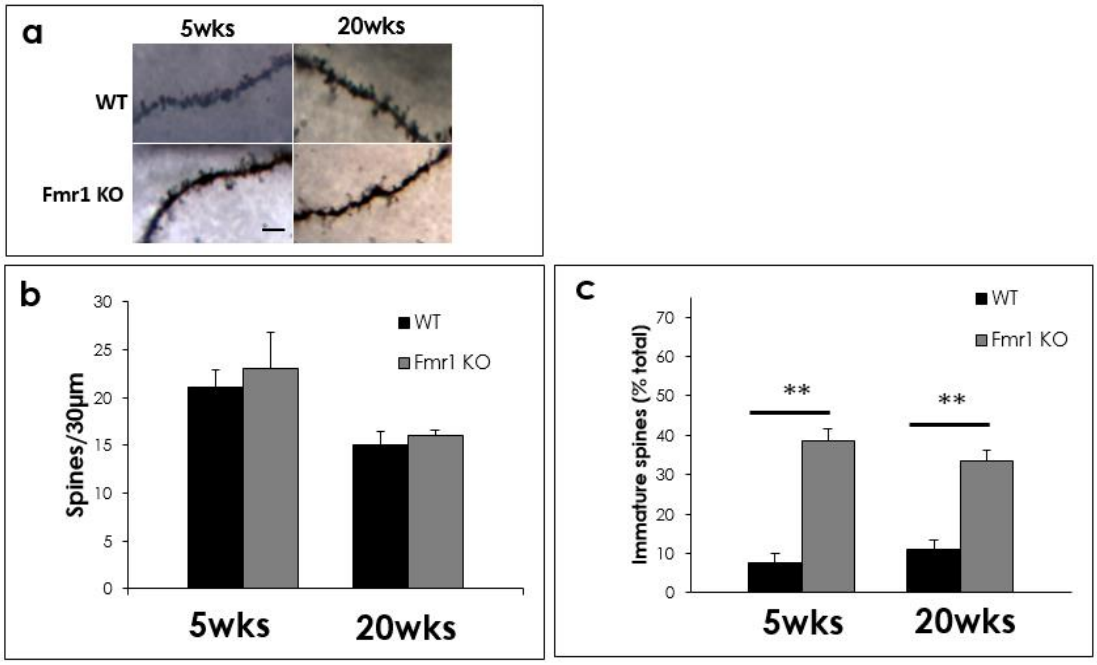

Figure 4. ***Fmr1 KO mice express increased thin dendritic spine density in hippocampal area CA1.*** Golgi stained hippocampal slices were examined for dendritic spine density in area CA1 pyramidal neurons at 5 and 20 weeks of age. (a) Representative images from area CA1 basal dendrites from pyramidal neurons at the stratum radiatum layer. (b) No difference was found in total dendritic density between wild type and Fmr1 KO (WT 5wks: 21±1.8, Fmr1 KO 5wks: 23±3.8; WT 20wks: 15±1.4, Fmr1 KO 20wks: 16±0.5). (c) Compared to wild type, Fmr1 KO mice expressed a higher density of immature, thin spines on apical dendrites of hippocampal area CA1 pyramidal neurons (WT 5wks: 7.7±2.2%, Fmr1 KO: 5wks: 38.4±3.2%; WT 20wks: 10.8±2.6%, Fmr1 KO 20wks: 33.5±2.6%). n=4 dendrites from 4 mice. Data represent mean ±sem. **p<0.01, Student's *t*-test. Scale bar is 5µm.

4.2 Fmr1 KO hippocampus area CA1 exhibits impaired TBS-LTP

A robust form of long term potentiation can be induced in hippocampal slices by applying bursts of pulses delivered at frequencies that mimic physiological firing patterns (i.e. 5Hz) (Larson et al, 1986). Theta burst stimulation is modeled after the theta rhythm, which was first detected as prominent local field potentials in rodents actively exploring a novel environment (Vanderwolf, 1969; Oddie and Bland, 1998). Area CA1 of the Fmr1 KO hippocampus exhibits an LTP deficit specifically induced by theta burst stimulation but not other patterned stimulation (Figure 5). This deficit is revealed under low stimulation intensity (Lauterborn et al, 2007; Lee et al, 2011; Boda et al, 2014). By reducing the number of bursts traditionally used from 10 to 5 theta bursts, Fmr1 KO exhibit a significantly lower magnitude of LTP compared to wild type (Figure 5a and 5d). To confirm the specificity of this form of LTP deficit, LTP was induced by different stimulation protocols. Delivering 50 pulses at 50Hz to induce short-term LTP did not reveal a difference in LTP magnitude between wild type and Fmr1 KO (Figure 5b and 5e). Induction of LTP by 4 pulses of high frequency stimulation (100Hz) produced similar robust LTP in slices from both genotypes. These results demonstrated the specificity of the theta frequency induced-LTP deficit (Figure 5b and 5c).

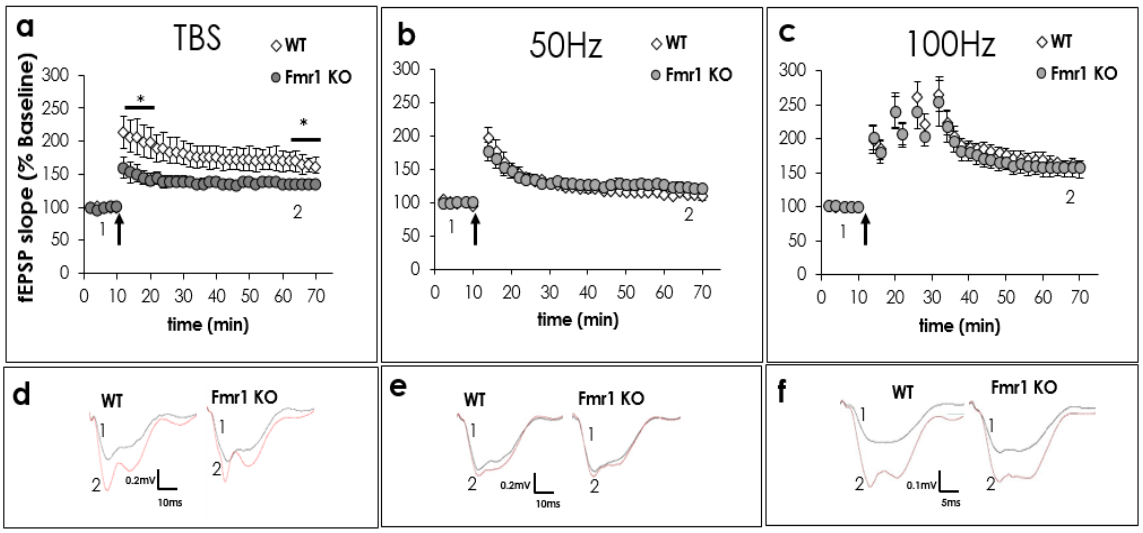

Figure 5. ***Fmr1 KO hippocampal area CA1 exhibits impaired TBS-LTP*****.** Theta Burst Stimulation-LTP was induced in area CA1 of acute hippocampal slices (black arrow; each burst is composed of 4 pulses given at 100Hz, inter-burst interval is 200ms). (a) Slices derived from Fmr1 KO exhibited a significant deficit in TBS-LTP induction (WT: 213±25.1%, Fmr1 KO: 159.7±15.9%) and maintenance (WT: 163.3±12.5%, Fmr1 KO:135.7±4.4%). (b) No difference was observed when LTP was induced by 50Hz (WT: 108±5.6%, Fmr1 KO: 119±4.7%. (C) LTP induced by 4 high frequency stimuli (100Hz, 1-minute inter-stimulus-interval) was not different between genotypes (WT: 158.8±13.1%., Fmr1 KO: 155.8±12.8%) (d, e, f) Sample traces from each LTP experiment indicating baseline time point (1) and post-LTP induction time point (2) labeled in the graph. Comparison between genotypes of the first four time points after LTP-induction. The final 4 time points after LTP-induction were also compared between genotypes. n=14 slices, 2 slices/mouse, 7 mice. Data represent mean ±sem.; * $p<0.05$ Student's *t*-test.

4.3 Fmr1 KO hippocampal area CA1 exhibits normal DHPG-LTD

Area CA1 of Fmr1 KO hippocampus has also been found to exhibit exaggerated metabotropic glutamate receptor-dependent long term depression (mGluR-LTD) (Huber et al, 2002; Choi et al, 2011). The exaggerated mGluR-LTD has been reported to result from excessive endocytosis of AMPA receptors (Nakamoto et al, 2007). mGluR-LTD in Fmr1 KO hippocampus is also believed to underlie the observed abundance of immature dendritic spines since LTD promotes spine shrinkage (Zhou et al, 2004; Wigert and Oertner, 2013). To determine the presence of LTD impairments, mGluR-LTD was induced by a brief application of DHPG, a group-5 mGluR agonist. Since LTD can also be induced by activation of NMDA receptors, occlusion by NMDA receptor-dependent LTD was prevented by concurrent application of APV, an NMDA receptor blocker. Fmr1 KO mice did not exhibit impaired metabotropic glutamate receptor dependent-LTD induced by DHPG (Figure 6a). Both wild type and Fmr1 KO hippocampus displayed synaptic depression to the same level. mGluR-LTD can also be induced by delivering low-frequency stimulus trains of paired pulses (PP-LFS). LTD by PP-LFS was induced to the same extent in both wild type and Fmr1 KO hippocampal slices (Figure 6b). mGluR-LTD impairments in Fmr1 KO hippocampus may be minor or may only be revealed in the presence of protein synthesis inhibitors (Nosyreva et al, 2006; Ronesi et al, 2012).

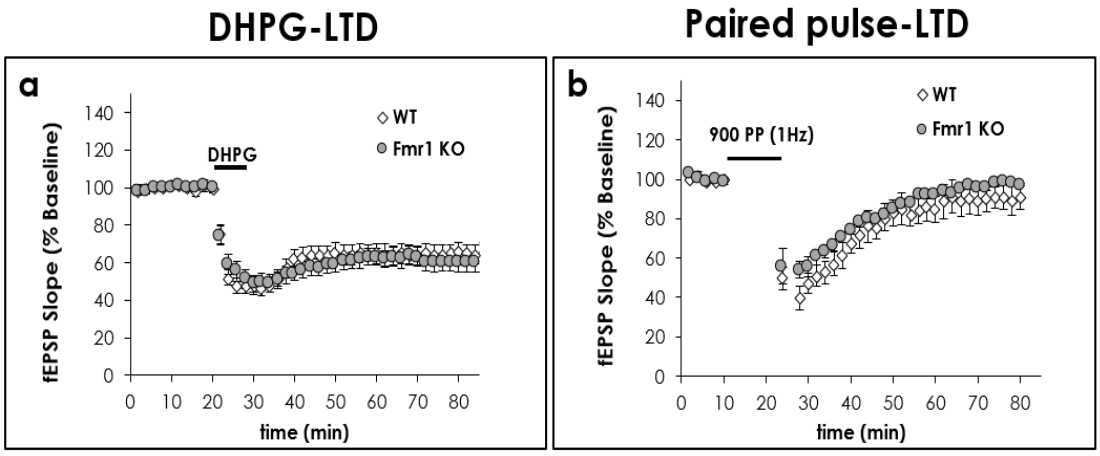

Figure 6. ***Fmr1 KO hippocampal area CA1 exhibits normal DHPG-LTD.*** A commonly cited phenotype in hippocampus from Fmr1 KO mice is enhanced mGluR-dependent LTD. However, the exaggerated LTD has been reported to be inconsistent across several studies. Therefore, it was necessary to determine its presence in the current study. (a) mGluR-dependent LTD was induced in acute hippocampal slices by incubation with the group 5 mGluR agonist DHPG for 5 minutes (horizontal black bar). No difference was detected in induction or maintenance of LTD induced by DHPG (WT: 64.4±5.3%, Fmr1 KO:59.6±4.8%). (b) Inducing mGluR-dependent LTD by 900 paired-pulses at 1Hz for 15 minutes with interpulse interval of 40ms (black arrow) also did not reveal a difference between wild type and Fmr1 KO (WT: 90.7±6.0%, Fmr1 KO: 97.2±0.3%. n=10-14, 2 slices/mouse, 5-7 mice. Data represent mean ±sem.

4.4 Fmr1 KO mice exhibit impaired fear conditioning (FC)

Fear conditioning was used to examine impaired associative learning in Fmr1 KO mice. During training, mice learn to associate an aversive, unconditioned stimulus (mild foot shock) with the training environment (context) or a cue (tone), the conditioning stimuli. The timing of the unconditioned stimulus (US) with the conditioning stimulus (CS) is an important factor that determines which areas of the brain are engaged and how the stimuli are processed for memory storage (Davis et al, 1989; Bevins et al, 1995; Misane et al, 2005; Maren, 2011). Fmr1 KO mice exhibit synaptic plasticity impairments in various areas important for fear learning such as the prefrontal cortex (Zhao et al, 2005), amygdala (Olmos-Serrano et al, 2011) and hippocampus (Zhao et al, 2005; Deacon et al, 2015; Oddi et al, 2015; Tian et al, 2015). By changing the timing between US and CS, these areas can be engaged and assessed separately. During *delay-fear conditioning*, US and CS overlap as a tone is presented along with a mild foot shock given during the final three seconds of the tone. This associative learning process depends on the amygdala and the hippocampus (Bast et al, 2003). During *trace-fear conditioning*, a short interval of 5 to 30 seconds separates the US from the CS. This associative learning process engages the frontal cortex (Runyan et al, 2004). In this study, one day after training, memory was tested by examining the fear response (freezing behavior) to the training context or the tone.

4.4.1 Fmr1 KO mice exhibit impaired delay-FC

During delay fear conditioning, a 30 second tone is presented and co-terminates with a 3 second foot-shock. Two separate forms of memory can afterward be assessed: contextual and cue memory (Curzon et al, 2009). Contextual memory, which depends on the hippocampus and amygdala, is tested by returning mice to the training environment. Cue memory, which relies heavily on the amygdala, can be assessed by putting mice into a novel environment where the tone is replayed. Fmr1 KO mice have been reported to express deficits in contextual memory (Deacon et al, 2015; Oddi et al, 2015; Tian et al, 2015).

In our study, 5 week old Fmr1 KO mice expressed normal exploratory (baseline) behavior when first introduced into the training context, but significantly reduced freezing behavior compared to WT after the last tone-shock pair (Figure 7a), suggesting a weaker acquisition of the aversive experience. The following day, when returned to the training chamber, Fmr1 KO mice exhibited significantly reduced contextual memory (Figure 7b). Additionally, when the context was modified, Fmr1 KO mice expressed similar baseline behavior compared to wild type but exhibited significantly less freezing behavior than WT mice during the presentation of the tone (Figure 7c). After the tone is turned off, wild type behavior returns to baseline levels, indicative of the strong influence of tone on freezing behavior. This effect is also observed in the Fmr1 KO mice.

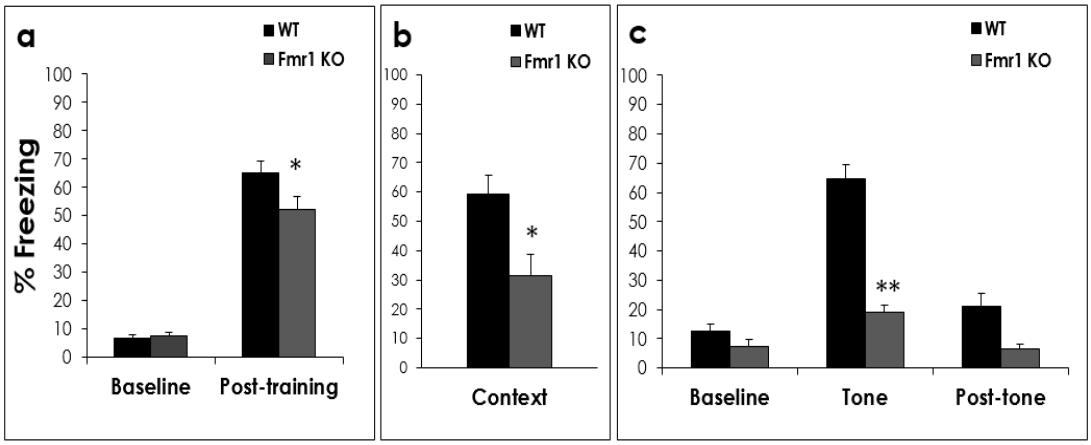

***Figure 7. Fmr1 KO mice exhibit impaired delay-FC at 5 weeks of age*.** (a) No difference is observed in exploratory behavior prior to conditioning (Baseline; WT: 6.9±1.1%, Fmr1 KO: 7.4±1.2%). Significantly less freezing behavior was measured in Fmr1 KO after the end of training (Post-training; WT: 65.1±4.1%, Fmr1 KO: 52.2±4.4%). (b, c) Twenty-four hours later, contextual memory (b, context; WT: 59.32±6.44%, Fmr1 KO: 31.2±7.2%) and cue memory (c, tone, WT: 64.7±4.6%, Fmr1 KO: 18.9±2.4%) deficits were measured in the Fmr1 KO mice. n=15. Data represent mean ±-sem. *p<0.05; **p<0.01. Student *t*-test.

The FVB strain of Fmr1 KO mice used in this study becomes susceptible to seizures induced by loud sound after 10 weeks of age (Chen and Toth, 2001). Hippocampal slices from Fmr1 KO mice have been shown to exhibit epileptiform activity (Chuang et al, 2005), which could potentially exacerbate hippocampus-dependent fear conditioning (Mao et al, 2009; Holley and Lugo, 2016). We examined delay fear conditioning at 20 weeks of age since Fmr1 KO mice of the FVB strain exhibit a high incidence of seizures at this age (Chen and Toth, 2001).

In the present study, relative to WT mice, Fmr1 KO mice at 20 weeks of age still exhibited a deficit in fear learning (Figure 8a). Contextual memory was still deficient in Fmr1 KO mice at 20 weeks of age (Figure 8b). Furthermore, relative to 5 week old Fmr1 KO mice, 20 week old Fmr1 KO mice did not exhibit worsened associative fear learning but rather a mild improvement in contextual memory (Figure 8b). This age-dependent improvement in contextual memory was also seen in the WT groups. In a novel environment baseline behavior was significantly different between wild type and Fmr1 KO mice at 20 weeks of age. Fmr1 KO mice exhibited significantly lower freezing behavior compared to wild type of 20 weeks of age and Fmr1 KO of 5 weeks of age. Although baseline behavior during training did not differ between genotypes, Fmr1 KO mice are reported to exhibit hyperactivity behavior in open-field tests (Mineur et al, 2002; Spencer et al, 2005), which may have been masked after the tone was played.

Although there is a mild increase in freezing behavior exhibited by 20 week old Fmr1 KO mice compared to 5 week old KO, cue memory was still significantly deficient compared to wild type (Figure 8c). These results indicate that, at 20 weeks of age, Fmr1 KO mice still express learning and memory deficits. Furthermore, mild developmentally-dependent improvements do not considerably impact memory deficits in Fmr1 KO mice.

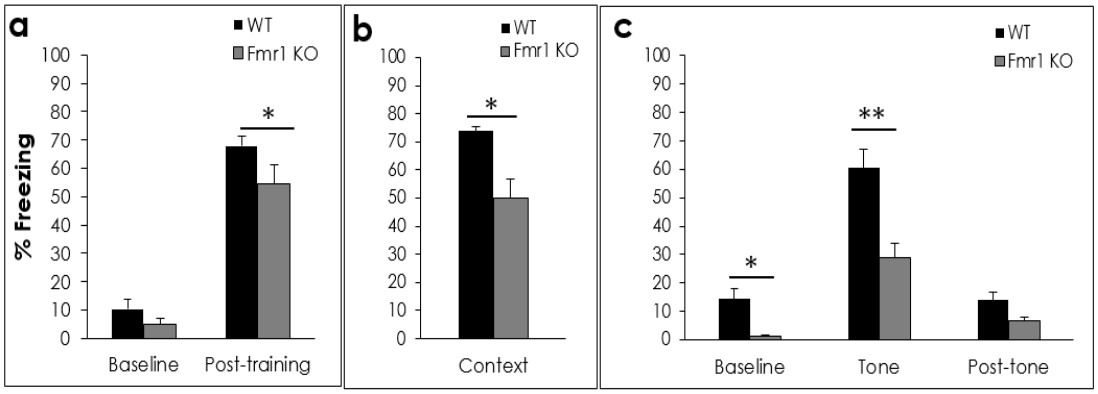

Figure 8. ***Fmr1 KO mice exhibit impaired delay-FC at 20 weeks of age*****.** (a) No difference was observed between genotypes in exploratory behavior (Baseline; WT: 10.3±3.3%, Fmr1 KO: 5.1±1.9%), but Fmr1 KO mice display significantly reduced freezing at the end of conditioning (Post-training; WT: 67.9±3.2%, Fmr1 KO: 54±6.6%). (b, c) Fmr1 KO mice exhibit reduced freezing behavior when tested for contextual (b; WT: 73.8±1.6%, Fmr1 KO:49.8±6.9%) and tone-memory (c; WT: 60±6.4%, Fmr1 KO: 28.9±5.0%). n=11-13. Data represent mean ±sem. *p<0.05; **p<0.01. Student's *t*-test.

4.4.2 Fmr1 KO mice exhibit impaired trace-FC

Fmr1 KO mice also exhibit memory deficiencies after trace-fear conditioning (Zhao, et al 2005), which requires the frontal cortex (Runyan, et al 2004; Han et al 2003) in addition to the hippocampus (McEchron, et al 1998; Bangasser et al, 2006; Yoon and Otto, 2007). A short interval between the tone and shock increases the requirement for attentiveness and allows assessment of cortical function. To provide a more direct comparison between delay fear conditioning and trace-fear conditioning with respect to the number of foot shocks, an attempt was made to carry our trace-fear conditioning with 3 tone shock pairs. However, mice did not exhibit freezing when the tone was replayed after undergoing this trace-fear conditioning protocol. This indicated that the interval also requires a minimum number of tone-shock pairs to be administered for the conditioned stimulus to predict the unconditioned stimulus.

In this study a total of six tone-shock pairs were given during trace-fear conditioning. Both genotypes expressed similar pre-training (baseline), post-training freezing behavior (Figure 9a) as well as contextual memory (Figure 9b). The absence of a contextual memory deficit may be explained by the difference in number of shocks. Mice received 3 shocks during the previous delay-fear conditioning, but received 6 shocks during trace fear conditioning. Increasing the number of shocks may be prolonging pain sensation

and may be strongly enhancing activation of fear centers, promoting stronger acquisition (Sacchetti et al, 1999; Baldi et al, 2004). Previous studies show similar graded freezing behavior after changing number of shocks (Maren, 1998). In parallel with acquisition, under these conditions contextual memory was also not revealed to be deficient in Fmr1 KO mice (Figure 9b). However, a highly significant deficit in tone memory still exists in Fmr1 KO mice after trace-fear conditioning (Figure 9c).

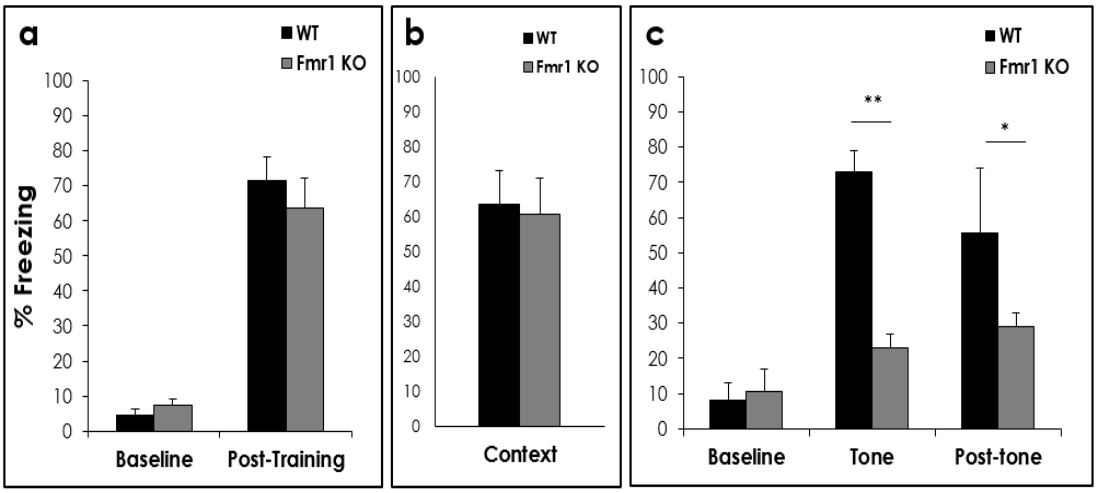

Figure 9. ***Fmr1 KO mice exhibit memory deficits after trace-FC.*** (a) Baseline exploratory behavior (WT: 4.3±1.9%, Fmr1 KO 7.3±1.6%) and post-training behavior (WT: 71.3±7.0%, Fmr1 KO 63.5±8.6%) was not different between wild type and Fmr1 KO mice indicating similar acquisition of the aversive experience. (b) Contextual memory was similar between wild type and Fmr1 KO (WT: 63.4±6.7%, Fmr1 KO 60.5±2.6%). (c) Exploratory behavior in a novel context did not differ between groups (WT: 7.9±5.2%, Fmr1 KO 10.7±6.3%). However, a significant difference in tone-induced freezing behavior was observed between genotypes (WT: 72.8±6.3%, Fmr1 KO 23.0±3.8%). Additionally, immediately following the end of the tone, wild type maintained a significant level of freezing compared to Fmr1 KO (WT: 55.4±18.4%, Fmr1 KO 29.1±3.9%). n=10 Data represent mean ±sem. *p<0.05; **p<0.01. Student's *t*-test.

4.5 Fmr1 KO exhibit decreased acoustic startle response and enhanced PPI

Prepulse inhibition (PPI) of startle is the attenuation of the startle response by a weaker pre-stimulus and occurs maximally with an interval of between 40-100ms (Norris and Blumenthal, 1996; Plappert et al, 2004). PPI is a purported filtering mechanism of background stimuli which permits focus on salient stimuli (Norris and Blumenthal, 1996; Braff et al, 2001). Studies show Fmr1 KO mice express enhanced PPI (Chen and Toth, 2001; Frankland et al, 2004) and a blunted acoustic startle response (Nielsen, et al 2003; Baker et al, 2010). PPI deficits may be related to dysfunctional brainstem auditory processing (Beebe et al, 2014; Rotschafer et al, 2015). Fmr1 KO mice used in this study also exhibit reduced startle response (Figure 10a). Fmr1 KO mice also exhibited significantly enhanced pre-pulse inhibition of the acoustic response after using 78, 82, 86 and 90db pre-pulse levels (Figure 10b). In this study, the tone used during fear conditioning was at 90dB. The level of acquisition during fear conditioning may be influenced by tone-induced over-filtering. Together with an inability to transmit tone-induced signaling accurately (Strumbos et al, 2010; Rotschafer et al, 2015), enhanced PPI may over-filter auditory stimuli and further degrade the magnitude of tone-specific signaling that reaches association areas.

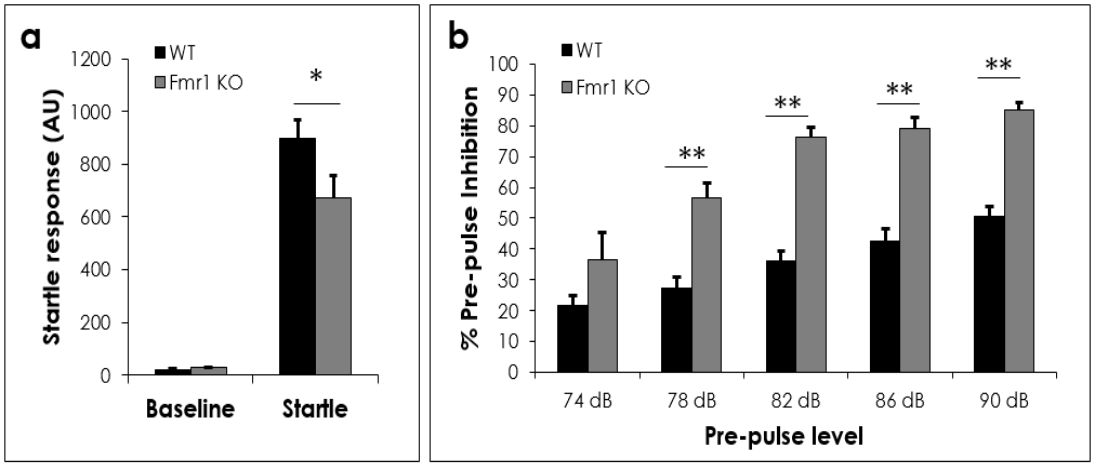

Figure 10. ***Fmr1 KO mice exibit decreased acoustic startle and enhanced PPI.*** (a) Baseline behavior within the acoustic startle chamber did not differ between groups (WT: 21.0±1.4, Fmr1 KO 27.6±2.7) but a startle response induced by a loud (120dB) pulse was significantly less in Fmr1 KO mice compared to wild type mice (WT: 899.9±70.1, Fmr1 KO: 671±87.0%). (b) Prepulse inhibition of the acoustic startle was significantly enhanced during the four higher prepulse levels in Fmr1 KO mice (WT: 74dB: 21.7±3.1%, 78dB: 27.3±3.7%, 82dB: 36.0±3.3%, 86dB 42.5±3.9%, 90dB: 50.7±3.0%; Fmr1 KO: 74dB: 36.5±8.8%, 78dB: 56.6± 4.7%, 82dB: 76.2±3.3%, 86dB: 79.1±3.6%, 90dB: 85.1±2.4%). n=10 Data represent mean ±sem. *p<0.05; **p< 0.01 compared to WT level. Student's *t*-test.

4.6 Fmr1 KO exhibit a mild spatial memory deficit

Since Fmr1 KO mice exhibited hippocampus-dependent learning and memory deficits after fear conditioning, mice were assessed using the Morris water maze for spatial memory, which is also dependent on hippocampus. Reports of Fmr1 KO mice on spatial memory performance are inconsistent (The Dutch Belgian Fragile X Consortium, 1994; Kooy et al, 1996; D'Hooge et al, 1997; Boda et al, 2014; Tian et al, 2015).

In our study, Fmr1 KO mice learned to locate a hidden platform at the same rate as wild type (Figure 11a). Both groups exhibited significantly reduced latencies between the first and last day of training in locating the platform. To test memory of platform location, mice were placed into the pool after the platform was removed. The amount of time spent searching for the platform in the target quadrant was less in Fmr1 KO mice compared to wild type, but did not reach statistical significance (Figure 11b). Both groups spent significantly more time in the target quadrant compared to other quadrants, indicating recall of the correct platform location. This was similar to studies by other laboratories which found only a mild spatial memory impairment in Fmr1 KO mice (D'Hooge et al, 1996). Discrepancies in learning and memory impairments between fear conditioning and spatial memory may be explained by motivation factors involving number of training days or differential levels of stress. Swim stress may provide an immediate and ongoing motivator to learn the location of the platform.

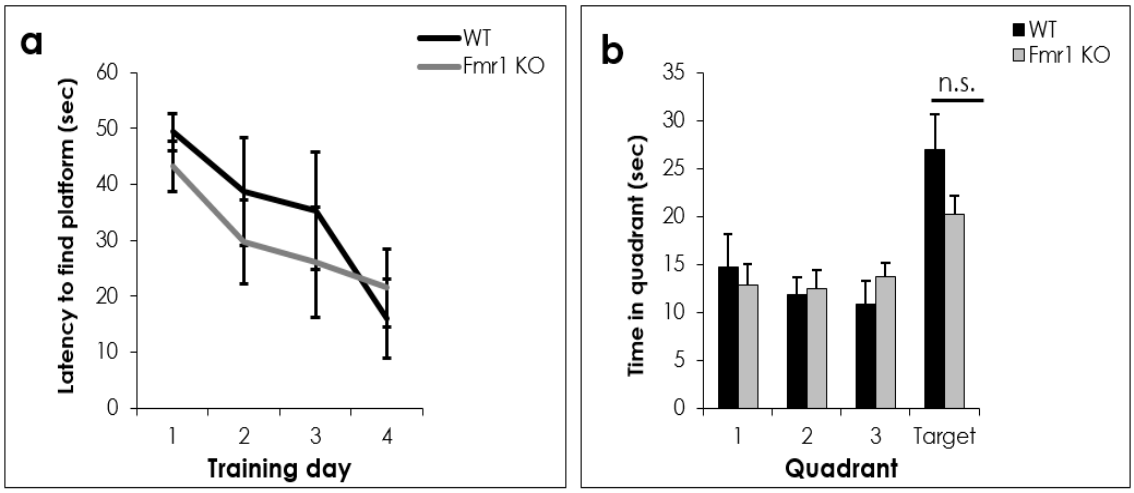

Figure 11. Fmr1 KO mice exhibit a mild spatial memory impairment. The Morris water maze was used to assess hippocampus-dependent spatial memory in Fmr1 KO mice. Training occurred over 4 days where mice learned the location of an escape platform in a pool of water by using spatial cues. (a) Fmr1 KO mice were able to learn the location of the hidden platform at the same rate as wild type mice. Both genotypes were able to significantly reduce the latency to locate the platform over the course of training. (b) A memory test consisted of removal of the platform and monitoring the amount of time mice spend in the platform quadrant (Target). A mild but non-significant spatial memory deficit was detected in Fmr1 KO mice indicated by less time spent in the target quadrant compared to wild type. n=10. n.s.= not significant $p>0.05$. One-way ANOVA.

EFFECT OF FXS ANIMAL MODEL ON RAC1

4.7 Rac1 localization in Fmr1 KO hippocampus is impaired across critical developmental stages

Rac1 undergoes isoprenylation due to its C terminal CAAX motif, a post-translational modification that targets Rac1 to the plasma membrane (Roberts et al 2009). A pool of inactive Rac1 exists in the cytosol where it is sequestered by RhoGDI, which binds to the isoprenyl group on Rac1 (Garcia-Mata et al, 2011). A major step in Rac1 activation is its translocation to the membrane after being dissociated from RhoGDI (Bustelo et al, 2012). In the hippocampus, migration between cytosol and membrane appears to be important during exposure to enriched environments (Yuan et al 2015) and fear learning and memory (Martinez and Tejada-Simon, 2007). Although high Rac1-GTP levels have been found in hippocampus of Fmr1 KO mice (Bongmba et al, 2011), it is not known what is the state of membrane bound Rac1.

Whole hippocampus was assessed for Rac1 levels, as well as distribution between cytosol and membrane upon sample fractionation. Given the dynamic nature of synapses and dendritic spines across development (Penzes et al, 2011) and the importance of Rac1 function in spinogenesis, whole hippocampus was examined at three developmental stages: postnatal day 5 (P5), 5 weeks and 20 weeks. Total Rac1 in

hippocampus appears similar in both WT and KO mice (Figure 12a). After fractionating the hippocampus into cytosol and membrane, Rac1 distribution in WT across the three ages reveals dynamic movement between fractions (Figure 12b). At P5 in WT mice, Rac1 is equally distributed between cytosol and membrane, but is found significantly higher in the cytosol at 5 weeks. At 20 weeks, there is a significant shift of Rac1 in WT hippocampus to the membrane fraction. In Fmr1 KO mouse hippocampi, Rac1 is found equally distributed between fractions the three ages tested (Figure 12b). These results suggest that faulty translocation of Rac1 is present in Fmr1 KO hippocampus. Comparing wild type and Fmr1 KO at 5 weeks reveals there is significantly more membrane-bound Rac1 in Fmr1 KO hippocampus. Increased membrane-bound Rac1 could perturb dendritic spine development by preventing proper actin remodeling after synaptic plasticity. Post-synaptic membrane reconstruction is associated with LTP maintenance (Geinisman et al, 1996; Toni et al, 2001), and therefore, Rac1 hyperactivity at the membrane may be contributing to the observed altered synapse and dendritic spine development. Rac1 has been shown to regulate addition of AMPA receptor to the synapse during spinogenesis (Wiens et al, 2005) and removal of AMPA receptors from the synapse after LTD (Benoist et al, 2013).

Two other Rho-GTPases that are structurally and functionally related to Rac1 (RhoA and CDC42) have been extensively studied in synaptogenesis and spinogenesis (Nakayama et

al, 2000; Schuster et al, 2015). In brain, Rac1, RhoA and CDC42 are isoprenylated by the same geranylgeranyl transferase (Roberts et al, 2008). It has also been demonstrated that impaired distribution of one Rho GTPase affects distribution of the others (Boulter et al, 2010). Examination of distribution of fractionated hippocampus revealed that RhoA and CDC42 were both found significantly higher in the membrane fraction at all ages and did not differ between genotypes (Figure 13 and 14). Overall, these results indicated impaired distribution of Rac1 in the Fmr1 KO mouse hippocampus which appears to be specific to just Rac1, and not a general effect on small Rho GTPases.

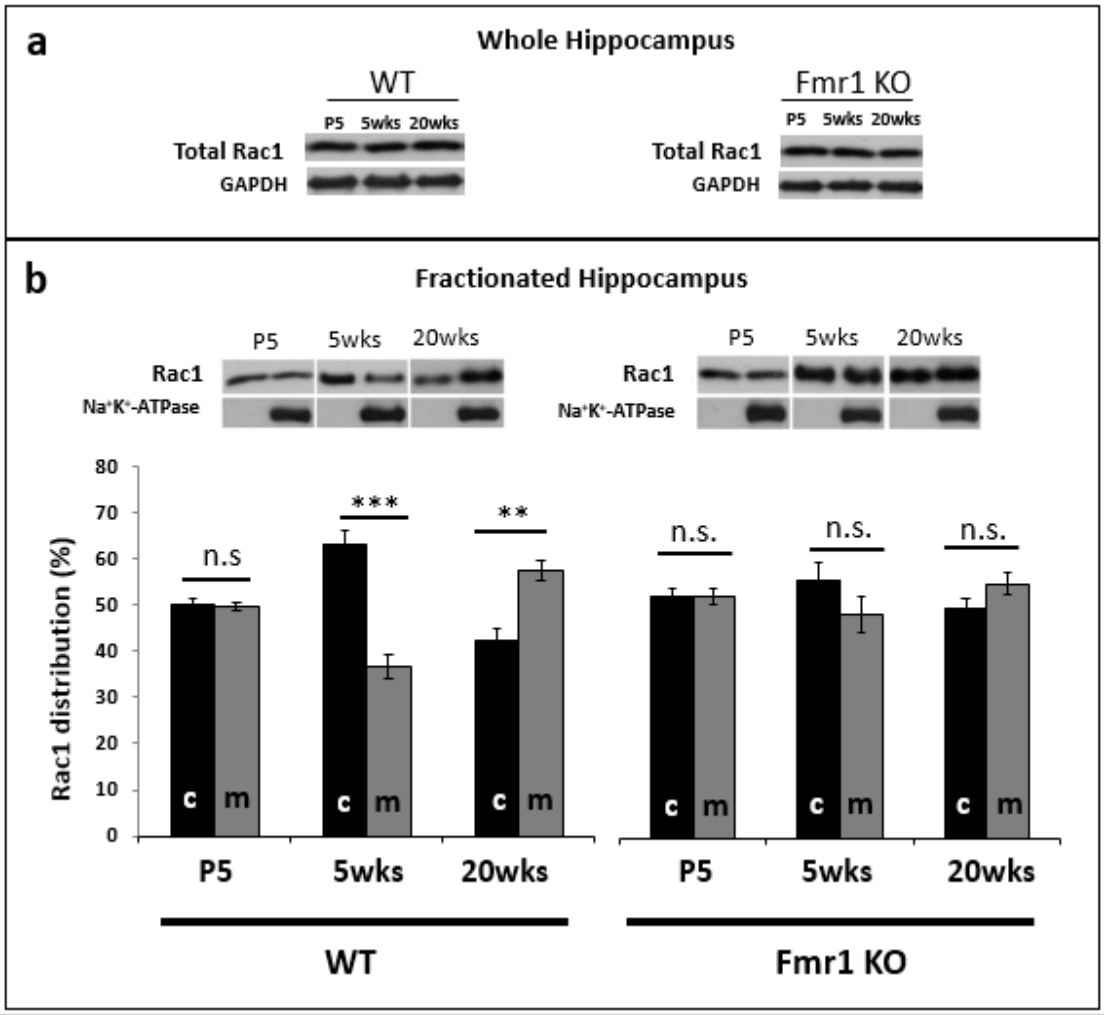

Figure 12. ***Rac1 localization in Fmr1 KO mouse hippocampus.*** **Whole hippocampi from three developmental stages were fractionated into cytosol and membrane.** (a) Representative western blots depicting total Rac1 in hippocampus of WT and KO animals at different developmental stages [postnatal day 5 (P5), 5 and 20 weeks] from total hippocampus. Total Rac1 did not differ across ages or between genotypes. (b) Representative western blots depicting Rac1 in cytosol and membrane fractions derived from hippocampus at different developmental stages. In WT and KO mice, Rac1 was equally distributed between cytosol (c inside bar

graphs) and membrane (m inside bar graphs) at P5 in wild type (cytosol: 50.2%, membrane: 49.7% ±0.9). In wild type at 5 weeks, Rac1 was found significantly higher in cytosol than membrane (cytosol: 63.3, membrane: 36.6%±2.7) but at 20 weeks Rac1 was found higher in membrane fractions (cytosol: 42.5%, membrane: 57.4%±2.1). In Fmr1 KO, Rac1 did not exhibit differences in distribution between cytosol and membrane at any of the three ages probed (P5: cytosol: 50.0%, membrane: 49.9%±1.6; 5wks: cytosol: 53.5%, membrane: 46.4%±3.7; 20wks: cytosol: 47.38, 52.62%±2.2). The integral membrane protein Na^+K^+- ATPase was probed to determine purity of fractionation. n=6. Data represent mean ±sem. **p<0.01. ***p<0.001. Student's *t*-test. n.s.: not significant.

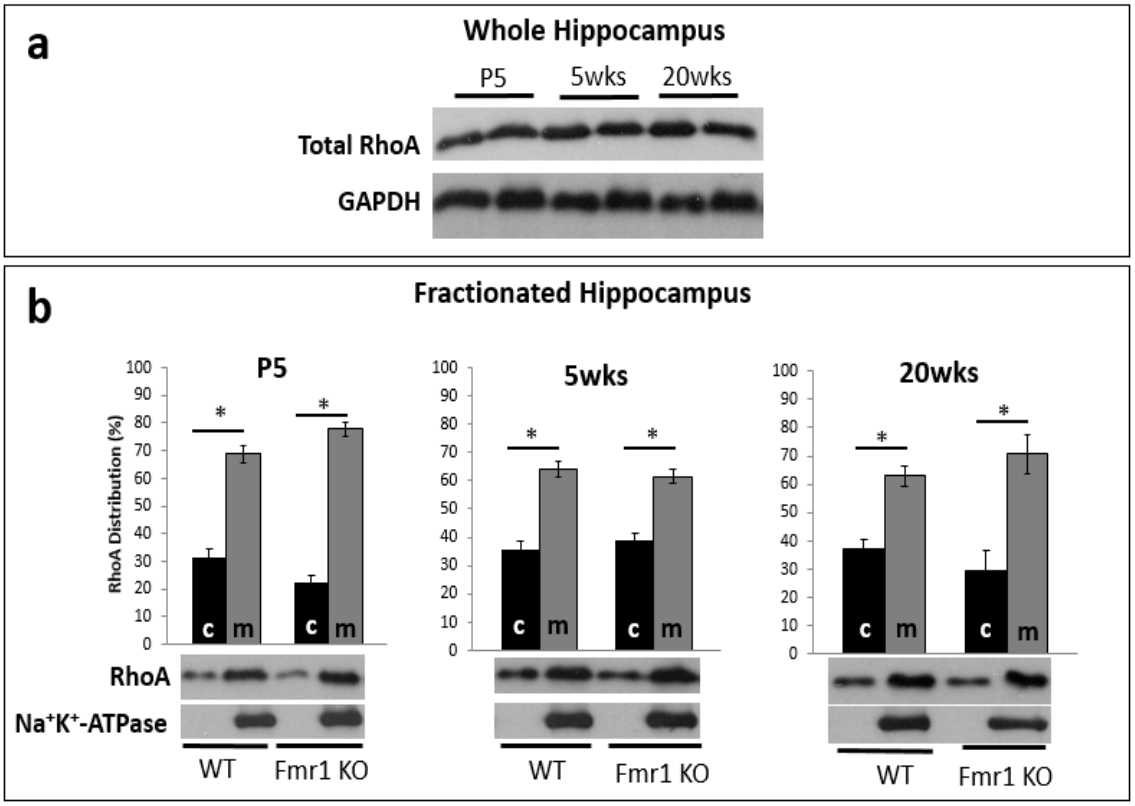

Figure 13. ***Localization of the associated small GTPase RhoA in Fmr1 KO mouse hippocampus is normal across critical developmental stages.*** Across all ages in both genotypes, RhoA was found to be significantly higher in the membrane (m inside bar graphs) compared to cytosol (c inside bar graphs). (WT P5: cytosol: 31.3%, membrane: 68.6%±3.1; 5wks: cytosol: 35.6%, membrane: 64.3%±2.7; 20wks: cytosol: 37.0%, membrane: 62.9%±3.6) (Fmr1 KO P5: cytosol: 22.3%, membrane: 77.6%±2.4; 5wks: cytosol: 38.6%, membrane: 61.3%±2.4; 20wks: cytosol: 29.3%, membrane 70.6%±7.0). Representative western blots depicting RhoA in cytosol and membrane fractions derived from hippocampus at different developmental stages. Data represent mean ±sem. n=6 *p<0.05. Student's *t*-test.

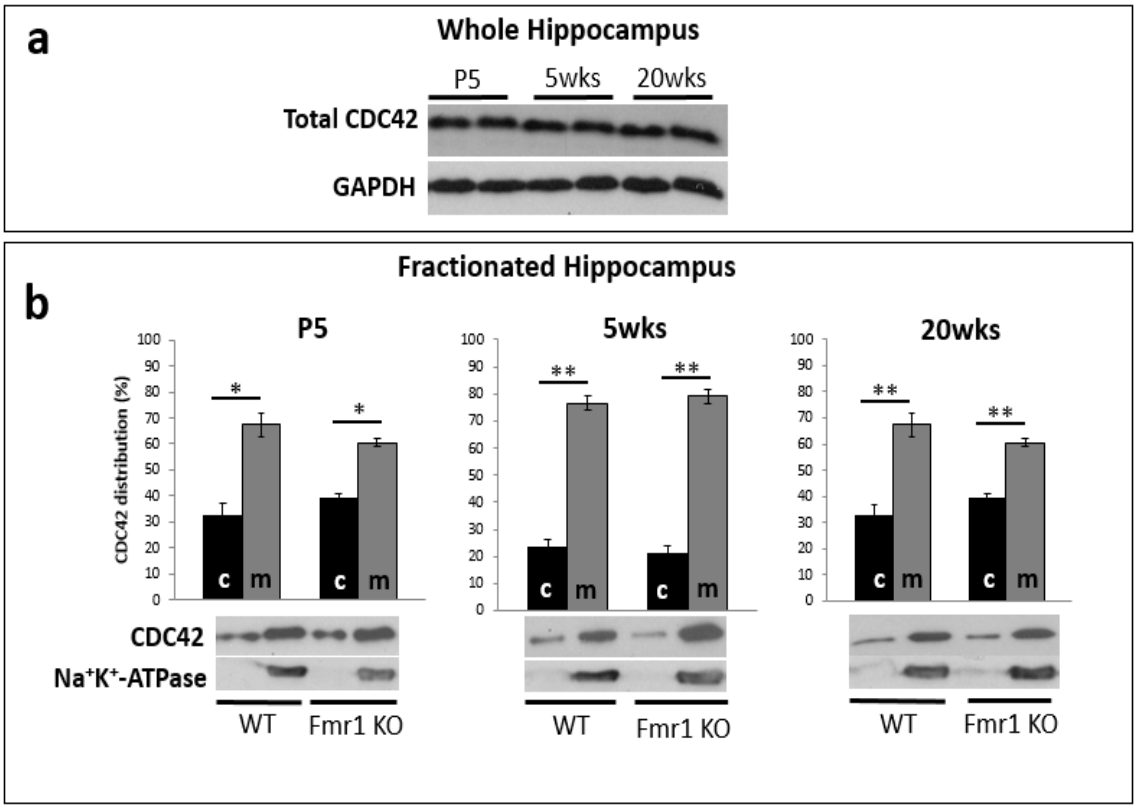

Figure 14. ***Localization of the associated small GTPase CDC42 in Fmr1 KO mouse hippocampus is normal across critical developmental stages.*** Fractionated hippocampal samples were probed for the small Rho GTPase CDC42. Across all ages in both genotypes CDC42 were found to be significantly higher in the membrane (m inside bar graph) fraction compared to cytosol (c inside bar graph). Representative western blots depicting CDC42 in cytosol and membrane fractions derived from hippocampus at different developmental stages. (WT P5: cytosol: 32.6%, membrane: 67.3%±4.3; 5wks: cytosol: 15.8%, membrane: 84.1%±3.0; 20wks: cytosol: 23.5%, membrane: 76.4%±2.6) (Fmr1 KO P5: cytosol: 39.4%, membrane: 60.5%±1.6; 5wks: cytosol: 14.2%, membrane: 85.7%±2.6; 20wks: cytosol: 21.0%, membrane 78.9%±2.8). Data represent mean ±sem. n=6 *p<0.05, **p<0.01. Student's *t*-test.

4.8 Increasing training intensity during delay-FC rescues contextual memory in Fmr1 KO

During characterization of the Fmr1 KO mouse model, contextual memory was impaired in Fmr1 KO mice after delay-fear conditioning using 3 tone-shock pairs (see Figure 7), but not after trace-fear conditioning using 6 tone shock pairs (Figure 9). This observation suggests that Fmr1 KO mice are capable of overcoming hippocampal-dependent learning and memory deficits if training intensity is increased.

To determine the extent of the memory impairment phenotype in the 5 week old Fmr1 KO mice, separate groups of mice were trained where three different levels of training intensities were given: 1, 3 or 6 tone-shock pairs, administered during delay-fear conditioning. Immediately after conditioning, Fmr1 KO mice exhibited significantly reduced freezing compared to WT when given a single tone-shock pair (Figure 15). Three tone-shock pairs once again revealed a learning deficit in Fmr1 KO mice (Figure 15). However, after 6 tone-shock pairs, both groups exhibited comparable freezing behavior, indicating similar levels of acquisition. Since during delay fear conditioning the tone and shock co-terminate, this observation also suggested that the tone-shock

separation during trace-fear conditioning did not explain the increased behavior in Fmr1 KO mice.

Long-term contextual memory was tested one day after graded intensity training. Performance of contextual memory paralleled levels of acquisition. Compared to wild type, Fmr1 KO mice that received 1 and 3 tone-shock pairs showed less freezing when placed back into the training chamber. However, freezing behavior exhibited by Fmr1 KO mice was similar to WT after 6 tone-shock pairs (Figure 16a). Two hours after contextual memory testing, mice were put into a modified training chamber to test for tone-dependent memory. During presentation of the tone, Fmr1 KO mice displayed significantly reduced freezing compared to WT mice regardless of the number of tone-shock pairs received (Figure 16b). Additionally, WT groups expressed similar freezing behavior across each of the three training intensities. This observation also applied to the decreased freezing seen Fmr1 KO groups.

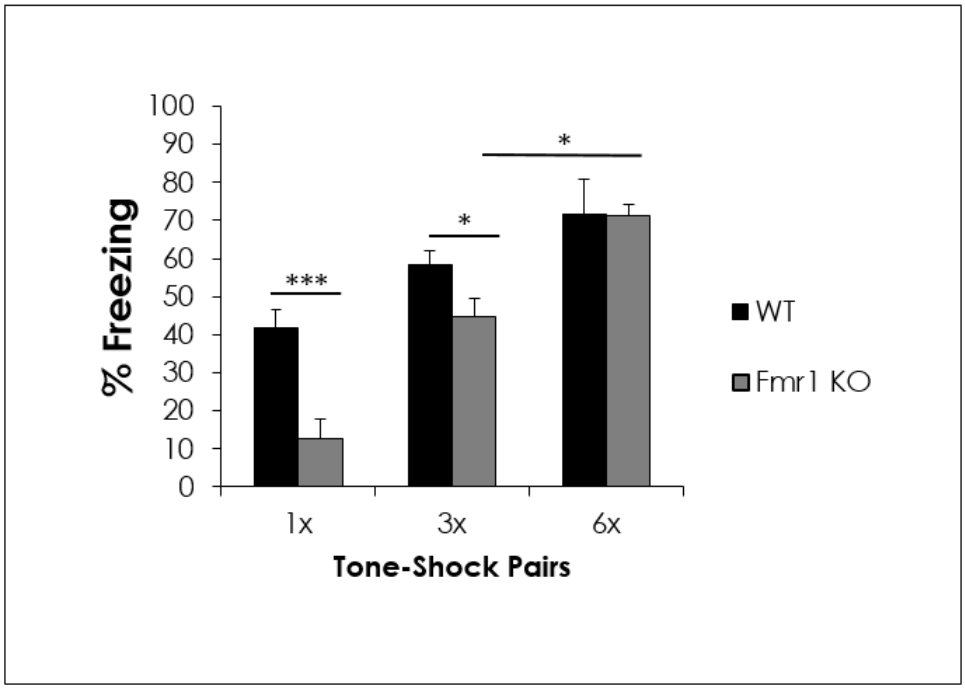

Figure 15. Increasing the number of tone-shock pairs (1x, 3x, and 6x) reveals that learning can be restored in Fmr1 KO mice. Training carried out with 1x and 3x tone-shock pairs shows Fmr1 KO mice freezing significantly less than WT at the end of conditioning (1x: WT:41.6±4.7%, Fmr1 KO: 12.6±5%; 3x: WT: 58.2±3.7%, Fmr1 KO: 44.8±4.5%) However, by using 6x tone-shock pairs, freezing behavior does not differ between groups, indicating similar acquisition of the aversive experience by Fmr1 KO as in WT mice (WT: 71.5±9.2%, Fmr1 KO: 71.4±2.7%). Data represent mean ±sem. n=10-15. *p<0.05; ***p<0.001. One-way ANOVA.

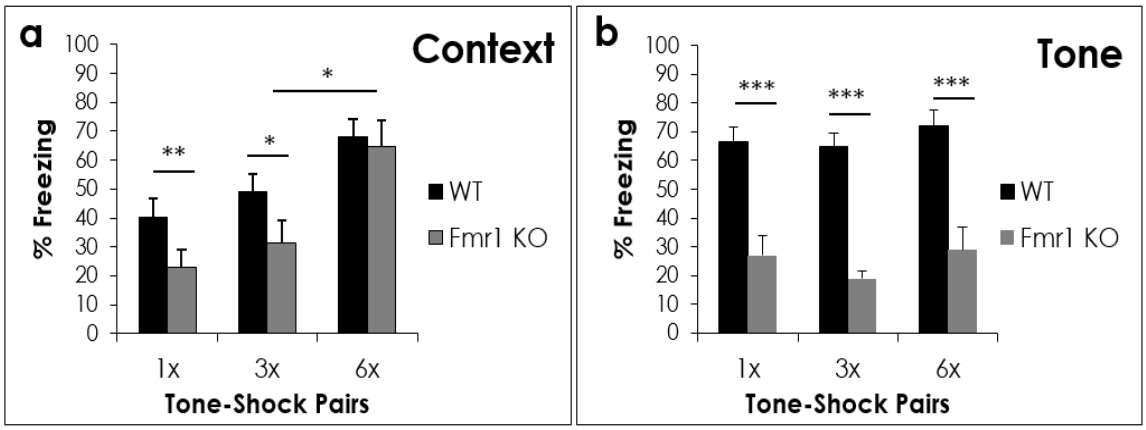

Figure 16. Increasing the number of tone-shock pairs (1x, 3x, and 6x) reveals that contextual memory but not tone-memory can be restored in Fmr1 KO mice. Twenty-four hours after training with either 1x, 3x or 6x tone-shock pairs contextual and cue memory were tested. (a) In parallel with training, contextual memory shows that Fmr1 KO mice are deficient after using 1x and 3x tone shock pairs (1x: WT: 40.0±6.8%, Fmr1 KO: 22.9±6.1%; 3x: WT: 49.0±6.2%, Fmr1 KO: 31.5±7.6%), but freezing behavior is comparable to WT after 6x tone shock pairs (WT: 67.9±6.4%, Fmr1 KO: 64.7±9.2%). (b) Fmr1 KO mice exhibit cue-memory deficits at all three training intensities (1x: WT: 66.3±5.2%, Fmr1 KO: 27.0±6.9%; 3x: WT: 64.7±4.6%, Fmr1 KO: 18.9±2.4%; 6x: WT: 72.2±5.2%, Fmr1 KO: 28.8±8.0%). Furthermore, Fmr1 KO cue-induced freezing does not increase with increasing intensity, a pattern that is also seen with WT mice. Data represent mean ±sem. n=10-15. *p<0.05; **p<0.01; ***p<0.001. One-way ANOVA.

4.9 Fmr1 KO mice express reduced membrane bound Rac1 after increased delay-FC training trials

To determine the localization of Rac1 after fear conditioning and its relation to the memory deficit seen in Fmr1 KO mice, whole hippocampus was collected and fractionated into cytosol and membrane in a new cohort of animals. Hippocampus was collected from mice 1 hour after delay fear conditioning with 0x, 3x or 6x tone-shock pairs. Chamber control mice receiving 0x tone-shock pair were allowed to explore the training context for seven minutes, the same duration that mice have in the chamber during conditioning (Figure 17a). No difference was seen between genotypes in exploratory behavior when comparing freezing behavior at the start and the end of exploration. Similar to previous reported results obtained during characterization (see Figure 15), in this new cohort, Fmr1 KO mice that received 3x tone shock pairs display learning deficits after fear conditioning (Figure 17b), whereas a learning deficit was not detected after 6x tone shock pairs (Figure 17c).

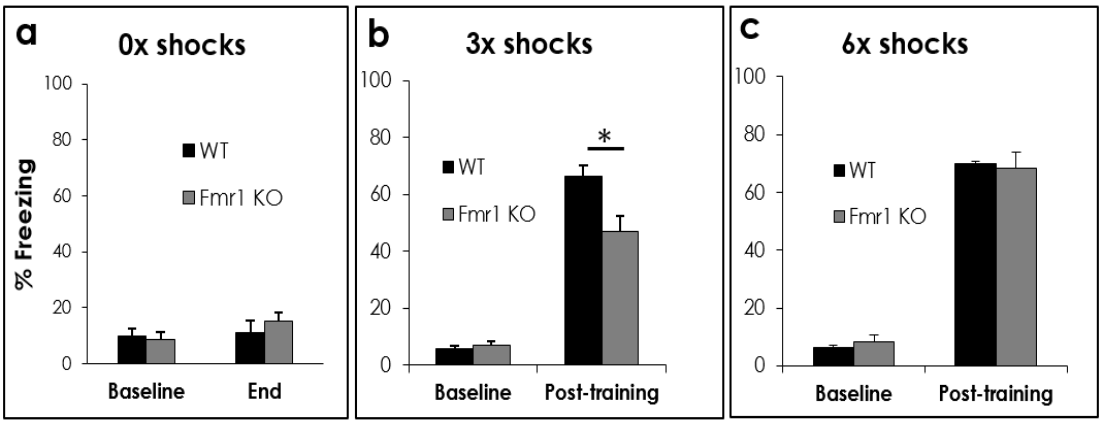

Figure 17. Delay FC training followed by collection of hippocampus. (a) Mice were placed into the training chamber and allowed to explore for seven minutes, the duration mice are in the chamber during fear conditioning. As previously found, baseline behavior was similar between groups (WT: 9.8±2.7%, Fmr1 KO: 8.5±2.6%). At the end of exploration, no difference was detected in exploratory behavior (WT: 11.0±4.4%, Fmr1 KO: 15.3±2.7%). (b) Once again, significantly less freezing behavior was recorded in Fmr1 KO mice during post-training period following fear conditioning with 3x tone shock pairs (Baseline: WT: 9.8±2.7%, Fmr1 KO: 5.7±1.2%; Post-training: WT: 66.3±3.9, Fmr1 KO: 47.0±5.5%). (c) No difference in freezing behavior was observed using 6x tone shock pairs (Baseline: WT: 6.1±1.2%, Fmr1 KO: 8.1±2.3%; Post-training: WT: 69.8±4.7%, Fmr1 KO: 68.3±5.7%). After being removed from the chamber, hippocampus was collected and processed for TBS-LTP, western blot or Golgi staining. n=5 for chamber exploration; Data represent mean ±sem. n=15 for 3x tone shock pair; n=10-13 for 6x tone shock pair. *p<0.05. Student's *t*-test.

After collection and fractionation, levels of total Rac1 were comparable for all samples (Figure 18a). Rac1 in WT hippocampus was found significantly higher in the membrane, and membrane-bound Rac1 was similar across the three WT groups (Figure 18b). This indicated that the level of training intensity did not alter Rac1 distribution in the WT hippocampus. In Fmr1 KO, Rac1 distribution in the hippocampus was not affected by 0x and 3x tone shock pairs (Figure 18b) and resembled the distribution found during previous characterization results (see Figure 12). However, significantly reduced membrane-bound Rac1 was found in hippocampus from Fmr1 KO mice that received 6 tone-shock pairs.

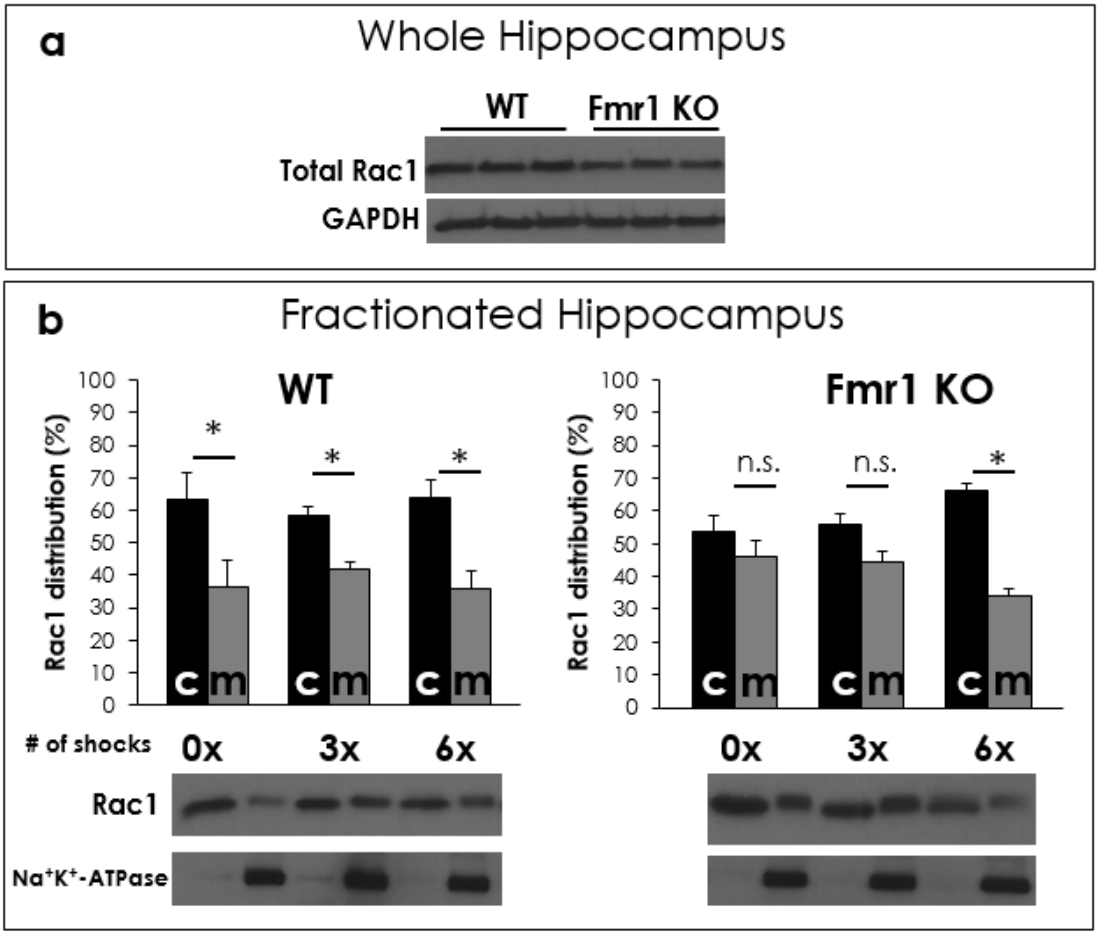

Figure 18. ***Increasing training intensity during delay FC alters Rac1 distribution.*** (a) total Rac1 levels in the whole hippocampus of WT and KO mice subjected to delay FC training. (b) One hour after delay-FC training with 0x, 3x or 6x tone-shock pairs, hippocampus was collected and fractionated into cytosol (c) and membrane (m).

Neither training intensity caused changes in Rac1 distribution in hippocampus from WT (1x: cytosol: 63.0, membrane: 36.4±8.0%; 3x: cytosol: 58.3, membrane: 41.6±2.5%; 6x: cytosol: 64.0, membrane: 35.9±5.3%). Fmr1 KO that received no foot shocks (0x) and 3x foot shocks showed no changes in Rac1 distribution (1x: cytosol: 53.7, membrane: 46.2±4.5%; 3x: cytosol: 55.8, membrane: 44.1±3.2%). However, after 6x tone shock pairs, Fmr1 KO membrane fractions showed a reduction in membrane-bound Rac1 (cytosol: 66.0, membrane: 33.9±2.1%). Control mice were allowed to explore the training chamber for seven minutes, the duration mice are in the chamber during fear conditioning. Data represent mean ±sem. n=3. *$p<0.05$. n.s.: not significant. Student's *t*-test.

Active Rac1 binds to p21-activated kinase (PAK), which induces its activation through auto-phosphorylation (Manser et al, 1997; Chong et al, 2001). Phosphorylated PAK (pPAK) promotes actin polymerization at membrane protrusions (Sells et al, 1997).

Hippocampi from wild type mice that receive 3x tone shock pairs exhibited a modest increase in pPAK compared to wild type that received 0x tone shock pairs. Hippocampus from wild type that received 6x tone shock pairs exhibited a reduction in pPAK compared to wild type that received 3x tone-shock pairs (Figure 19). Fmr1 KO mice also expressed significantly reduced pPAK in hippocampus after receiving 6 tone-shock pairs compared to Fmr1 KO mice that received 3x tone-shock pairs. These results indicated that whereas 6x tone shock pairs was associated with a reduction in active pPAK in both genotypes, a mechanism specific to Fmr1 KO mouse hippocampus may have induced translocation of Rac1 from the membrane to the cytosol.

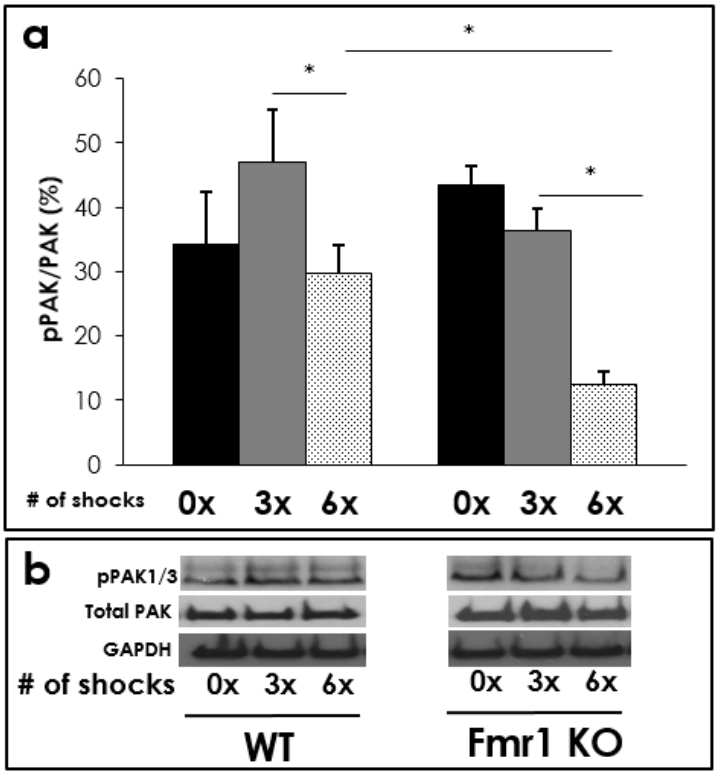

Figure 19. ***Increasing training intensity during delay FC reduces pPAK levels in hippocampus.*** (a) One hour after delay-FC training with 0x, 3x or 6x tone-shock pairs, hippocampus was collected and processed to obtain homogenates for quantification of PAK and pPAK. Both WT and Fmr1 KO hippocampi exhibited significantly reduced phosphorylated PAK after undergoing delay-FC with 6x tone-shock pairs, compared to 3x tone-shock pairs from WT (WT 3x: 46.9±8.1%, 6x: 29.8±4.4%; Fmr1 KO 3x: 36.4±3.4%, 6x: 12.5±1.8%). Mice that received no shocks (0x) were allowed to explore the training chamber for seven minutes, the duration mice are in the chamber during fear conditioning. (b) Representative western blots with levels of pPAK, total PAK in both WT and Fmr1 KO mice subjected to 0, 3, or 6x tone-shock pairs. n=3. Data represent mean ±sem. *$p<0.05$. One-way ANOVA.

4.10 TBS-LTP deficit in FMR1 KO mice is corrected after delay-FC using 6x tone shock pairs

To determine the effect of fear conditioning on TBS-LTP in the hippocampus, mice underwent delay-FC followed one hour later by collection of hippocampal slices and TBS-LTP induction. Hippocampal slices from control mice that were exposed to the training chamber for seven minutes did not exhibit differences in TBS-LTP between genotypes (Figure 20a). This was initially surprising given the difference seen in TBS-LTP from naïve mice (see Figure 5a). However, it has been previously reported that exploration of novel environments can reduce LTP (Xu et al, 1998) and facilitate LTD (Manahan-Vaughan and Braunewell 1999) in the hippocampus. This would have a net diminishing effect on LTP magnitude as observed in wild type hippocampi. TBS-LTP induced in hippocampi from naïve mice and mice that received 0x tone shock pairs does not differ. This observation suggests that exploration of a novel environment does not induce changes in synaptic plasticity in the Fmr1 KO hippocampus. Furthermore, whereas wild type hippocampus from mice that received 3x tone-shock pairs exhibited a significant increase in TBS-LTP compared to naïve and 0x tone shock pair, no such increase was observed in Fmr1 KO (Figure 20b). When mice received 6x tone shock pairs, TBS-LTP was similar in hippocampi from both genotypes (Figure 20c).

Comparisons of input-output curves in hippocampi from both genotypes revealed that training intensity did not affect basal synaptic responses to increasing strengths of stimuli (Figure 21). This indicated that differences were activity-dependent and were specifically affecting TBS-LTP (Song et al, 2012).

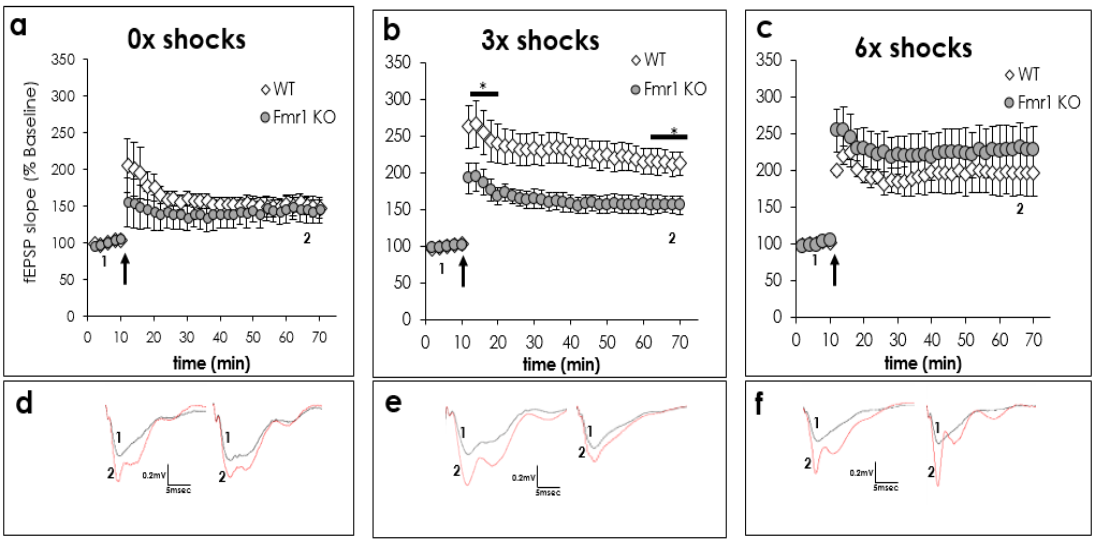

Figure 20. ***TBS-LTP observed deficit in FMR1 KO mice can be corrected after delay-FC when using increased tone shock pairs.*** (a) Mice received 0x, 3x or 6x tone-shock pairs and one-hour later hippocampus slices were collected for TBS-LTP. (a) No difference in LTP was observed in hippocampus after 0x tone-shock pairs (WT: 143.6±11.5%, Fmr1 KO: 142±5.7%). (b) Robust LTP was induced in WT hippocampus after 3x tone-shock pairs (212.7±16.2%) that was significantly higher than in Fmr1 KO hippocampus (156±12.4%). (c) Hippocampus from WT and Fmr1 KO mice exhibited similar enhanced LTP after 6x tone shock pairs (WT: 195.5±31.6%, Fmr1 KO: 227.4±31.4%). (d, e, f) Insets are representative traces from TBS-LTP experiment (black: pre-LTP induction; red: post-LTP at minute 70). Data represent mean ±sem. n=6, 2 slices/mouse, 3 mice. Black arrow= Theta burst. Student's *t*-test.

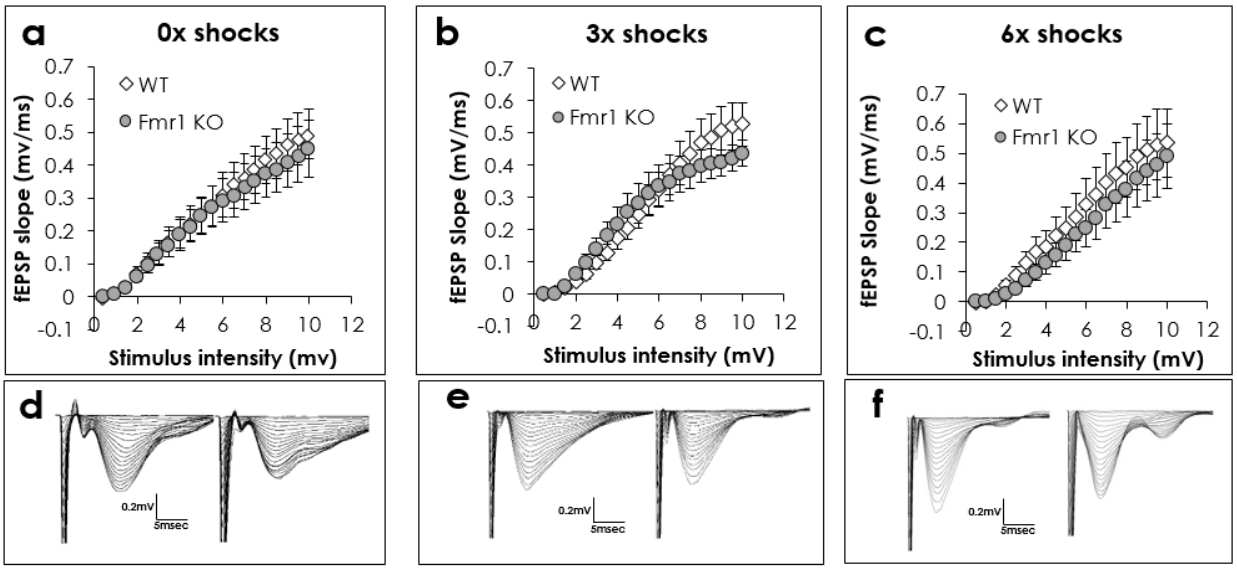

Figure 21. ***Input-Output curves in wild type and FMR1 KO mice after delay-FC when using increased tone shock pairs.*** Mice received 0x, 3x or 6x tone-shock pairs and one-hour later hippocampus slices were collected for electrophysiology. Input-output curves show there was no difference in the response of slices to a) 0x tone-shock pair, (b) 3x tone-shock pair, or 6x tone-shock pair with increases in stimulus intensities. (d, e, f) Insets representative input/output traces for each genotype. N=6, 2 slices/mouse, 3 mice. Black arrow: Theta burst. Data represent mean ±sem.

RESCUE OF OBSERVED DEFICITS IN FXS ANIMAL MODEL BY REGULATING RAC1

4.11 Treatment of Fmr1 KO hippocampus slices with Rac1 inhibitor increases TBS-LTP

Our experiments point to the possibility that a deficit in localization of Rac1 could be associated to the observed deficits in plasticity and behavior in the FXS animal model. To determine whether regulation of Rac1 activity might revert these deficits, the specific Rac1 inhibitor NSC23766 was used (Gao et al, 2004).

When slices derived from WT hippocampus were used, TBS-LTP was not affected by pretreatment with NSC23766 (Figure 22a). However, the LTP deficit previously observed in the hippocampal slices derived from Fmr1 KO mice receiving 6x tone shock pairs was corrected (Figure 22b), which also was linked to a reduction in membrane-bound Rac1. This suggested that Rac1 inhibition may be contributing to LTP increase. Fmr1 KO hippocampus slices pretreated with NSC23766 exhibited a modest but significant increase in TBS-LTP (Figure 22b) compared to untreated Fmr1 KO slices.

To determine whether NSC23766 had a general effect on LTP, HFS-LTP was induced after pre-treatment with the Rac1 inhibitor. NSC23766 has previously been shown to inhibit HFS-LTP in hippocampus from wild type mice (Martinez and Tejada-Simon, 2011). HFS-LTP in wild type hippocampus was only modestly diminished by pre-treatment with

NSC23766 (Figure 23a) which may be a result of mouse strain effect. HFS-LTP in the Fmr1 KO hippocampus was induced robustly in both controls and inhibitor treated slices (Figure 23b). This suggested that the effect of the Rac1 inhibitor on the increased LTP magnitude observed after theta burst stimulation was specific for this LTP-induction stimulus pattern.

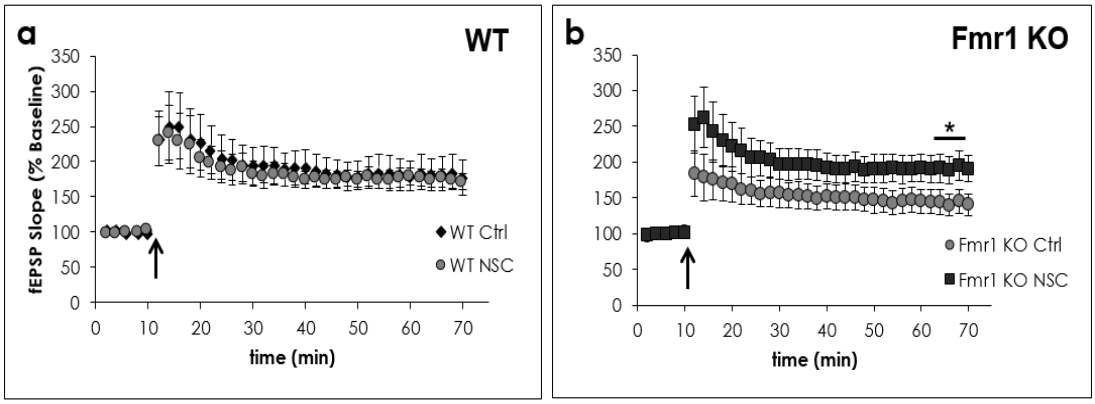

Figure 22. ***Treatment with NSC23766 increases TBS-LTP in Fmr1 KO hippocampus*****.** Hippocampal slices were pretreated with 100uM NSC23766 prior to induction of TBS-LTP. (a) Rac1 inhibitor did not affect LTP in hippocampus from WT (Control: 177.4±25.6%; NSC: 171.7±11.1%). (b) Fmr1 KO slices pretreated with Rac1 inhibitor exhibited a modest but significant increase in LTP maintenance (Control: 140.4±14.6%; NSC: 190.5±18.3%). Data represent mean ±sem. n=6 slices, 2 slices per mouse. Black arrow: Theta burst. *p<0.05. Student's *t*-test. Ctrl: Control.

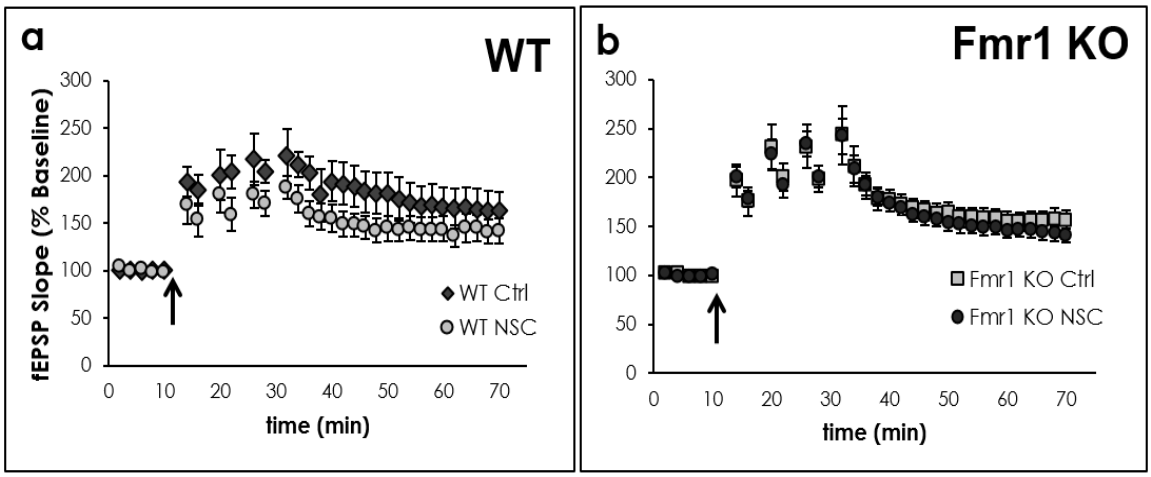

Figure 23. ***Treatment with NSC23766 does not significantly affect HFS-LTP*****.** Hippocampal slices were pretreated with 100uM NSC23766 prior to induction of HFS-LTP with 4x 100Hz with 1-minute inter-train interval. (a) Rac1 inhibitor affected LTP in hippocampus from WT mice modestly but non-significantly (Control: 162.4±20.7%; NSC: 141.9±13.5%). (b) Fmr1 KO-derived slices pretreated with Rac1 inhibitor showed similar behavior than Fmr1 KO untreated slices (Control: ±%; NSC: ±% Control: 158.8±13.1%; NSC: 155.6±12.8%).). Data represent mean ±sem. n=6 slices, 2 slices per mouse. Black arrow: Theta burst. *p<0.05. Student's *t*-test.

4.12 Treatment of Fmr1 KO with Rac1 inhibitor improves contextual memory after delay-FC

Fmr1 KO mice have been previously shown to express high levels of active Rac1 in the brain (de Diego-Otero et al, 2009), specifically in cortex and hippocampus (Bongmba et al, 2011). Furthermore, upregulated Rac1 activity has been demonstrated to impair other forms of hippocampus dependent memory in mice (Oh, et al, 2010). In this study, fear conditioning using 6x tone shock pairs resulted in a reduction of hippocampal membrane-bound Rac1 in association with memory improvement. This suggests that inhibiting Rac1 during fear conditioning using lower intensity training (1x or 3x tone shock pairs) may improve the memory deficits in Fmr1 KO mice.

To test the idea that hyperactive Rac1 in the hippocampus might contribute to these memory deficits in Fmr1 KO mice, a single dose of the Rac1 inhibitor NSC23766 (5mg/kg b.w.) was intraperitoneally given two hours prior to training. NSC23766 did not affect baseline behavior or post training behavior in WT or Fmr1 KO mice, as compared to the corresponding saline treated group (Figure 24a). Similar deficits in learning (see Figure 7 and 8) were observed between WT and Fmr1 KO mice untreated groups. Even though it did not reach statistical significance, an increased freezing behavior was observed in WT and Fmr1 KO groups treated with inhibitor (Figure 24a, post-training).

When contextual memory was tested, Fmr1 KO mice treated with Rac1 inhibitor exhibited significantly increased freezing compared to Fmr1 KO treated with saline (Figure 24b). The amount of freezing expressed by Fmr1 KO mice treated with inhibitor reached similar levels as observed for both WT groups (untreated and treated), indicating a rescue of the learning deficit observed without pharmacological treatment. Two hours later mice were tested for tone memory. Our results indicated that the inhibitor did not produce changes in tone-induced freezing, either in WT or Fmr1 KO mice (Figure 24b). The level of freezing during the tone however was still significantly reduced in Fmr1 KO mice compared to the WT groups. These results indicate that the inhibitor did not alter tone-dependent memory, but had a specific effect on contextual memory.

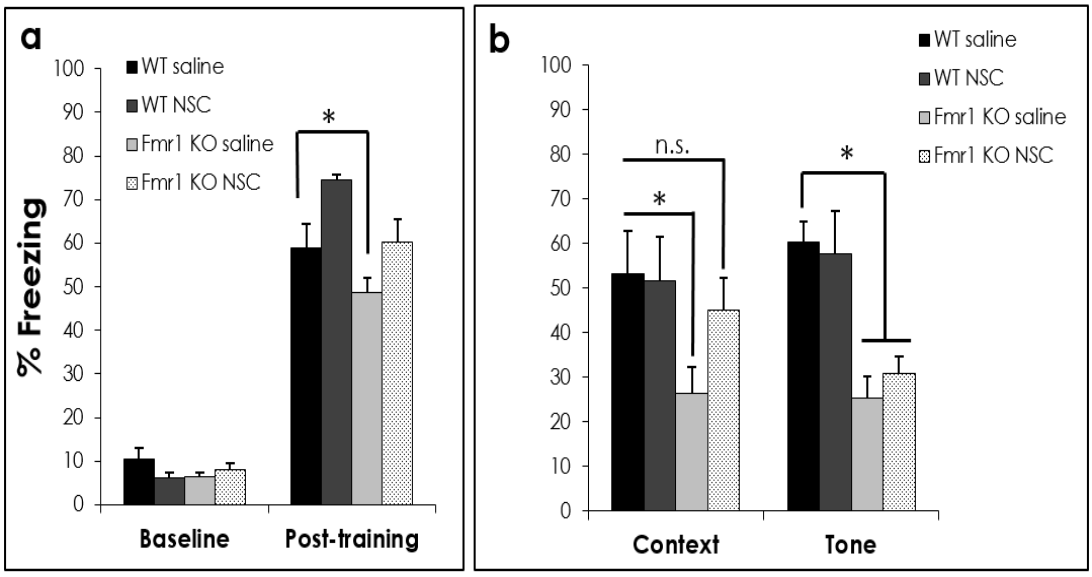

Figure 24. ***Fmr1 KO mice treated with NSC23766 exhibit increased contextual memory.*** Mice were treated with a single dose of NSC23766 (5mg/kg b.w.) and 2 hours later went through delay-FC with 3x tone-shock pairs. (a) NSC23766 did not significantly affect baseline behavior (WT Sal: 10.5±2.3%, WT NSC: 6.25±1.4%, Fmr1 KO Sal: 6.5±0.9%, Fmr1 KO NSC: 8.0±1.4%). A trend towards increased freezing behavior was observed in NSC23766-treated mice from both genotypes during post-training measurements, but was not statistically significant (WT Sal: 59.0±5.3%, WT NSC: 74.6±5.9%, Fmr1 KO Sal: 48.5±3.5%, Fmr1 KO NSC: 60.3±5.2%). (b) Contextual memory was not affected in wild type mice, however freezing behavior was significantly increased in Fmr1 KO mice treated with inhibitor (WT Sal: 53.2±9.4%, WT NSC: 51.5±9.8%, Fmr1 KO Sal: 26.3±5.9%, Fmr1 KO NSC: 45.0±7.2%). Tone-induced freezing behavior was unaffected in both groups (WT Sal: 60.2±4.6%, WT NSC: 57.6±9.5%, Fmr1 KO Sal: 25.3±4.9%, Fmr1 KO NSC: 30.8±3.8%). Data represent mean ±sem. n=10-12 *$p<0.05$. n.s.: not significant. One-way ANOVA with Bonferroni *post hoc* test.

Memory can be separated into phases of formation, acquisition, consolidation and retrieval (Abel and Lattal, 2001). Therefore, to test whether the effects described on treated groups are the results of an alteration during acquisition or consolidation of learning, mice were injected right after training and tested similarly to the process described previously. Treatment after training did not affect contextual or tone memory in either genotype (Figure 25a and 25b). This indicated that the effect of the inhibitor on the Fmr1 KO mice was affecting learning acquisition and not consolidation.

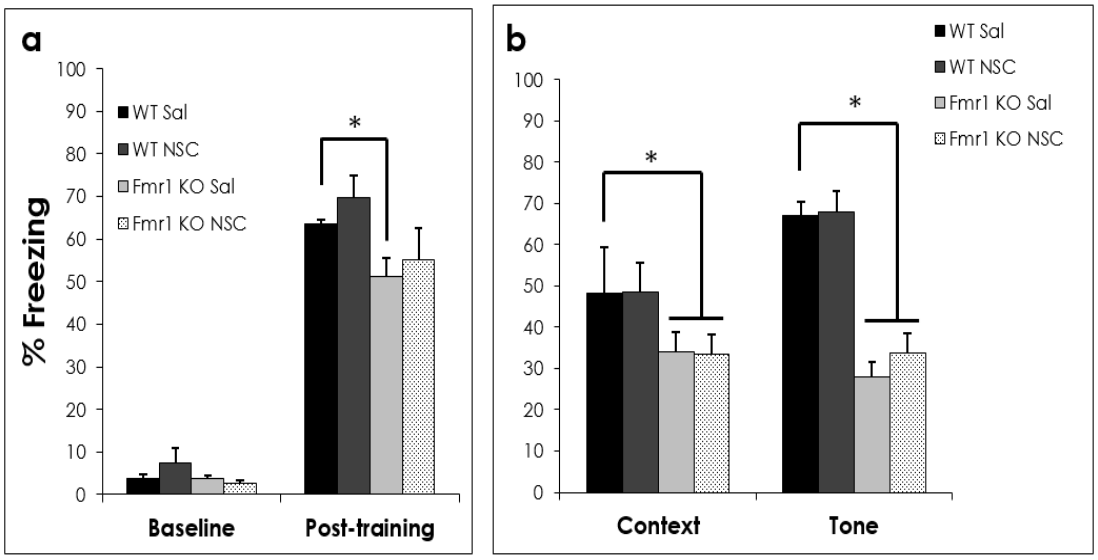

Figure 25. ***Post-training treatment with NSC23766 does not affect fear learning and memory in Fm1 KO mice.*** Mice were injected with a single dose of NSC23766 (5mg/kg b.w.) (a) 2 hours after delay-FC training with 3x tone-shock pairs. Post-training freezing behavior is shown to be reduced in Fmr1 KO groups. (b) Twenty-four hours after training and Rac1 inhibitor treatment, contextual (WT Sal: 48.0±11.3%, WT NSC: 48.4±7.1%, Fmr1 KO Sal: 33.9±4.7%, Fmr1 KO NSC: 33.4±4.8%). and tone-memory (WT Sal: 67.1±3.3%, WT NSC: 67.8±5.1%, Fmr1 KO Sal: 27.9±3.7%, Fmr1 KO NSC: 33.6±4.7%) tests reveal no effect on either forms of memory. This indicated that treatment with inhibitor did not affect consolidation of the aversive experience. Data represent mean ±sem. n=10-11. *$p<0.05$ compared to wild type control. One-way ANOVA with Bonferroni *post hoc* test.

4.13 Treatment of Fmr1 KO with Rac1 inhibitor improves cue-memory after trace-FC

A separate cohort of mice was then treated with NSC23766 (5mg/kg b.w. I.P.) or 50µl 0.9% saline 2hrs prior to trace-fear conditioning. Pre-training exploratory and post-training freezing behavior was not affected by treatment with the Rac1 inhibitor (Figure 26a). All groups displayed similar levels of freezing behavior immediately after the final tone-shock pair. Tone memory was again deficient in Fmr1 KO mice treated with saline and was unaffected in WT. Interestingly, freezing behavior in Fmr1 KO mice treated with NSC23766 was significantly higher during the tone when compared to baseline behavior (Figure 26b). Moreover, a significantly higher level of freezing was observed in the inhibitor-treated, Fmr1 KO group compared to saline-treated Fmr1 KO, immediately after the termination of the tone (post-tone), a moment that would represent the interval during trace-fear conditioning. As expected, no difference in contextual memory was detected since a total of 6x tone shock pairs were given during trace-fear conditioning (Figure 27).

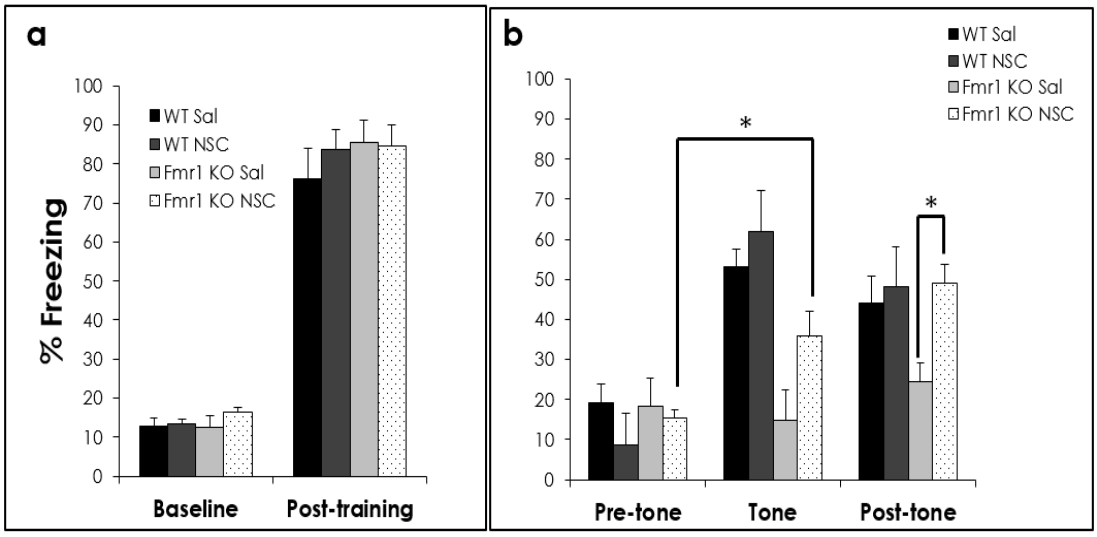

Figure 26. ***NSC23766 treatment increases cue memory after trace-FC in Fmr1 KO mice*****.** Mice were treated with a single dose of NSC23766 (5mg/kg b.w.) followed 2 hours later by trace-FC training using 6x tone-shock pairs. (a) Treatment with Rac1 inhibitor did not significantly affect baseline (WT Sal: 12.8±1.9%, WT NSC: 13.5±1.2%, Fmr1 KO Sal: 12.5±3.1%, Fmr1 KO NSC: 16.4±1.2%) or post-training freezing behavior (WT Sal: 76.1±7.8%, WT NSC: 83.5±5.1%, Fmr1 KO Sal: 85.5±5.7%, Fmr1 KO NSC: 84.7±5.3%) in either genotype. (b) Twenty-four hours after training, mice were put into a novel environment to test tone-memory. Rac1 inhibitor treatment did not affect exploratory behavior in the novel context in either genotype (Pre-tone) (WT Sal: 19.0±4.9%, WT NSC: 8.7±7.6%, Fmr1 KO Sal: 18.4±6.9%, Fmr1 KO NSC: 15.3±2.1%). When the tone was played both wild type groups exhibited similar freezing behavior that was significantly higher when compared to baseline in the same wild type group (tone) (WT Sal: 53.0±4.4%, WT NSC: 48.0±10.0%). Significantly high freezing behavior was also observed in both

wild type groups immediately after the tone was stopped (post-tone) (WT Sal: 44.0±6.6%, WT NSC: 48.0±10.0%). When the tone was played, the freezing behavior exhibited by Fmr1 KO mice treated with saline did not differ from baseline freezing behavior for the same group (Fmr1 KO Sal: 14.8±7.5%). After the tone ended (post-tone), the freezing behavior in saline-treated Fmr1 KO mice was not different from baseline or tone-induced freezing (Fmr1 KO Sal: 24.5±4.4%). However, during the tone, Rac1 inhibitor-treated Fmr1 KO mice exhibited freezing significantly higher above baseline (Fmr1 KO NSC: 35.7±%).). Freezing behavior exhibited by treated Fmr1 KO mice was sustained to similar levels as wild type and significantly higher than saline Fmr1 KO group, when the tone was terminated (post-tone) (Fmr1 KO NSC: 48.9±4.6%). Data represent mean ±sem. n=10-12. n.s.: not significant. *$p<0.05$ One-way ANOVA with Bonferroni *post hoc* test.

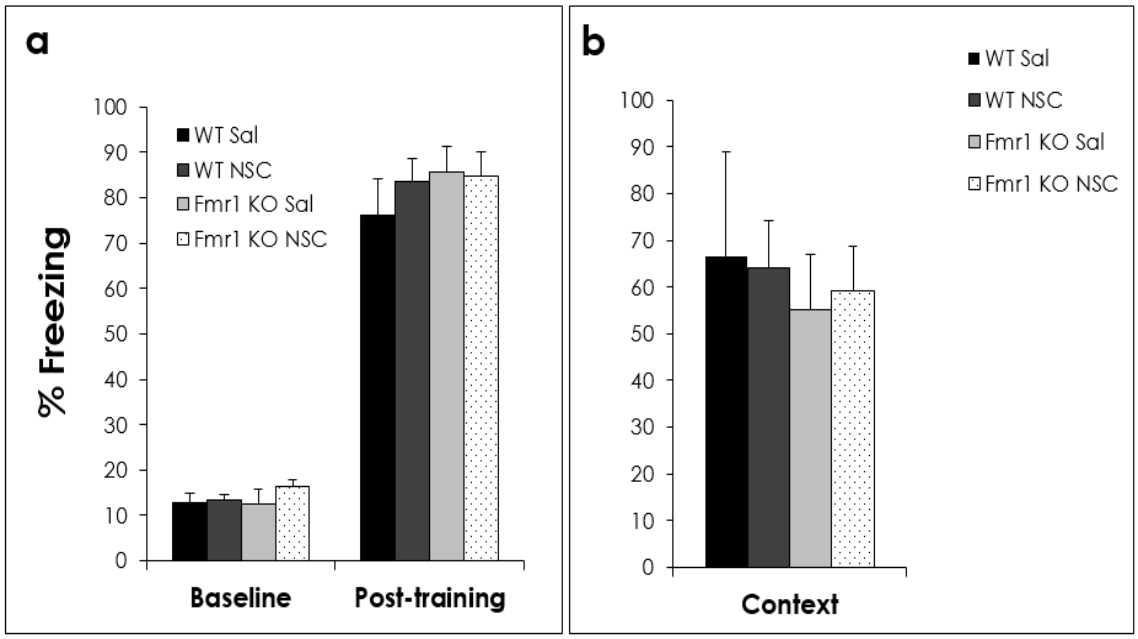

Figure 27. ***Treatment with a single dose of NSC23766 (5mg/kg b.w.) does not affect contextual memory after trace-FC.*** Mice were treated 2 hours prior to trace-FC training. (a) Treatment with Rac1 inhibitor did not affect baseline or post-training freezing behavior in either genotype. (b) Twenty-four hours after training, mice were put into a novel environment to test for contextual memory. Contextual memory tests did not reveal a difference in freezing behavior between saline and inhibitor treater groups (WT Sal: 66.4±22.4%, WT NSC: 64.1±10.1%, Fmr1 KO Sal: 55.1±11.8%, Fmr1 KO NSC: 59.1±9.6%). A difference was also not detected between genotypes. Data represent mean+/-sem. n=10-12. One-way ANOVA.

4.14 Rac1 inhibitor treatment does not affect acoustic startle or PPI in Fmr1 KO mice

To determine if the Rac1 inhibitor affected auditory sensory integration, PPI of acoustic startle was tested in WT and Fmr1 KO mice treated with a single dose of NSC23766 (5mg/kg b.w. I.P.) or 50µl 0.9% saline. Two hours after treatment PPI of the acoustic startle was assessed. The blunted acoustic startle response expressed by Fmr1 KO mice was not affected by inhibitor treatment (Figure 28a). Similar to results reported previously (see Figure 10), Fmr1 KO mice treated with saline exhibited enhanced pre-pulse inhibition of acoustic startle compared to the WT saline group. Furthermore, NSC23766 did not affect PPI in WT or Fmr1 KO (Figure 28b). Taken together, our results show that a lack of changes on PPI or the startle response may exclude these neurological processes as targets of NSC23766 during learning and memory tests.

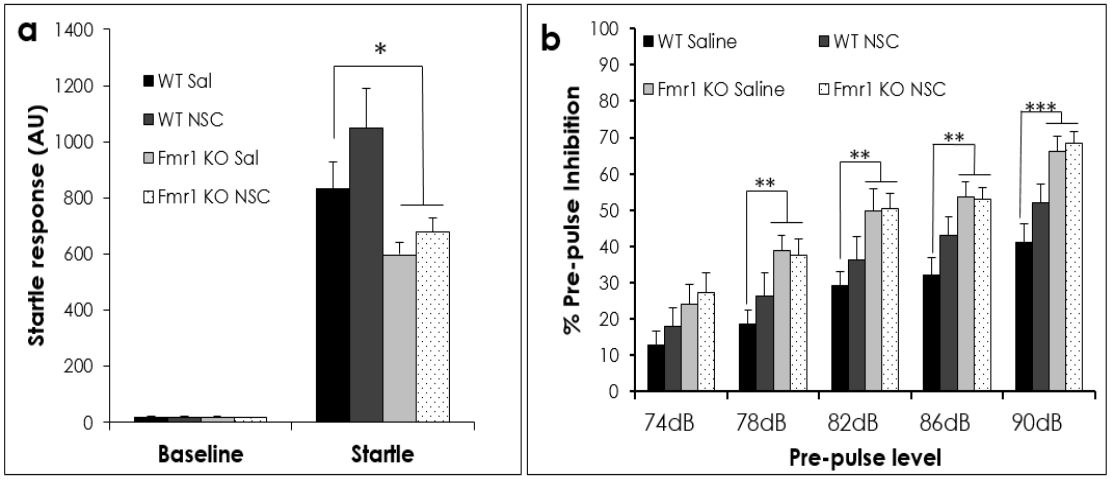

Figure 28. ***NSC23766 treatment does not affect acoustic startle and PPI in Fmr1 KO mice.*** Treatment with NSC23766 does not alter acoustic startle response or PPI in WT and Fmr1 KO mice. (a) Two hours after receiving a single dose of saline or NSC23766 (5mg/kg b.w.), mice were measured for an acoustic startle response. Baseline behavior in the enclosure was similar between all treatment groups (WT Sal: 19.2±0.5, WT NSC: 18.0±2.3, Fmr1 KO Sal: 18.5±1.4, Fmr1 KO NSC: 16.5±2.3). Wild type mice treated with Rac1 inhibitor exhibited a modest increase in startle response to a sound burst (120dB), which was not significantly different from saline-treated wild type (WT Sal: 833.8±93.9, WT NSC: 1050.2±139.3). A significantly reduced startle response was measured in both Fmr1 KO groups (Fmr1 KO Sal: 597.3±44.6, Fmr1 KO NSC: 680.0±50.1). (b) Prepulse inhibition of the acoustic startle

response was not affected by Rac1 inibitor treatment. Rac1 inhibitor-treated wild type showed a trend towards increased pre-pulse inhibition, but was non-significant compared to saline-treated wild type (WT Sal: 74dB: 12.7±4.0%, 78dB: 18.5±3.8%, 82dB: 29.3±3.6%, 86dB: 32.1±4.7%, 90dB: 41.0±5.3%; WT NSC: 74dB: 17.8±5.3%, 78dB: 26.4±6.2%, 82dB: 36.3±6.2%, 86dB: 43.1±4.9%, 90dB: 51.9±5.1%). Both Fmr1 KO treatments exhibited significantly higher pre-pulse inhibition compared to wild type groups at the four higher prepulse levels. but no significant difference was measured between KO groups (Fmr1 KO Sal: 74dB: 24.0±5.3%, 78dB: 38.8±4.1%, 82dB: 49.7±6.0%, 86dB: 53.6±4.0%, 90dB: 66.2±3.9%; Fmr1 KO NSC: 74dB: 27.2±5.3%, 78dB: 37.5±4.1%, 82dB: 50.4±4.0%, 86dB: 52.9±3.2%, 90dB: 68.3±3.2%). Data represent mean ±sem. N=12-17. *p<0.05, **p<0.01, ***p<0.001 compared to WT saline. One-way ANOVA with Bonferroni *post hoc* test.

4.15 Treatment with Rac1 inhibitor does not affect pain response

Alterations in pain sensory processing could conceivably affect formation of proper associations between a foot shock and context or tone, essential for fear conditioning paradigms. In previous reports, measurements of pain from thermal stimuli in Fmr1 KO mice have demonstrated inconsistencies, showing reduced sensitivity (Veeraragavan et al 2012), no differences compared to wild type (Zhao et al, 2005; Spencer, et al 2006) or a non-thermal nociceptive impairment (Price et al, 2007). Although NSC23766 has been shown not to affect pain threshold in naïve mice (Tan et al, 2012), NSC23766 has been shown increase pain threshold under neuropathic conditions (Tan et al, 2008; Tan et al 2012). Therefore, we wanted to determine whether NSC23766 produced any analgesic effect that could influence the formation of association with the tone or context during our learning and memory tests. The present study did not detect a difference in pain threshold between WT and Fmr1 KO groups as demonstrated in the hotplate test (Figure 29). Additionally, NSC23766 treatment did not alter the latency to respond in either group.

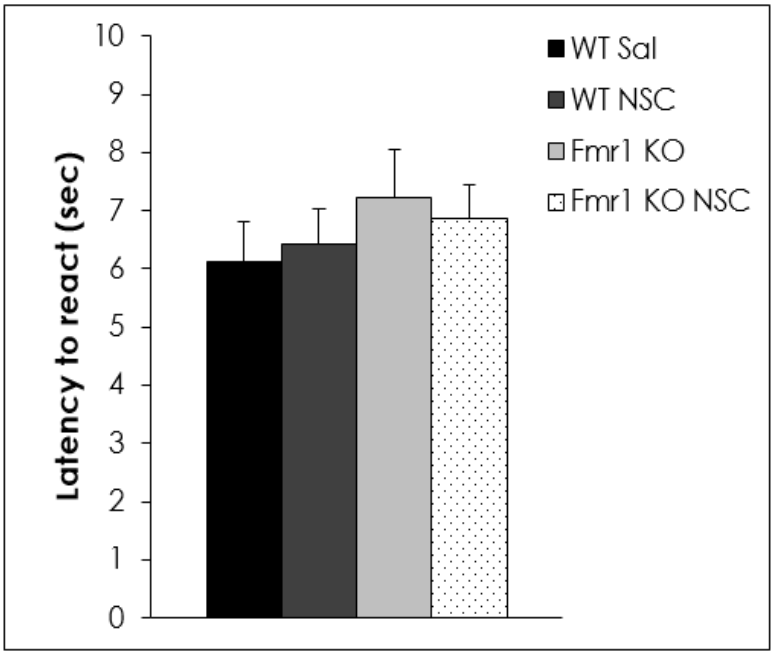

Figure 29. ***NSC23766 treatment does not alter pain response in hotplate*****.** Mice were treated with saline or a single dose of NSC23766 (5mg/kg b.w.), then placed into a hotplate analgesia meter 2 hours later. The latency to react to the thermal stimuli as indicated by paw flinch or licking, was similar between all treatment groups (WT Sal 6.1±0.6sec; WT NSC 6.4±0.6sec; Fmr1 KO 7.2±0.8sec; Fmr1 KO NSC 6.8±0.5sec). This indicated that the dose of Rac1 inhibitor used did not affect this form of nociception. Data represent mean ±sem. n=10. One-way ANOVA.

To further confirm that pain threshold was unaltered by this pharmacological treatment, we measured whether nociception was disrupted in the foot shock administered during fear conditioning. The mild foot shock administered during fear conditioning induces a transient flailing reaction that can be recorded and quantified as a motion index. Treatment with NSC23766 does not alter the foot shock induced flailing (Figure 30). These results point to undisturbed nociception during fear conditioning by NSC23766 treatment.

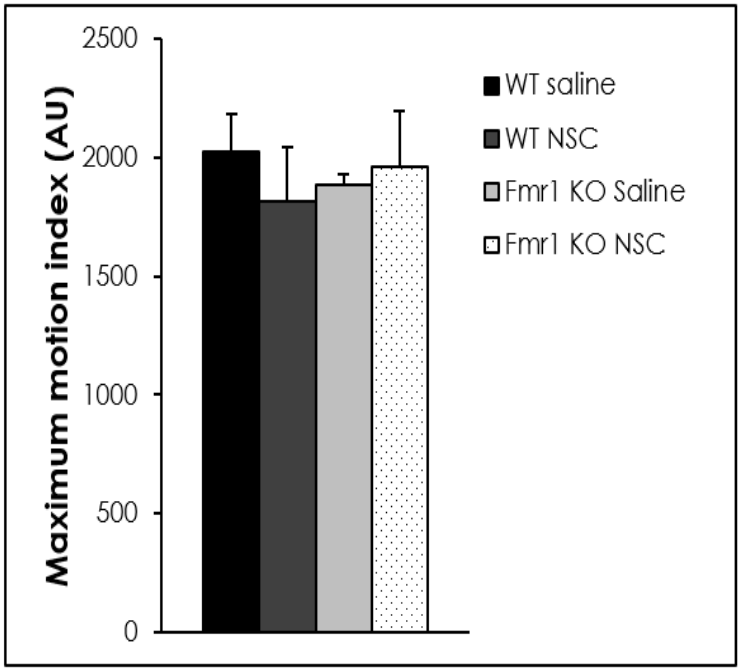

Figure 30. ***Treatment with NSC23766 does not alter the flailing reaction during the fear conditioning foot-shock.*** To establish an aversive memory in the fear conditioning chamber during training, mice receive a mild, 0.7mA foot shock lasting 3 seconds. The flailing response provides an overt indication that the mouse senses the foot-shock and is a measureable parameter. All groups displayed similar magnitude of flailing during the foot-shock (WT Sal 2024.3±161.2; WT NSC 1812.2±232.2; Fmr1 KO Sal 1882.4±48.0; Fmr1 KO NSC 1960.1±237.9). Data represent mean ±sem. n=10-12. One-way ANOVA.

5. DISCUSSION

Integral to learning processes are repetition and formation of associations, which induces proper synaptic changes to occur in specialized areas of the brain. This is known as the process of neuroplasticity, which is believed to be impaired in autism. Fragile X Syndrome (FXS), a leading cause of autism and intellectual disability, also exhibits impairments in neuroplasticity. In a mouse model of FXS, Rac1 hyperactivation has previously been found in the hippocampus (Bongmba et al, 2011), an area critical for learning and memory. It is hypothesized here that enhanced Rac1 activity may be impairing hippocampus-dependent synaptic plasticity and memory in FXS (Oh et al, 2010), or may be erasing memory traces (Hayashi-Takagi et al, 2015). Interestingly, inhibiting Rac1 activity can impair forgetting (i.e. prolong memory retention) (Jiang et al, 2016). These observations implicate hyperactive Rac1 in FXS and indicate Rac1 may be a suitable therapeutic target for cognitive impairments.

Excitatory neurotransmission predominantly impinges on dendritic spines (Megias et al, 2002). Silent synapses or immature dendritic spines have significantly reduced density of AMPA receptors (Busseto et al, 2008) which may be due to the shape of the spine (Kusters et al, 2013). FXS patients and Fmr1 KO mice exhibit an over-abundance of immature dendritic spines. It is not known if these are residual immature spines from an early proliferation phase or mature spines that underwent activity-induced regression.

Morphological properties of spines are tightly linked to effective synaptic transmission and compartmentalization of signaling (Araya et al, 2006; Harnett et al, 2012; Araya et al, 2014; Tonnesen et al, 2014). Therefore, immature spines are not expected to exhibit specificity of synaptic transmission or properly transduce molecular signaling cascades. Additionally, the increase in immature dendritic spines may also be a result of filopodia proliferation. Filopodia are the precursors of spines; weakened or blocked synaptic transmission causes an increase in filopodia number (Petrak et al, 2005). Hyper-connectivity in Fmr1 KO has been shown to weaken synaptic input in the cortex (Testa-Silva et al, 2012) which may also induce filopodia formation.

The small Rho GTPase Rac1 regulates changes in dendritic spine structure by tightly controlling actin cytoskeleton remodeling. In early postnatal development a proliferation of filopodia transitions into dendritic spines (Fiala et al, 1998), many of which are pruned in adulthood (Bourne and Harris, 2007). Therefore, across developmental stages, Rac1 activity and localization at the synapse are critical factors that determine spine structure.

In a novel finding, we show that Fmr1 KO mice, a model of FXS, exhibit impaired Rac1 membrane localization in the hippocampus at three critical developmental stages: postnatal day 5, 5 weeks and 20 weeks. In humans, these developmental stages have been approximated to early postnatal development, puberty and adulthood,

respectively (Dutta and Sengupta, 2016). These developmental stages coincide with periods of large changes in the number of synaptic connections (Purves and Lichtman, 1980; Tropea et al, 2010; Petanjek et al, 2011). Whereas wildtype hippocampus shows changes in Rac1 cytosolic and membrane localization across the three stages, Fmr1 KO mice exhibit equal distributions between these two cellular compartments during the three stages.

During early development (P5), a proliferation of synapses and dendritic spines would require membrane rearrangements along with actin remodeling. This process is promoted by increased active Rac1 at the synapse (Penzes et al, 2003; Zhang et al, 2005; Xie et al, 2007) and may explain the membrane-bound Rac1 at P5 wild type and Fmr1 KO hippocampi. At 5 weeks of age, Rac1 is found significantly higher in the cytosol compared to membrane, which may signal the spine pruning phase of synapse development in the hippocampus. In line with this thought, it has been reported that expression of dominant negative Rac1 in cultured hippocampal neurons leads to a reduction in dendritic spines (Van de Ven et al, 2005), and suggests that Rac1 inhibition would be required to progress through the spine elimination phase.

At 20 weeks of age, Rac1 is found significantly higher in the membrane fraction of wild type hippocampus. Rac1 is also known to function in the maintenance of dendritic spines (Nakayama et al, 2000), which may explain the increased levels of membrane-

bound Rac1 in wild type hippocampus. Stable spines are associated with long-term memories (Yang et al, 2009); thus active, membrane-bound Rac1 may regulate actin cytoskeleton stability. These shifts of Rac1 between cytosol and membrane are, however, not observed in the Fmr1 KO hippocampus. Although in Fmr1 KO at P5 (during spine proliferation) Rac1 distribution in the hippocampus resembles wild type, Rac1 distribution profiles at 5 weeks and 20 weeks of age, (when spine elimination and maintenance is occurring, respectively), resembles that of P5.

High levels of Rac1 protein have been found in post-mortem brain tissue of children with autism (Fatemi et al, 2013), although cellular distribution was not measured. Total levels of Rac1 in adults with autism were reduced relative to children with autism, but were still higher than age-matched controls (Fatemi et al, 2013). These results argue for the need to investigate Rac1 distribution mechanisms in human subjects, which may provide insights to regarding Rac1 in autism.

Localization of the closely related Rho GTPase, CDC42, did not differ between wildtype and Fmr1 KO hippocampus. RhoA, another member of the small Rho GTPase subfamily, which functions in the elimination of dendritic spines during early development (Nakayama et al, 2000), was also found similarly distributed in wild type and Fmr1 KO hippocampi. These observations suggested an impaired localization mechanism selective for Rac1. It has been reported that FMRP forms complexes with Rac1 through

SRA-1 protein (specific Rac1 associated 1) among others (Schenck et al, 2003; Castets et al, 2005). This interaction is important for dendritic spine stability (De Rubeis et al, 2013). In wild type mouse hippocampus, FMRP is expressed at high levels throughout PO to P55 (Gholizadeh et al 2015). However, the expression levels of the interacting protein SRA-1 peaks at three weeks of age (Bonaccorso et al, 2015), which may impact levels of the FMRP-SRA-1-Rac1 complex. In Fmr1 KO mice, because of the absence of FMRP, it may prevent proper Rac1 distribution during critical developmental phases such as spine pruning.

Fmr1 KO mice at 5 weeks of age displayed impaired low-intensity TBS-LTP. The relevance of theta frequency to other forms of behavior has been reported. For example, theta frequencies have also been found to be necessary for spatial memory (Winson, 1978) and also undergoes synchronizing changes during retrieval and consolidation of fear memory (Seidenbecher et al, 2003; Narayanan, et al, 2007). Theta also exhibits changes in strength depending on the context, becoming weakened after sudden exposure to novelty (Jeewajee et al 2008) but increasing in strength during correct spatial decision making (Belchior et al, 2014). In humans, theta band power is increased during successful consolidation of information (Klimesch et al, 1996) while artificially increasing theta frequency by transcranial magnetic stimulation enhances hippocampus dependent-memories (Kirov et al, 2009). The Medial septum-diagonal

band of Brocca (MS-DB) in the basal forebrain converts continuous input from brainstem reticular formation into the discontinuous output at the theta frequency (King et al, 1998; Oddie and Bland, 1998) which is received by the hippocampus. During exploration of novel contexts, theta frequency originating from basal forebrain nuclei may prime synapses of the hippocampus which could be strengthened and maintained by salient coincident stimuli. Area CA1 of the Fmr1 KO exhibits an abundance of immature dendritic spines and changes in spines are associated with synaptic plasticity (Muller et al, 2000; De Roo et al, 2008). Therefore, LTP induction in Fmr1 KO mice may remain subthreshold in response to stimuli that induces robust LTP in wild type. A subthreshold response may fail to alter dendritic spine structure and insufficiently prime synapses. TBS-LTP requires NMDA receptors that activate calcium calmodulin-dependent kinase II (αCamKII). Importantly, GEFs are activated by CaMKII prior to their effect on Rac1 (Lai and Ip, 2013), leading to synaptic membrane remodeling (Toni et al, 2002). Others have reported that expression of LTP results from increases in AMPA receptor density at the postsynaptic density (Makino and Malinow, 2009). Moreover, Rac1 promotes increases of AMPA receptors at the post-synaptic density (Wiens et al, 2005). Additionally, deficits in TBS-LTP can be corrected by increasing the number of theta bursts (Lauterborn et al, 2007). Increasing stimulation intensity appears to engage a Rac1 regulatory mechanism (Nikolic et al, 1998) that permits proper LTP expression. Correction of TBS-LTP by increasing stimulation magnitude parallels the impaired

associative memory that can be restored after increasing training intensity (i.e. by increasing number of training trials).

Examination of low-intensity TBS-LTP after high intensity training reveals enhanced LTP magnitude in Fmr1 KO mice. Furthermore, membrane-bound Rac1 is reduced in hippocampus of Fm1 KO mice after high-intensity training compared to low-intensity training. This reduction of membrane-bound Rac1 is accompanied by reduced levels of pPAK in both wild type and Fmr1 KO hippocampus. This suggests that Rac1 inhibition in the hippocampus may be contributing to the corrected hippocampus-dependent fear learning and memory in Fmr1 KO mice. Indeed, Rac1 regulation has been shown to enhance memory retention (Jiang et al, 2016). Binding of Rac1 to p35/Cdk1 kinase induces feedforward inhibition of PAK (Nikolic et al, 1998) which may provide a means in Fmr1 KO hippocampus to suppress Rac1 hyperactivation. Together, these results indicate that pharmacological regulation of Rac1 may support a mechanism conducive to learning in Fmr1 KO mice.

To follow up on this idea, Fmr1 KO mice that were treated with a specific Rac1 inhibitor, NSC23766, immediately before low-intensity training exhibited increased contextual memory. Additionally, TBS-LTP in Fmr1 KO hippocampus was increased after treatment with NSC23766. While Fmr1 KO mice treated with NSC23766 showed normalized contextual memory after delay-fear conditioning, tone memory was not restored,

indicating a task-specific effect of the inhibitor. Fmr1 KO mice also exhibit deficits in tone-memory after trace-fear conditioning which requires frontal cortex function in addition to the hippocampus. Interestingly, Fmr1 KO mice treated with NSC23766 displayed significantly increased tone-induced freezing behavior above baseline. Furthermore, immediately following the end of the tone, which would coincide with the trace between tone and shock during training, freezing behavior significantly increased above Fmr1 KO control.

Theta frequency is a neuronal firing pattern detected in the rodent hippocampus during exploration of novel environments, states of increased alertness (Penley, et al 2013) and retrieval and consolidation of fear memory (Seidenbecher et al, 2003; Narayanan, et al, 2007). The Medial septum-diagonal band of Brocca (MS-DB) in the basal forebrain is a brain region that converts continuous input from the brainstem reticular formation into the discontinuous output at the theta frequency (King et al, 1998; Oddie and Bland, 1998) being received by the hippocampus. During exploration of novel contexts, theta frequency originating from basal forebrain nuclei may prime synapses of the hippocampus (Larson and Munkácsy, 2015), which could be strengthened and maintained by salient coincident stimuli.

Fmr1 KO mice exhibit a blunted acoustic startle response (Nielsen, et al 2003; Baker et al, 2010) that further implicates dysfunctional brainstem auditory processing and output

(Beebe et al, 2014; Rotschafer et al, 2015) during associative learning. Sensory integration in Fmr1 KO mice is impaired as demonstrated by pre-pulse inhibition data reported here and elsewhere (Nielsen et al, 2002; Frankland et al, 2004). Additionally, studies find that Fmr1 KO mice have increased response thresholds to tone stimuli (Rotschafer et al, 2015). FMRP is reportedly highly expressed in auditory processing neurons of the brainstem in humans (Beebe et al, 2014). Studies have also shown that FMRP controls expression and localization of Kv3.1b potassium channels in brain stem auditory centers (Strumbos et al, 2010). Kv3.1b potassium channels impart neurons with high firing rates (Rudy and McBain, 2001), which are necessary for high fidelity coding of auditory information (Macica et al, 2003). Auditory processing defects in the brainstem of Fmr1 KO mice may produce subthreshold signaling to higher processing centers such as the hippocampus, where US and CS associations are encoded (O'Reilly and McClelland, 1994; Cutsuridis et al, 2010). The number of theta bursts applied to the Fmr1 KO hippocampus *in vitro* can influence LTP magnitude (Lauterborn et al, 2007). In our study, pharmacological regulation of did not alter the reduced startle response amplitude in Fmr1 KO mice, excluding effects of the inhibitor on this system. *In vitro*, hippocampal slices pre-incubated for at least 1 hour with the Rac1 inhibitor NSC23766 displayed a modest but significantly enhanced maintenance of theta-burst LTP. This at least indicates that the capacity for theta-burst LTP in the Fmr1 KO hippocampus can be increased, which could then be reinforced *in vivo* by other systems. Others have

reported that Fmr1 KO mice also display LTP deficits in the auditory cortex induced by high frequency stimulation, HFS (Yang et al, 2014), which along with impairments in primary auditory processing, could explain the inability of Fmr1 KO mice to appropriately associate the tone with the foot shock, regardless of number of pairings, giving validity to the results reported here.

In summary, this study reveals that Rac1 signaling is impaired in a mouse model of autism, possibly linking this small GTPase to associated learning disabilities. This study also shows that learning and memory impairments in Fmr1 KO mice can be rescued by increasing the intensity of training. This parallels results obtained from behavior treatments in patients with learning disabilities. Furthermore, by increasing training intensity it is found that Rac1 undergoes localization changes that implicate an inhibitory mechanism specific to the Fmr1 KO. Whereas typically it is reasonable to examine impaired signaling in the pathophysiological state, it may be of equal value to examine naturalistic mechanisms that may, under certain conditions, correct the impairment. These investigations may unveil therapeutic targets that would never have been considered. In post-mortem brain tissue of autistic individuals, Rac1 appears to be upregulated (Fatemi et al, 2013), as well as positioned in an integral place within dysfunctional molecular signaling nodes in autism (Zeidán-Chuliá et al, 2013). Therefore,

Rac1 may contribute to learning deficiencies in autism and ID, and features an intriguing modulatory target that may complement behavior interventions.

6. Summary of Findings and Conclusions

1. The Fmr1 KO mouse model of Fragile X syndrome (FXS), a leading cause of autism and intellectual disability, exhibits an abundance of immature dendritic spines in pyramidal neurons of the hippocampus.
2. The hippocampus of the Fmr1 KO displays impaired localization of the small Rho GTPase Rac1 between cytosol and membrane across three critical developmental stages. Compared to wild type, Fmr1 KO hippocampus show higher levels of membrane-bound Rac1, suggesting increased activity.
3. Fmr1 KO mice perform poorly in hippocampus-dependent associative learning and memory compared to wild type. Fmr1 KO hippocampus also exhibits deficits in theta-burst stimulation long term potentiation (TBS-LTP), a form of synaptic plasticity linked to learning and memory.
4. Associative learning and memory deficits in Fmr1 KO mice can be corrected by increasing training intensity. Furthermore, hippocampi derived from Fmr1 KO mice that received increased training intensity express increased TBS-LTP that is comparable to wild type.
5. Increasing training intensity in the Fmr1 KO is also associated with distribution of Rac1 between cytosol and membrane in the hippocampus that resembles that of wild type.

6. Treatment of Fmr1 KO hippocampal slices with the Rac1 inhibitor, NSC23766, increases TBS-LTP.
7. Treatment of Fmr1 KO mice with the Rac1 inhibitor rescued hippocampus-dependent forms of memory.
8. Based on these findings in a mouse model of Fragile X Syndrome, Rac1 dysregulation appears to be an important contributor of impaired synaptic plasticity and learning and memory. Furthermore, these investigations are highly suggestive of a self-regulating mechanism induced under conditions favorable to the learning process. These conditions potentially invoke Rac1 inhibition that is conducive to learning, an observation which introduces one of perhaps many therapeutic targets in the treatment of FXS. The pathophysiological state, which by definition is responding differently to the environment, will induce compensatory mechanisms uniquely different from the healthy state. Therefore, it is equally valuable to study self-correcting pathways that function beyond the deficit. Many more animal models of autism have been developed that consistently display similar impairments in synaptic structure and function to those observed in the FXS mouse model. Therefore, other animal models of autism may further help to support the therapeutic utility of Rac1 inhibitors in autism-related cognitive impairment.

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