ABSTRACT

Oiling the Brain: Omega-3 Fatty Acid Supplementation in a Mouse Model of Autism

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Previous work has supported the role of omega-3 fatty acids in the attenuation of certain aspects of the behavioral phenotype of the male *Fmr1* knockout mouse model, a mouse model of ASD. The current study aimed to address important questions regarding the therapeutic efficacy of omega-3 fatty acids for various behavioral and neuroimmunological aspects of the *Fmr1* phenotype. To address these questions, our experimental design utilized two different omega-3 fatty acid administration paradigms, compared to both standard laboratory chow controls as well as a diet controlling for the increase in fat content. In the first paradigm, post-weaning supplementation (after postnatal day 21) with omega-3 fatty acid diet reversed hyperactivity and deficits in startle threshold, but not deficits in pre-pulse inhibition, though the effect on startle threshold was not specific to the omega-3 diet. However, post-weaning supplementation with both experimental diets also impaired acquisition of a fear response, recall of the fear memory and contextual fear conditioning. As hypothesized, post-weaning treatment with omega-3 fatty acids reduced hippocampal expression of IL-6 and this reduction of IL-6 was significantly associated with diminished performance in the fear conditioning

task, specifically implicating this signaling molecule in these behavioral deficits. In the second experimental paradigm, prenatal supplementation with omega-3's attenuated hyperactivity, pre-pulse inhibition, and acquisition of a fear response. While prenatal omega-3 treatment had a positive impact on behavior, prenatal exposure to the control fat diet (i.e. "Western" diet) exacerbated diminished nonsocial anxiety in the *Fmr1* knockout. The improvements in acquisition of a fear response seen in this paradigm were significantly associated with diminished hippocampal expression of BDNF and IL-1β. Finally, as preliminary evidence of the potential sex-specificity of this manipulation, prenatal treatment with both dietary manipulations improved diminished spectral purity of ultrasonic vocalizations in female homozygous *Fmr1* knockouts, but not male knockouts, on PD9. Taken together, this study provides significant evidence that dietary fatty acids throughout the lifespan can significantly impact the behavioral and neuroimmune phenotype of the *Fmr1* knockout model and supports the further development of this novel pharmaceutical alternative.

Oiling the Brain: Omega-3 Fatty Acid Supplementation in a Mouse Model of Autism

by

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A Dissertation

Approved by the Department of Psychology and Neuroscience

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Charles A. Weaver III, Ph.D., Chairperson

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Doctor of Philosophy

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August 2019

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACTH	Adrenocorticotropic hormone
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
ASD	Autism Spectrum disorder
ASR	Acoustic startle response
CARS	Childhood autism rating scale
CS	Conditioned stimulus
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
D6D	Delta-6 desaturase
EPA	Eicosapentaenoic acid
FDA	Food and Drug Administration
FMRP	Fragile X mental retardation protein
FXS	Fragile X syndrome
GPCR	G-protein coupled receptor
HPA	Hypothalamic-pituitary-adrenal axis
Ig	Immunoglobulin

IL	Interleukin
ISI	Inter-syllable interval
ITI	Inter-trial interval
KO	Knockout
LPS	Lipopolysaccharide
MCI	Mild cognitive impairment
MECP	Methyl CpG binding protein
mGLUR	Metabotropic glutamate receptor
MIA	Maternal immune activation
NIH	National Institutes of Health
PBL	Peripheral blood leukocyte
PBS	Phosphate buffered saline
PD	Postnatal day
PPAR	Peroxisome proliferator-activated receptor
PPI	Prepulse inhibition
PTEN	Phosphatase and tensin homolog
PUFA	Polyunsaturated fatty acids
qRT-PCR	Quantitative reverse transcriptase PCR
RAR	Retinoic acid receptor
ROS	Reactive oxygen species
RXR	Retinoic x receptor
TGB-β	Transforming growth factor beta

TSC	Tuberous sclerosis complex
TTBS	Tris-tween buffered saline
US	Unconditioned stimulus
USDA	United States Department of Agriculture
USV's	Ultrasonic vocalizations
UTR	Untranslated region
VMAT	Vesicular monoamine transporter
WT	Wild type

ACKNOWLEDGMENTS

I would like to thank my mentor Dr. Joaquin Lugo for his continued support and guidance during my time at Baylor University and throughout my project. I would also like to thank my colleagues and fellow graduate students that I have worked with including Samantha Hodges and Matt Binder. Additionally, I would like to recognize the multitude of undergraduate students that have help me on this project including Conner Reynolds, Taylor Jefferson, Brianna Escobar, and James Okoh as well as the many other talented undergraduates that I worked with while at Baylor. I would like to also acknowledge and thank my family, for their never-ending love and support. Additionally, I would like to thank Baylor University and the faculty and staff of Baylor for all the support I received. Finally, I would like to thank Lee Lowe and Natashia Howard, and their staff, for their support and dedication to taking care of the animals.

CHAPTER ONE

Introduction

Fragile X syndrome (FXS) is a neurodevelopmental disorder resulting from a trinucleotide (CGG) repeat mutation in the fragile X mental retardation (*FMR1)* gene, ultimately coding for the Fragile X mental retardation protein (FMRP). Mutations in *FMR1* are one of the most prevalent genetic contributors to inherited intellectual disability (Turner, Webb, Wake, & Robinson, 1996), and the most common genetic contributor to Autism spectrum disorder (ASD) (Kaufmann et al., 2004). In clinical studies, individuals with FXS display a myriad of behavioral abnormalities, including significant cognitive dysfunction, autistic behaviors, and hyperactivity (Hagerman & Sobesky, 1989). These phenotypes are often mirrored in the *Fmr1* knockout, which demonstrates significant learning impairments, hyperactivity, social impairments, and increased repetitive behavior, among other behavioral phenotypes (Liu & Smith, 2009; Nolan et al., 2017; Veeraragavan et al., 2011). Alongside these behavioral phenotypes, recent clinical investigations have also demonstrated significant evidence for aberrant proinflammatory signaling as a key feature in both FXS and its comorbid condition, ASD (Ashwood et al., 2011; Ashwood, Nguyen, Hessl, Hagerman, & Tassone, 2010). Previous pre-clinical studies have shown similar evidence of changes in both proinflammatory and anti-inflammatory cytokine signaling following homozygous loss of *Fmr1* (Hodges, Nolan, Reynolds, & Lugo, 2017; Pietropaolo et al., 2014). Aspects of these immune

phenotypes are receiving mounting attention as potential avenues for therapeutic development.

Recently, in the field of *Fmr1* research, there has been strong preclinical evidence for several treatments, including metabotropic glutamate receptor 5 (mGluR5) antagonism and GABA receptor agonism, and this has facilitated a strong effort to conduct clinical trials (Berry-Kravis et al., 2017). One treatment that received considerable attention was minocycline, a broad-spectrum antibiotic that attenuates inflammatory signaling in the brain (Cheng et al., 2015). In the *Fmr1* mouse model, treatment with minocycline has been demonstrated to ameliorate a host of behavioral and physiological changes, including improved synaptic maturation, reduced hyperactivity, rescued social recognition memory and improved communication phenotypes (Bilousova et al., 2009; Dansie et al., 2013; Dziembowska et al., 2013; Rotschafer, Trujillo, Dansie, Ethell, & Razak, 2012; Yau, Chiu, Vetrici, & Christie, 2016). However, clinical trials to date have been inconclusive regarding the long-term efficacy, overall safety and tolerability of minocycline in human populations (Leigh et al., 2013; Paribello et al., 2010; Schneider et al., 2013; Utari et al., 2010). Therefore, other treatments that work under similar anti-inflammatory mechanisms may present as additional preferable treatment options. One such therapy, omega-3 fatty acids, has also been investigated for the potential to alleviate symptoms associated with many neurodevelopmental disorders, including ASD (Amminger et al., 2007) and Rett syndrome (De Felice et al., 2012). In the *Fmr1* model, post-weaning treatment with omega-3 fatty acids resulted in improvements in novelty exploration, social interaction, and object recognition in

association with reductions in neuroinflammatory signaling markers in the adult *Fmr1* mouse model (Pietropaolo et al., 2014).

Treatment with omega-3 fatty acids incorporates several potential mechanisms that may be relevant to the current model. First, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two of the primary omega-3 fatty acids, are significant components of the neuronal membrane, and their composition in this membrane regulates functioning of key transmembrane receptors and other proteins (Frisardi, Panza, Seripa, Farooqui, & Farooqui, 2011). Previous work has demonstrated that even one substitution of a single-bonded PUFA for a double-bonded PUFAs exerts changes in membrane properties that in turn alter constitutive activity of integral membrane proteins, such as rhodopsin (Eldho, Feller, Tristram-Nagle, Polozov, & Gawrisch, 2003). Mechanistically, increasing the bioavailability of long chain polyunsaturated fatty acids may also enable important anti-inflammatory mechanisms (Kang & Weylandt, 2008). These may be accomplished through a variety of methods, including interactions with G-protein coupled receptors or downstream production of small anti-inflammatory compounds (Oh et al., 2010). There is also significant evidence for an influence of DHA on markers of synaptogenesis and expression of glutamate receptors (Cao et al., 2012).

While the aforementioned mechanistic evidence supports a role of omega-3s as a potential therapeutic for the *Fmr1* knockout phenotype, it is unknown if administration of this intervention at an earlier timepoint would result in differential effects on the behavioral and neuroinflammatory phenotypes studied. Accumulation of fatty acids is fundamental for the formation of neuronal membranes, particularly early in development (Yehuda, 2012). Studies of fatty acid incorporation demonstrate that essential long chain

polyunsaturated fatty acids, such as DHA and EPA, are not absorbed and incorporated appropriately during development in children with ASD (Vancassel et al., 2001). Moreover, increased bioavailability of fatty acids during early development can induce discernable phenotypic changes as early as the weaning point (Bongiovanni, Depeters, & Van Eenennaam, 2007). While no studies have examined the therapeutic potential of prenatal administration in the *Fmr1* model, previous studies have supported the potential for prenatal omega-3 fatty acids in the reversal of autistic-like deficits in other ASD rodent models (Fortunato et al., 2017; Garay, Hsiao, Patterson, & McAllister, 2013; Li et al., 2015; Malkova, Yu, Hsiao, Moore, & Patterson, 2012).

The current study aimed to address questions regarding the therapeutic efficacy of omega-3 fatty acids for various aspects of the *Fmr1* phenotype, such as how the behavioral effects are mediated through changes in inflammatory gene expression, whether timing matters, and how previous effects compare when referenced to a standard dietary control. In the first experiment, we exposed male *Fmr1* knockouts to dietary manipulations in the post-weaning period and examined various aspects of adult behavior and cytokine signaling markers. In the second experiment, we administered similar dietary manipulations during the prenatal period to investigate how prenatal dietary manipulations prior to weaning will influence these same behaviors and cytokine signaling markers during adulthood in male *Fmr1* KO mice. Additionally, we collected ultrasonic vocalizations on postnatal day (PD) 9 during this prenatal paradigm to determine the impact of omega-3's on early communicative behaviors in *Fmr1* KO mice.

CHAPTER TWO

Literature Review

Autism Spectrum Disorder

Introduction and History of ASD

Autism spectrum disorder (ASD), also formerly known as autism, is neurodevelopmental disorder, characterized by repetitive behavior and deficits in social behavior and communication. The word "autism" is derived from the Greek word, *autos*, meaning self. Autism was originally referred to as Kanner's syndrome, named after the doctor who described it in 1943. There is some controversy about when it was first described, as other reports insist it was first described as early as 1887 by Dr. John Langdon Down, who characterized Down's syndrome. Similarly, Dr. Hans Asperger described a similar disorder characterized by social and emotional limitations, and overall withdrawn behavior in 1944, which he named Asperger's syndrome. In 1949, Kanner next identified a small sample of families with children with Kanner's syndrome, and from this study, he proposed his "refrigerator mother" theory, suggesting that children with this syndrome were products of "a cold mothering style". This theory has since been discredited. Later, findings from twin studies lent credence to the hypothesis that this set of disorders had a genetic basis (Folstein & Rutter, 1977). The diagnosis of autism, known at the time as "infantile autism," wasn't added to the Diagnostic and Statistical Manual of Mental Disorders (DSM) until the third edition published in 1980. The definition of the diagnosis remained the same until 2013, when the DSM-V changed the

criteria for autism from 3 features to 2 features, and the diagnostic category no longer has any subheadings (i.e. Asperger syndrome). Today, all former autism diagnoses fall on a spectrum, now known under the heading "Autism Spectrum" disorder (ASD). The prevalence of this disorders is estimated to be 1 in 68 children. There is a significant difference in the prevalence of ASD between sexes, with a higher prevalence in males, around 1 in 42 males, compared to 1 in 189 females (CDC, 2014). There is concern regarding the rising rates of diagnosis, though a portion of this increase is believed to be accounted for by changes in diagnostic criteria and increased awareness (King & Bearman, 2009). Even today, there is still considerable debate about the causes of autism. Moreover, the significant heterogeneity among behavioral and genetic phenotypes of individuals with ASD has led to difficulty in treatment options that persists today.

Characteristics of ASD

Autism is a disorder that appears in infancy, and is most commonly diagnosed in school-age children (Barbaro & Dissanayake, 2009). The behavioral phenotype of ASD is highly variable among individuals with this diagnosis, and the severity of these phenotypes can also vary significantly. According to the DSM the two major symptom categories have been identified as consistent across individuals (American Psychiatric Association, 2013). The first category involves social abilities, such as deficits in social interaction and deficits in social communication. Examples of social deficits include social-emotional reciprocity, poor integration of verbal and nonverbal communication, and difficulty developing and managing social relationships. These social deficits manifest themselves early in development, but become more evident as children with ASD fail to socialize with other children (Lord, Cook, Leventhal, & Amaral, 2000).

Deficits in social communication are especially prevalent and have been studied in detail. Aside from obvious deficits in verbal communication later in life, these communication deficits may be distinguished as early as 1 year of age, using comparative analysis of infant crying (Esposito & Venuti, 2009). In the same study, maternal infant reactions were also examined, and found that maternal reactions to crying episodes were qualitatively different as well (Esposito & Venuti, 2009). The purposes of nonverbal communication, prior to the emergence of language, in children with ASD between the ages of 2 and 5 years old are also qualitatively different (Maljaars, Noens, Jansen, Scholte, & van Berckelaer-Onnes, 2011).

The other major diagnostic criterion includes restricted interests or repetitive patterns of behavior (American Psychiatric Association, 2013). For repetitive behavior, there are several types of behavior individuals with ASD engage in that fit this criterion. These include: stereotypy (i.e. handflapping), self-injurious behavior (i.e. head banging), compulsions and tics (Bodfish, Symons, Parker, & Lewis, 2000). Compared to individuals with intellectual disability alone, individuals with ASD are more likely to engage in compulsive behaviors, stereotypy and self-injury, and the severity of these is significantly associated with the severity rating of ASD itself (Bodfish et al., 2000). Restricted interests, the other aspect of this diagnostic criterion, is a commonly reported characteristic among parents, as children with ASD often focus in on one or a small number of toys, or restrict their food preferences (Baron-Cohen & Wheelwright, 1999). The severity of this diagnostic criterion is significantly associated with the presence of anxiety symptoms or anxiety-related pathologies, such as obsessive compulsive disorder, in individuals with ASD (Spiker, Lin, Van Dyke, & Wood, 2011).

Aside from the major two categories, there are many other symptom categories that can arise in this condition. Another symptom cluster also common to individuals with ASD includes hypo- or hyperreactivity to sensory input. This is most commonly manifested as deficits in sensorimotor gating in clinical evaluations. Prepulse inhibition of the acoustic startle reflex is a phenomenon wherein a smaller decibel stimulus (prepulse) precedes the startle stimulus, and results in attenuation of the startle reflex. Increased prepulse inhibition and sensitization of the startle reflex are two symptoms of children with ASD (Madsen, Bilenberg, Cantio, & Oranje, 2014). ASD is often comorbid with other psychiatric or medical conditions. Intellectual disabilities are a common comorbidity among these individuals and can be an accompanying diagnosis, with an estimated prevalence of 38% of individuals with ASD (CDC, 2014). Other comorbidities include epilepsy, ADHD, anxiety, sleep disorders, and gastrointestinal difficulties (Adams, Johansen, Powell, Quig, & Rubin, 2011; Klukowski, Wasilewska, & Lebensztejn, 2015; van Steensel, Bögels, & Perrin, 2011; Volkmar & Nelson, 1990). The severity of some of these issues, including sleep and gastrointestinal difficulties can predict the intensity of the main diagnostic criterion (Adams et al., 2011; Schreck, Mulick, & Smith, 2004).

The Origin of ASD

Efforts to understand that underlying neural mechanisms of these disorders have not yielded understanding of brain abnormalities underlying these behaviors. While some results have been promising, such as changes in white matter structure and functional imaging, to date, they have all be inconclusive. Much of the focus has since been on understanding the complex genetics of ASD.

Genetics of ASD. Recent estimates suggest that the heritability of ASD is as high between 90 to 95% in some samples (Colvert et al., 2015; Sandin et al., 2014). In population based samples ASD heritability is estimated to be 0.50 (Sandin et al., 2014). Given the high heritability, the predominant theory of the underlying cause of ASD is a genetic. The genetic architecture of ASD is highly heterogenous, as one fully penetrant mutation is enough to cause ASD, while a different individual with an ASD diagnosis may have an accumulation of several low-risk alleles (Bourgeron, 2015). Over 1,000 single-nucleotide polymorphisms have been identified, and together these are estimated to account for between 17 and 60% of ASD cases (Gaugler et al., 2014; Klei et al., 2012; Lee et al., 2013). The largest monogenic cause to date associated with ASD is copy number variation in the *FMR1* gene, which accounts for 2 – 6 percent of cases of ASD (Kaufmann et al., 2004). The other most common of these mutations includes: tuberous sclerosis complex (TSC) (Baker, Piven, & Sato, 1998), phosphatase and tensin homolog (PTEN) (Buxbaum et al., 2007), and methyl-CpG-binding protein 2 (MECP2) (Moretti & Zoghbi, 2006).

Efforts to understand the genetic underpinnings of ASD have yielded no conclusive evidence of any unifying gene family. The distinction between causes of ASD with known genetic causes and those with unidentified causes leads to the classification of two major types of ASD: syndromic and idiopathic. The former category includes those with associated neurological disorders (i.e. syndromes), such as Fragile X syndrome or Rett syndrome. Idiopathic ASDs are those for which genetic causes have not been characterized. In the aforementioned study, in addition to heritability being estimated at 0.50, the nonshared environmental influence was also estimated to be 0.50

(Sandin et al., 2014). Moreover, the concordance rate for fraternal twins (31-36%) is higher than the observed rate for siblings of different ages (3-14%) (Constantino, Zhang, Frazier, Abbacchi, & Law, 2010; Hallmayer et al., 2011). These results suggest that aspects of the fetal environment may contribute to some of the variance. There are three main factors hypothesized to contribute to the development of autistic-like symptoms covered here: immune factors, stress and dietary features.

Environmental factors and ASD. In the search for biomarkers of ASD, empirical reports of inflammatory activity have been demonstrated to be altered in ASD. The most common empirical biomarker for altered immune system activity is expression of cytokines. Cytokines are small peptides secreted by several cells throughout the body, including immune cells, such as macrophages and T cells, as well as cells of the nervous system, i.e. neurons and microglia. These signaling molecules can serve both autocrine and paracrine functions. Typically, they are classified as either proinflammatory or anti-inflammatory, however, often they can be both. With respect to ASD, upregulated plasma levels of several proinflammatory cytokines, including IL-4, IL-5 and IL-13, have been demonstrated (Molloy et al., 2006). This study also showed that the severity of the behavioral symptoms of ASD is significantly correlated with such aspects of immune system functioning. In one sample, children with ASD had lower plasma levels of the transforming growth factor beta-1 (TGFβ1), and these levels were associated with maladaptive behaviors, including hyperactivity, irritability, lethargy and stereotyped behaviors (Ashwood et al., 2008). Reduced levels of immunoglobulins (Ig), particularly IgG, indicative of altered humoral immunity, found in individuals with ASD are also associated with these same behavioral abnormalities (Heuer et al., 2008). Increased levels

of several plasma proinflammatory cytokines, including IL-1β and IL-6, have been noted in subgroups of children with regressive ASD (Ashwood et al., 2011). In addition to alterations in baseline expression, the cytokine response of the immune cells isolated from individuals with ASD is also exaggerated when these cells are stimulated with an immune stimulant, lipopolysaccharide (LPS) (Jyonouchi, Sun, & Le, 2001). Taken together, these plasma analyses indicate that ASD is associated with dysfunction of the humoral immune response, which is important for initiating an adaptive immune response against invading pathogens or antigens. Evidence of immune irregularities have also been localized to post-mortem brain tissue in individuals with ASD (Li et al., 2009), as well as fresh tissue from living individuals, suggesting that immune system activity in the brain is also altered (Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005). While these studies provided the basis for immune involvement in the ASD phenotype, the directionality of this relationship, whether causal or simply associated, is not yet clear.

Given the evidence of an immune component in individuals with ASD, maternal infection has also been theorized to contribute to the development of autistic-like symptoms in offspring. One study estimated that 20,000 to 30,000 children in the US were born with congenital malformations linked to the 1964 US rubella epidemic (Chess, 1971). This seminal study has been expanded upon by more recent studies. Using information from the Danish National Hospital Register, calculation of hazard ratios indicate that viral infection requiring hospitalization during the first trimester is associated with increased risk of ASD diagnosis in infants, as well as bacterial infection during the second trimester (Atladottir et al., 2010). Other studies indicate this relationship may be less specific to the trimester or type of infection. In a Swedish

sample, maternal inpatient diagnosis of infection during pregnancy was associated with increased risk of ASD in the child (Lee et al., 2015). This included a broad spectrum of infections that included bacterial, viral and others, across all three trimesters (Lee et al., 2015). Similarly, in US samples, in-patient diagnosis of maternal infection significantly increased ASD risk (Zerbo et al., 2015). A systematic meta-analysis of the topic suggested that maternal infection during pregnancy is associated with a 12% increase in ASD risk. However, when interpreting the results for those infections requiring hospitalization, the percentage jumped to a 30% increase in risk of developing ASD in offspring (Jiang et al., 2016). Altogether, these results indicate that the severity of the infection may be an important mediator of the relationship between maternal infection and the risk of ASD in offspring.

The utility of animal studies has facilitated understanding of mechanisms for how maternal immune activation, such as infection, may influence the development of autistic-like symptoms. The maternal immune activation (MIA) model is a commonly used animal model, wherein some bacterial or viral mimetic is administered during pregnancy to induce an inflammatory response. Behaviorally, results of the MIA model display autistic-like robust alterations on a variety of behavioral paradigms (Garay et al., 2013; Malkova et al., 2012; Schwartzer et al., 2013; Xuan & Hampson, 2014). For example, pups born to dams with MIA produce fewer ultrasonic vocalizations in response to a brief separation period, and these communication deficits persist into adulthood (Malkova et al., 2012). Deficits in social behavior on the three-chambered social task and increased marble burying have also been noted (Malkova et al., 2012; Schwartzer et al., 2013). Therefore, the evidence supports a directional relationship between immune

aberrations and the development of autistic-like traits related to widespread alterations in cytokine signaling homeostasis.

Mechanistically, these autistic-like behavioral changes are thought to be due to chronic changes in cytokine expression (Arrode-Brusés & Brusés, 2012; Smith, Li, Garbett, Mirnics, & Patterson, 2007). Using proteomic analysis of cytokine levels in the MIA model, one study analyzed the expression of 23 different cytokines across 3 brain regions (frontal cortex, cingulate cortex, and hippocampus), and across five time points (P0, P7, P14, P30 and P60). Results indicated that in healthy developing offspring, cytokine expression displays region-specific and age-specific patterns, not related to peripheral immune cell infiltration. For example, in frontal cortex, levels of proinflammatory cytokines, including IL-1β, IL-10, IL-12, and IL-6, are elevated around the time of birth, decrease during synaptogenesis periods, and then increase again during adulthood. However, in the offspring of MIA dams, there were widespread changes in these patterns, as determined by bioinformatic analysis. In the hippocampus, IL-6 is significantly elevated in response to MIA at birth, while at P30, IL-6 is significantly decreased in these same MIA-exposed offspring (Garay et al., 2013). This work also determined that brain levels of cytokines were not related to peripheral immune cell infiltration, as assessed using immunostaining. It is therefore hypothesized that microglia or other glia cells are producing these cytokines. Exposure to infection early in postnatal development, as well as prenatally, results in increases in microglia numbers in the hippocampus and parietal cortex, associated with decreases in neurogenesis in the dentate gyrus of the hippocampus (Bland et al., 2010). Overall, work from animal models suggests that the relationship between immune biomarkers and autistic-like behavior is

directional, as early-life perturbations in the balance of the inflammatory system lead to robust changes in the behavioral and molecular phenotype related to ASD.

Another factor that is empirically shown to contribute to ASD etiology is maternal stress. High levels of stress during early prenatal development is considered a risk factor for several neurodevelopment disorders including ASD and ADHD (Ronald, Pennell, & Whitehouse, 2010). However, the specifics about the most sensitive period during gestation to stress and which type of stress has the largest impact are not quite clear. Longitudinal studies have demonstrated that stressful life events, such as divorce or moving, show a small, but statistically significant relationship with autistic traits in a large American sample (Ronald et al., 2010). In retrospective studies, it has been demonstrated that third-trimester prenatal stress (in this case, bereavement stress) increased the risk of ASD in offspring (Class et al., 2014; Walder et al., 2014). Stressful experiences may have some commonalities with the previously mentioned effects of maternal immune activation. The experience of stress and subsequent glucocorticoid release directly suppresses the immune system, leading altered cytokine expression (Selye, 1955). Furthermore, the psychological experience of an infection, typically "sickness behavior," has also been associated with increased plasma concentrations of stress hormones, such as adrenocorticotropic hormone (ACTH) and corticosterone, thereby categorizing infection as a "stressor" (Dunn, 1993; Dunn, 2000). Thus, it is difficult to disentangle the impact of stress and inflammation.

One other environmental factor of note, and the main interest of the current prospectus, is the impact of diet on the etiology of autism. During fetal development and during early postnatal development, the fetus receives all nutrition from the mother.

Several dietary factors have been theorized to relate to the development ASD, including certain vitamins, minerals and fatty acids. Fatty acids are a major component of neuronal cell membranes, and are incorporated in large amounts, during early brain development, and may be involved in neuronal cell migration. The temporal specificity of these factors is variable, and as such, prenatal and postnatal factors will be reviewed separately. In a small cohort, pregnant mothers in the lowest 5% of omega-3 fatty acid intake had a significant increase in offspring ASD risk (Lyall, Munger, O'Reilly, Santangelo, & Ascherio, 2013). Moreover, low prenatal omega-3 to omega-6 ratio, as assessed by maternal plasma levels mid-pregnancy, was associated with autistic-like phenotypes in a cohort of children in the Netherlands (Steenweg-de Graaff et al., 2016).

In addition to longitudinal studies of birth outcomes, several studies have reported reduced plasma levels of omega-3 fatty acids in individuals with ASD, further supporting a role for these compounds in the ASD phenotype (Brigandi et al., 2015; Vancassel et al., 2001). Incorporation of these fatty acids into the brain has been demonstrated to continue during the early postnatal period in rats, suggesting that the early postnatal period is still vulnerable to dietary modifications of fatty acid status (Sinclair, 1975). Several studies have focused on the role of postnatal supplementation with the polyunsaturated fatty acid DHA. One study demonstrated in a sample of 30 children (age range 3 – 11 years) with ASD that DHA supplementation twice daily for three months resulted in improvement of the Childhood Autism Rating Scale (CARS) score in 66% of individuals, associated with increased plasma levels of omega-3 fatty acids, as assessed by mass spectrometry (Meguid, Atta, Gouda, & Khalil, 2008). In a different sample of children aged 5 to 17 years, 6-week supplementation of DHA/EPA reduced hyperactivity and stereotypy in a

cohort of individuals with ASD (Amminger et al., 2007). These studies have provided evidence of the potential for DHA and EPA to attenuate some aspects of the ASD phenotype. Prior to understanding this relationship, it's also important to understand how omega-3 fatty acids influence behavior in normally developing ("neurotypical") individuals.

The Role of Omega-3 Fatty Acids in Neurotypical Individuals

Omega-3 Deficiency

Human studies. In developed countries, despite the overwhelming availability of food, there has been a steep rise in the rate of childhood and maternal obesity, associated with increase in inexpensive high-calorie/high-fat foods (Kearney, 2010). This shift in food dynamics has left many without proper intake of several key nutrients, including omega-3 fatty acids. Meta-analyses indicate that plasma levels of EPA and DHA are low ($\leq 4\%$) in Western countries like the United States, compared to $> 8\%$ in populations not adapted to the Western diet (Stark, Van Elswyk, Higgins, Weatherford, & Salem Jr, 2016). With the decline in consumption of these key nutrients, it is especially important to understand how this particular type of malnutrition can impact development (Wang, Monteiro, & Popkin, 2002). Another contributor to the decline in omega-3 fatty acid consumption is the concern among pregnant women is the risk of methyl mercury, which is found in high amounts in seafood. In 2004, the Food and Drug Administration (FDA) advised all pregnant women to limit seafood consumption to 2 to 6-oz servings per week, thereby limiting fetal exposure to mercury (EPA-FDA, 2004). The recommendation had an unintended consequence of a overall reduction in consumption of omega-3 fatty acids

by pregnant women. This advice has been superseded by new advice from 2017, which recommends 2 – 3 servings of 8 - 12 oz of fish low in methylmercury (EPA-FDA, 2017). The new advice also details the levels of methylmercury found in various species of seafood and makes instructions on which to avoid/ consume based on these levels (EPA-FDA, 2017). Moreover, systematic reviews on the topic suggest that consuming appropriate amounts of seafood during prenatal development may confer benefits that outweigh many negative consequences of methylmercury (Starling, Charlton, McMahon, & Lucas, 2015). Observation-based studies have demonstrated that maternal seafood consumption (the major dietary source of omega-3 fatty acids) of less than 340 g during each week of pregnancy was associated with performance in the lowest quartiles for verbal intelligence, prosocial behavior, fine motor coordination, and communication (Hibbeln et al., 2007). While these support to the necessity of omega-3 fatty acids in early development, it is difficult to accurately assess consumption in these types of retrospective studies, and experimental manipulations of these dietary parameters in pregnant women are against the policy of the National Institutes of Health (Institute of Medicine Committee on the & Legal Issues Relating to the Inclusion of Women in Clinical, 1999).

Animal studies. Studies using rodents allow more experimental manipulations that are not ethical in pregnant women. The impact of dietary omega-3 fatty acid insufficiency has been examined on several different behavioral facets. With respect to activity levels, the findings are mixed. Some studies have found that omega-3 deficiency during early prenatal development does not impact locomotive behaviors, including novelty-induced exploration in rats (Bourre et al., 1989; Nakashima et al., 1993), while one reported

increased locomotion (Umezawa et al., 1995). However, Enslen and colleagues (1991) reported decreased exploratory behavior in a novel environment, though total locomotor activity was unchanged in adult rats raised on diminished omega-3 diets (Enslen, Milon, & Malnoe, 1991). Increased anxiety following omega-3 deficiency has also been demonstrated using the elevated plus maze, but only when the deficiency was combined with a stress manipulation (Fedorova & Salem, 2006). Perturbations in sensorimotor gating behavior have also been demonstrated following rearing on an omega-3 deficient diet (Fedorova, Alvheim, Hussein, & Salem, 2009). Moreover, male offspring reared by damns kept on omega-3 deficient diets (0.05% of total fatty acids) and maintained on that diet throughout life, demonstrated deficits in motor coordination, sensory integration and spatial memory (Janssen et al., 2015).

Exposure to high levels of other fatty acids, such as omega-6 fatty acids, has been shown to produce autistic-like sociability deficits in offspring (Jones, Will, Hecht, Parker, & Beversdorf, 2013). These findings highlight that it may be the ratio of omega-3 to omega-6 fatty acids, rather than the omega-6 themselves that may be impacting development, as increases in omega-6 intake lowers the aggregation of omega-3 in the brain (Raygada, Cho, & Hilakivi-Clarke, 1998). This relationship may be due to diminished incorporation of omega-3 fatty acids into the brain, as empirical studies have related this to in increased aggression towards a conspecific in the resident intruder test (Raygada et al., 1998). However, this diminished incorporation is potentially reversible. For example, in the previous study, mice on the low omega-3 diet were then switched to a regular diet and the levels of these fatty acids were assessed in post-mortem brains using mass spectrometry. Individuals from the dietary repletion group had significantly higher

levels of omega-3s and lower levels of omega-6s than the omega-3 deficient group. In line with this idea, the negative behavioral consequences of omega-3 fatty acid deficiency have also been ameliorated by dietary repletion of DHA and EPA. In one study, deficits in spatial cognition performance in offspring reared on omega-3 fatty deficient diets can be subsequently rescued with dietary reinstatement (Moriguchi & Salem, 2003). Taken together, these results suggest a role for deficiency of omega-3 fatty acids in the development of several autistic-like behaviors. Given the value of omega-3 fatty acids during early development, it is also necessary to examine how dietary supplementation may positively impact development of these behaviors in neurotypical individuals.

Dietary Enhancement with Omega-3 Fatty Acids

Human studies. Intake of seafood varies significantly in the U.S. population, particularly among different socio-demographic groups (Jahns et al., 2014). As such, assessing how different levels of supplemented polyunsaturated fatty acids relate to differences in neural development in offspring is important. In one study, increased maternal second-trimester fish intake correlates with improvements in visual motor abilities and vocabulary testing in the offspring (Oken et al., 2008). Like findings of prenatal development, in supplementation paradigms, studies have demonstrated positive impacts on cognition when supplemented during early childhood development. For instance, in neurotypical children, combined supplementation of DHA and other fatty acids significantly improves cognitive and motor development in infants (Birch, Garfield, Hoffman, Uauy, & Birch, 2000). These results suggest that early exposure to dietary supplementation may positively impact behavior.

Touted as the magic bullet for all aspects of brain development and functioning in the popular press, several studies have assessed whether omega-3 fatty acid supplementation is beneficial for cognitive performance in healthy adult populations. However, supplementation of DHA/EPA enhanced fish oil in both young and older adults results in no improvement in cognitive performance or aspects of mood (Jackson, Deary, Reay, Scholey, & Kennedy, 2011; van de Rest et al., 2008). While results fail to find impacts in neurotypical adults, there is mounting evidence that supports the beneficial impact of increased intake of omega-3 fatty acids for cognition among certain neurological conditions. For example, in populations of adults showing signs of mild cognitive impairment (MCI), some studies have found beneficial effects of fish oil supplementation on both cognitive function and aspects of mood (Lee, Shahar, Chin, & Yusoff, 2013; Sinn et al., 2011). In metanalytic studies, only patients with MCI seem to benefit from this supplementation, not healthy adults or those with Alzheimer's disease (Mazereeuw, Lanctôt, Chau, Swardfager, & Herrmann, 2012). Moreover, the only domains that showed a positive impact in the MCI patients included attention and processing speed. No changes were noted in composite memory, delayed recall, recognition memory, working memory, and executive function.

Animal studies. While human studies failed to find a positive effect of supplementation, evidence from animal models provides positive evidence for longer-term supplementation protocols. As previously mentioned, recent evidence suggests that the possible benefit of omega-3 fatty acids are a direct product of the ratio of fatty acids (omega-6 to omega-3). Long-term (from age 3 – 7 months) supplementation with a high omega-3:omega-6 ratio diet improves performance on the hippocampal-dependent

Barnes maze task, and decreases hippocampal expression of the proinflammatory cytokine, TNF-α (Grundy, Toben, Jaehne, Corrigan, & Baune, 2014). Moreover, higher intake of omega-3 PUFAs, resulting in a lower ratio of omega-6 to omega-3, results in increased neurogenesis in the dentate gyrus and CA3 regions of the hippocampus (Fan et al., 2015; Grundy et al., 2014).

Similarly, the effect of omega-3 fatty acid supplementation can also be impacted by when the behavior of interest is tested in non-human animals. In one study, animals were exposed to a high-fish oil diet, enriched with both DHA and EPA, from pregnancy through adulthood, and maintained on the diet throughout the lifespan (including the testing period). Three separate groups were tested to examine the possibility of an age-related effect: young (2 -3 months old) mice, mature (9 – 10 months old), mature (17 – 19 months old) (Carrie, Guesnet, Bourre, & Frances, 2000). Exposure to a high fish oil diet increases exploratory behavior in young mice, compared to mice on a palm oil diet. This effect dissipated as the offspring aged and reversed when the mice reached old age. Meanwhile, retention on the probe trial for the MWM was improved in the mature (9 – 10 months old) group and not affected in the young or old groups (Carrie et al., 2000). Impairments in active avoidance learning in the old and mature (17 – 19 months old) groups. Overall, the findings of this study indicate that long-term supplementation to enhanced levels of omega-3 fatty acids is more beneficial in younger mice and this effect dissipates with age. In a similar study in juvenile rats, perinatal supplementation from gestational day 8 until weaning with omega-3 fatty acids enhanced motor skills assessed early in development, however in adulthood there was no effect on learning and memory performance (Coluccia et al., 2009).

Omega-3 Fatty Acids and Neurodevelopmental Disorders

Introduction

The putative significance of fatty acids as a pharmaceutical alternative for developmental aberrations stems from the observation that fatty acids accumulate rapidly in the brain during development, comprising a major component of the neuronal membrane. Deficiency of these fatty acids during the prenatal period in both human and animals results in a variety of unfavorable outcomes, including intellectual disability, prosocial behavior and perturbed sensorimotor gating behavior. Moreover, a diet high in seafood consumption with these substrates during pregnancy correlates with improved vocabulary and visual motor abilities. In animals, prenatal dietary enhancement with DHA and EPA results in improvements on cognitive spatial tasks early in the lifespan, but these effects are lost by old age. In clinical studies, postnatal supplementation with DHA and EPA in infants improves development of both cognitive and motor skills, however studies in adults have failed to find any impact. The exception to these findings is that when the adults are impaired, such as in cases of mild cognitive impairment, DHA and EPA supplementation results in improvements in cognitive functioning, as well as aspects of mood. With respect to the current project, prior studies in children with ASD has supported the hypothesis that supplementation of omega-3 fatty acids in individuals with ASD may mitigate some or all the symptoms. Taken together, these results suggest that DHA and EPA exert their effects during early development or when neural functioning is at a disadvantage, supporting a role as a therapeutic in ASD. To further evaluate the potential efficacy, it is next important to understand the mechanism of this intervention.

The Proposed Mechanism of Omega-3 Fatty Acids for ASD

Structure and function of dietary fats. Lipids are a key macronutrient used by the body to create biological membranes, such as neuronal membranes. It is imperative to first examine the form and function of lipids. The biochemical properties of lipids are essential to their function, specifically their hydrophobic properties. Lipids are made up of fatty acids, to which most lipids owe their hydrophobic properties. Fatty acids are long hydrocarbon chains of various lengths. These fatty acid chains can be saturated or unsaturated, and the degree of saturation refers to the number of hydrogen atoms bound to the structure, with four hydrogens for every one carbon constituting a "saturated" state. The presence of double bonds among carbons prevents full saturation status, allowing for a fatty acid to become "unsaturated." Moreover, these fatty acid chains are numbered starting at the carboxyl terminus, and named for the parent hydrocarbon, with a denotation of "oic" or "ic" instead of "ate" on the end of the word (for example: oleate becomes oleic acid). Fatty acids typically contain an even number of carbon atoms, between 14 and 24, with the 16 and 18 carbon fatty acids being the most common. The properties of the fatty acids are derived from their chain length and degree of saturation. For instance, unsaturated fatty acids have lower melting points than do saturated fatty acids.

The omega-3 PUFA family consists of α-linolenic acid (ALA), DHA and EPA. Both omega-3 and omega-6 fatty acids constitute about 30 – 35 % of total brain fatty acids, while lipids in general constitute about 50 – 60 % of dry weight of the mammalian brain (Youdim, Martin, & Joseph, 2000). The human body cannot produce these compounds, however can enzymatically synthesize both DHA and EPA from ALA (see

Figure 1.1 from (Scorletti & Byrne, 2013)). Thus, ALA is considered the only essential omega-3 fatty acid. These fatty acids can also be derived from ALA. However, the capacity of this pathway is limited in humans, with less than 5% of available ALA converted to EPA and less than 0.5% converted to DHA (Plourde & Cunnane, 2007). There are many dietary sources of these fatty acids. One common source for these fatty acids is marine fish. Work in rodents has suggested that DHA from marine sources like shellfish are incorporated into the rat brain with an order of magnitude more efficiency that plant sources of DHA and EPA (Sinclair, 1975). Notably, fish do not actually produce EPA and DHA, but single-celled marine organisms (i.e. phytoplankton) eaten by fish are excellent producers of DHA and EPA.

Adequate intake for omega-3 fatty acids can be found in the table below (Table 2.1), adapted from the National Institutes of Health (NIH) Office of Dietary Supplements. These numbers were derived from a report from the United States Department of Agriculture (USDA) Institute of Medicine, referring to dietary intake of ALA, as the only essential PUFA, with the exception of the category for 0 – 6 months, which refers to total omega-3's (Trumbo, Schlicker, Yates, & Poos, 2002). With respect to fish consumption (DHA and EPA), the 2017 guidelines from the FDA state that women and children over the age of 10 should eat two to three servings of fish and shellfish each week (8 – 12 ounces).

Metabolism of omega-3 fatty acids. It is also important to consider how omega-3 fatty acids compounds are broken down and utilized by the body. Following oral ingestion, dietary lipids are hydrolyzed, yielding monoglycerides and other free fatty acids, in the intestinal lumen (Ross, Caballero, Cousins, Tucker, & Ziegler, 2012).

Table 2.1

Institute of Medicine's Report on Dietary Reference Intake

Age	Male	Female	Pregnancy	Lactation
0-6 months	0.5 g	0.5 g		
7-12 months	0.5 g	0.5 g		
1-3 years	0.7 g	0.7 g		
4-8 years	0.9 g	0.9 g		
9-13 years	1.2 g	1.0 g		
14-18 years	1.6 g	1.1 g	1.4 g	1.3 g
19-50 years	1.6 g	1.1 g	1.4 g	1.3 g
51+ years	1.6 g	1.1 g		

Note. (from Trumbo et al, 2002). Reported in grams/day.

In the liver, ALA is first metabolized into stearidonic acid. The end products of the pathway shown Figure 1.1 are then incorporated into the bile salt via passive diffusion. Free fatty acid products are then incorporated into aspects of the lymphatic system and enter circulation. From the circulation system, these fatty acids can be incorporated into various metabolic processes throughout the body.

As demonstrated in the figure below (Scorletti & Byrne, 2013), these fatty acids can also be further broken down within specific cells, for use in different capacities. Generally, the conversion goes from smaller to longer chains, and repeatedly undergoes a process of desaturation and elongation. This metabolism is also a competitive process, as EPA and DHA are more efficiently produced than ARA when ALA is more abundant (Cetin, Alvino, & Cardellicchio, 2009). There are many aspects about the breakdown of omega-3 and omega-6 fatty acids that are similar, including the types of enzymes used in

the process. Moreover, docosapentaenoic acid (DPA), one of the products of a reaction between elongase and EPA, is also a product of the metabolism of the omega-6 family.

Pregnancy is a unique time for maternal metabolism, as well as a critical period of brain development. Given the need for DHA/EPA availability during pregnancy, it is important to consider how these nutrients are passed from mother to fetus early in development. The first trimester is characterized by an anabolic period of metabolism wherein the mother increases net body weight through hyperphagia and energy storage in adipose tissue, including essential fatty acids. Fatty acid storage, in particular, is facilitated by increased insulin responsiveness in the adipose tissue (Knopp, Saudek, Arky, & O'Sullivan, 1973). With respect to omega-3 fatty acids, there is a steady increase in mobilized levels of DHA until 18 weeks gestation, at which point there is a steady decline in these levels (Al et al., 1995). In the third trimester, metabolism shifts to catabolism due to decreased tissue responsiveness to insulin, as the needs of the fetus intensify, particularly as the brain undergoes rapid expansion during this final period (Knopp et al., 1973). While dietary availability is important, pre-pregnancy storage of omega-3's, like DHA and EPA, may also help to promote optimal levels of omega-3 fatty acid incorporation during pregnancy and through birth (Coletta, Bell, & Roman, 2010).

Interestingly, there are also age-specific and sex-specific differences in the activity of the enzymatic reactions that convert smaller chain omega-3's to longer chains like DHA and EPA, mediated by the activity of desaturase enzymes. For example, the activity of delta-6 desaturase (D6D) decreases with age in women, possibly due to declining estrogen levels (Horrobin & Bennett, 1999). This step is critical for proper conversion, as this is the rate limiting step for the rest of the pathway. Estrogen has also

been known to enhance this conversion process, thereby possibly underlying the deceleration of this conversion in post-menopausal women, suggesting that males need more substrate to achieve the same levels of longer-chain fatty acids (Burdge & Calder, 2005). Findings have also demonstrated that female children are also born with more omega-3 fatty acids, suggesting a disparity in fetal needs or incorporation of omega-3 fatty (Colquhoun & Bunday, 1981). Taken together, there is significant support for sex differences present in homeostatic mechanisms of omega-3 fatty acid metabolism.

Mechanism of omega-3 fatty acids. With a foundational understanding of how these compounds are taken up and utilized in the body, it is next important to examine the pharmacology of these compounds. There are four major mechanisms reviewed here: membrane functionality, expression of genes related to inflammation and synaptic function, neurochemical changes and production of small bioactive molecules. The first mechanism of omega-3 fatty acids involves the role of phospholipids, produced from free fatty acids like DHA and EPA, in the neuronal membrane. The lipid bilayer of the neuron, and other cells, exists at a state between gel and liquid crystal, and this property is referred to as fluidity. Membrane fluidity regulates aspects of functioning of transmembrane receptors and other proteins that in turn regulate cellular functioning. Previous work has demonstrated that even one substitution of a single bonded PUFA for a double bonded PUFAs exerts changes in membrane properties that can in turn alter constitutive activity of integral membrane proteins, such as rhodopsin (Eldho et al., 2003). The fatty acid composition of the neuronal membrane is regulated by several factors, including age, disease status and dietary composition.

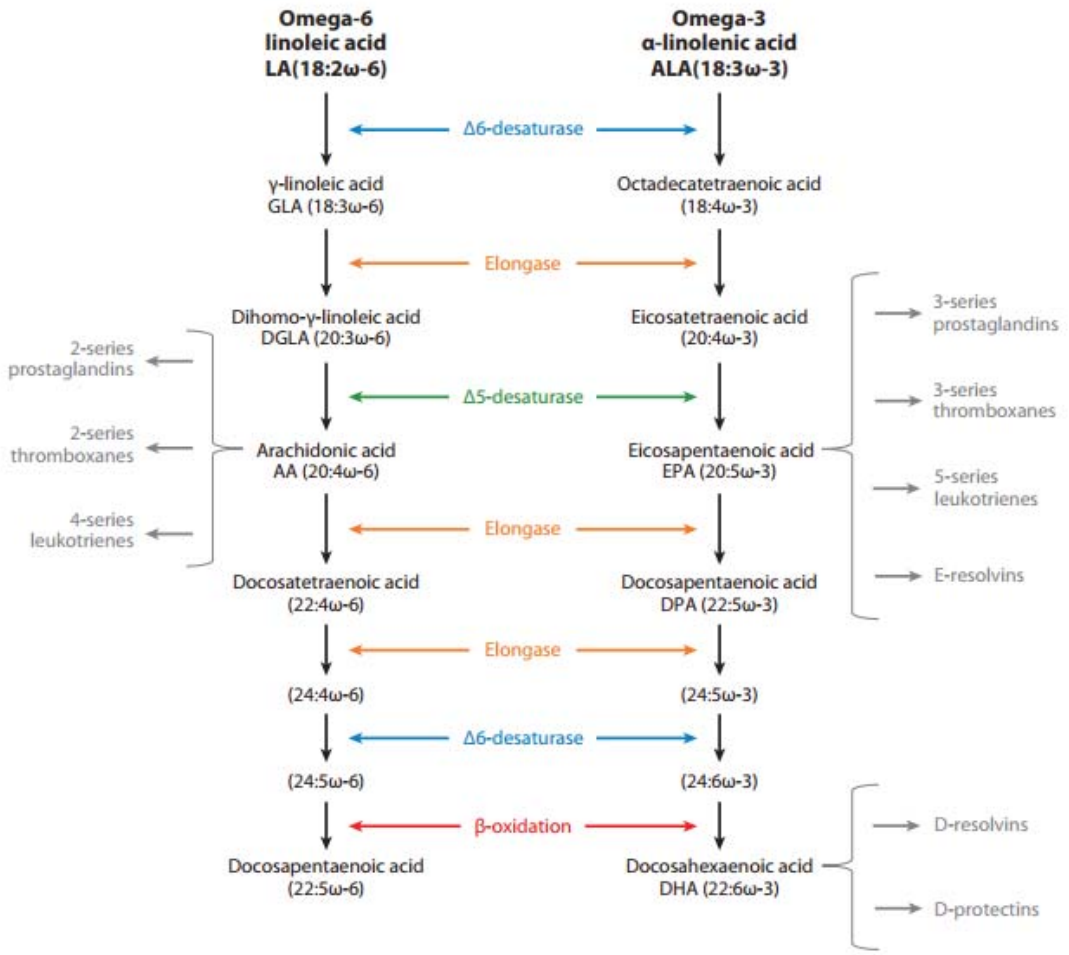

Figure 1.1. Omega-3 and Omega-6 Fatty Acid Metabolism.

For example, dietary modifications can increase content of cholesterol and reactive oxygen species (ROS) in the membrane, altering membrane fluidity thereby constitutive activity of inhibiting ion-channels (Frisardi et al., 2011). Dietary modifications of fatty acid composition also have been directly related to downstream changes in neuronal membrane fluidity (Zérouga, Beaugé, Niel, Durand, & Bourre, 1991). Thus, changes to behavior following dietary modification could be related to this mechanism.

The second mechanism by which treatment with omega-3 fatty acids may alter behavior concerns the effect on gene expression related to synaptic function and inflammation. Both free fatty acids and their metabolites have been demonstrated to activate several transcription factors. One group of targets, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), is involved in learning and memory in rodent models, as activation results in increased neurogenesis in the hippocampus (Dyall, Michael, & Michael-Titus, 2010). Peroxisome proliferator-activated proteins (PPARs) are another target of omega-3 fatty acids. PUFAs are known to bind to PPARγ directly downregulates inflammatory gene expression (Scorletti & Byrne, 2013). Moreover, PPARγ potently inhibits the activation of NFκB, which is responsible for induction of the proinflammatory cytokine response, as well as COX-2 synthesis (Scorletti & Byrne, 2013). In the periphery, free fatty acids can also bind to the G-protein coupled receptor 120 (GPR-120) found on macrophages, a phagocytic immune cell, reducing expression of several markers of inflammation (Oh et al., 2010). In vivo, it has been demonstrated that higher levels of neural inflammation following dietary deficiency of omega-3 fatty acids are directly related to variations in neuronal plasticity-associated genes, as well as balance of the innate immune system in the brain (Madore et al., 2014). Moreover,

aberrant, and in particular, increased cytokine production can be attenuated with dietary supplementation with DHA and EPA (Kang & Weylandt, 2008). Taken together, the support suggests that omega-3 fatty acids confer beneficial effects through a reduction in inflammatory gene expression and subsequent synthesis of inflammatory signaling molecules.

DHA and EPA, as well as other PUFAs, can also alter neural functioning through changes in synaptic functioning and brain neurochemistry. DHA specifically promotes synaptogenesis and expression of synapsin in embryonic mouse hippocampal cultured neurons, as well as expression of glutamate receptors in rat hippocampal neurons (Cao et al., 2009). Another PUFA, arachidonic acid, stimulates the uptake of glucose in cortical astrocytes, playing a critical role in the energy metabolism regulation within the cortex, in in-vitro studies (Yu, Martin, Stella, & Magistretti, 1993). Furthermore, dietary changes in omega-3 fatty acid supplementation has also been demonstrated to alter monoamine concentrations, particularly the dopaminergic and serotonergic systems in the CNS. Many parameters have been demonstrated to be affected including vesicular stores of dopamine and serotonin, accompanied by changes in the vesicular monoamine transporter (V-MAT-2) (Chalon, 2006). Moreover, there is evidence of involvement of cholinergic neurochemistry, as both AA and DHA modulate long-term potentiation through enhanced acetylcholine release, resulting in improved learning (Willis, Shukitt-Hale, & Joseph, 2009). Dietary repletion with omega-3 PUFAs can restore neurochemical abnormalities associated with dietary manipulations such as ALA deficiency (Chalon, 2006), as well as dopamine perturbations in a Parkinsonian model (Meng et al., 2010). Taken together,

perhaps through changes to membrane fluidity and potentially expression, dietary intake of omega-3 fatty acids alter several neurotransmitter pathways.

A final mechanism of how omega-3 fatty acids can impact brain functioning is the production of eicosanoids and other small molecules. Eicosanoids such as prostanoids and leukotrienes are key mediators of immunity. They can be produced from either AA or EPA (both 20-carbon chains). Those produced from AA, an omega-6 fatty acid, are typically more proinflammatory in nature, though some can also be solely anti-inflammatory. Among those produced by EPA, called resolvins, help to resolve sources of acute inflammation and promote return to homeostasis (Serhan, Chiang, & Van Dyke, 2008). Those produced by DHA, called docosanoids, lead to production of d-protectins, and inhibition of the NFκB pathway, thereby reducing inflammation (Asatryan & Bazan, 2017). Altogether, long chain PUFAs can alter neural functioning through production of small molecules that can either serve as ligands to induce expression of proinflammatory cytokines, as well as mediate expression of inflammatory pathways.

Support from other models of ASD. Given the numerous possible mechanisms for omega-3 fatty acids to impact neural functioning and subsequent behavior, it is next important to focus on evidence from other models of ASD to see what behavioral paradigms were affected by this treatment. Overall, there is significant support from different rodent models of ASD to suggest that maintenance on diets enriched with omega-3 fatty acids can attenuate autistic-like behavioral deficits in adult animals. As previously mentioned, the maternal immune activation (MIA) model is one animal model of ASD. In this model, an inflammatory agent, either bacterial (lipopolysaccharides [LPS]) or viral (polyI:C), is administered during pregnancy, inducing an inflammatory

response, and resulting in the development of autistic-like traits in offspring. Dietary supplementation with omega-3 fatty acids from weaning through adulthood ameliorates increased anxiety and impairments in prepulse inhibition following prenatal exposure to polyI:C (Li et al., 2015). These findings have translational relevance to humans, as the dietary enrichment was administered beginning on the weaning date. By definition, ASD onset is by 3 years of age, but the average age of diagnosis in children is age 4, most often when the child goes to school, making early adolescent treatments probable (Mandell, Novak, & Zubritsky, 2005) Others have noted similar findings in rat models exposed during development to lipopolysaccharides (LPS), demonstrating that post-weaning dietary enrichment with omega-3 fatty acids attenuated changes in stereotyped movements and social behavior following prenatal LPS exposure (Fortunato et al., 2017). While these results are promising, several methodological factors of the MIA model vary between studies, including the timing of the agent administration and the inflammatory agent chosen (i.e. polyI:C or LPS), in turn altering the behavioral results. Syndromic ASDs (i.e. those with associated genetic markers), like FXS, often have stable and well-characterized phenotypes, and can be studying using genetic knockout techniques in rodents. As such, these rodent models may provide a better template for understanding how environmental factors, such as diet, may influence the development and manifestation of autistic-like symptoms.

Fragile X Syndrome

Introduction. Fragile X syndrome is one of the syndromic ASDs, due to mutations in the *FMR1* gene, with 2 – 6 percent of individuals with ASD having a mutation in the gene (Kaufmann et al., 2004). Similar to ASD, there is a higher prevalence of FXS in males, with 1:4000 males and 1:8000 females (Crawford, Acuña, & Sherman, 2001). Moreover, an estimated 21 – 50% of individuals with FXS meet criteria for ASD (Moss & Howlin, 2009). FXS is caused by an overexpansion in the X-chromosome 5'-untranslated region (UTR) at position Xq27.3 due to a trinucleotide (CGG) repeat (Verkerk et al., 1991). Currently there are two classes of mutations: the pre-mutation (55 – 200 CGG repeats) and the full mutation (> 200 CGG repeats). This disorder does not follow the typical pattern of Mendelian inheritance, as maternal X chromosomes that carry the pre-mutation expansion are likely to expand into the full mutation during meiosis (Jin & Warren, 2000). Overexpansion in this region, as in the full mutation, due to trinucleotide repeats in the 5' UTR leads to hypermethylation of the *FMR1* gene and functional silencing of the promoter for the protein, fragile X mental retardation protein (FMRP). Methylation status of this promoter is directly related to *FMR1* mRNA expression and subsequent FMRP expression (Pretto et al., 2014).

Functionally, FMRP has been implicated in synthesis-dependent synaptic plasticity, where it acts as an mRNA-binding protein involved with the translation of synaptic plasticity proteins (Fatemi & Folsom, 2011). More generally, loss of FMRP following mutations in *FMR1* is related to deficiencies in activity-dependent synaptic change. For example, FMRP suppresses rapid activity-regulated transport of mRNA.

Loss of FMRP has also been linked to altered glutamatergic signaling, as MAP1b and CAMKIIa mRNA trafficking following metabotropic glutamate receptor (mGluR) stimulation are common FMRP targets. In addition to impeding mRNA trafficking, FMRP also stalls the translocation of ribosomes to mRNA transcripts, such as *Map1b*, by binding to the coding region of the transcript (Darnell et al., 2011). Deficits in trafficking of specific mRNA transcripts results in subsequent immature filopodial spine morphologies that are a major hallmark of FXS (Dictenberg, Swanger, Antar, Singer, & Bassell, 2008).

Behavioral phenotype. Fragile X Syndrome is most commonly characterized by significant intellectual disability (Hagerman & Hagerman, 2002). The severity of the intellectual impairment can range from mild to severe, correlating with the amount of constitutive FMRP expression, which is in turn dependent on the number of repeats and degree of *FMR1* gene methylation (Saldarriaga et al., 2014). Among other comorbidities, there is a high rate of ASD comorbidity in those with FXS, with mutations in the *FMR1* gene representing the largest monogenetic contributor to ASD, accounting for 2 – 6% of individuals with ASD (Reddy, 2005). Several autistic-like features have been reported, including hand-flapping, gaze avoidance, tactile defensiveness, communication impairments and hyperarousal to sensory stimuli (Hagerman, Jackson, Levitas, Rimland, & Braden, 1986).

In addition to variations in prevalence between the sexes, both the quantitative and qualitative aspects of deficits noted in FXS are also dependent on the sex of the individual. Generally, the symptoms in males are considered typically more severe, and this disparity in symptom severity is believed to be attributed to compensation in FMRP

expression in females by the second non-affected X chromosome (Kazdoba, Leach, Silverman, & Crawley, 2014). However, the relationship of gene dosage and the sex-specificity of the phenotype is unclear, given that the male and female phenotypes are also qualitatively different. For example, in males with mutations in *FMR1,* higher prevalence of autistic-like symptoms and hyperactivity is reported (Hagerman & Sobesky, 1989; Reiss & Freund, 1992). In females with the full mutation, intellectual disability is much less likely to be reported, though they demonstrate increased rates of schizophrenia (Freund, Reiss, & Abrams, 1993).

There is also significant variation in levels of FMRP within individuals. In females, given the two X chromosomes, one X chromosome is always silenced, a process called "random X inactivation". Due to this phenomenon, the levels of FMRP expression vary from cell to cell within the same individual, known as "mosaicism" (Pretto et al., 2014). Generally, this process leads to significant variation in FMRP expression between females with full mutations. This process can also take place within individual males with *FMR1* full mutations, as levels of methylation can vary significantly within an individual male, leading to subsequent variation in FMRP production (Nolin, Glicksman, Houck, Brown, & Dobkin, 1994; Pretto et al., 2014). Functionally, IQ scores can be inversely correlated with the percent of methylation, and positively correlated with FMRP expression (Pretto et al., 2014). Thus, like ASD, there is a significant degree of heterogeneity in the behavioral phenotype that can be attributed to several factors.

Immune abnormalities in FXS. Given that the environmental factors examined with respect to ASD all converged on immune mechanisms, it is necessary to review evidence of immune abnormalities in FXS. While there is considerable evidence of

immunological abnormalities in ASD, there is less evidence of this phenotype in FXS. Some upregulations, as in IL-1α, and some downregulations, as in IP-10 and RANTES, have been shown in children with FXS (Ashwood et al., 2010). Moreover, in subgroups of children with both ASD and FXS, plasma levels of IL-1α and IL-12 are increased, while levels of eotaxin, MCP-1α, RANTES and IP-10 are decreased compared to controls (Ashwood et al., 2010). Beyond these findings, other reports of immune deficiency in *FMR1* full mutation cases have been tangentially related to immune functioning. For example, one study found that a subgroup of male children with FXS had more early childhood ear infections (Hagerman, Altshul-Stark, & McBogg, 1987). Similarly, persistent gastrointestinal complications mirror those seen in ASD, including abdominal pain and gastroesophageal reflux (Kidd et al., 2014). These immune and gastroinstetinal difficulties typically correlate with severity of the ASD phenotype including stereotypy and hyperactivity (Adams et al., 2011). In female carriers with *FMR1* premutation (number of repeats between 55 and 200), deficiencies in isolated monocyte production of GM-CSF and IL-12p40 has also been found (Careaga et al., 2014). Moreover, in cultured peripheral blood leukocytes (PBLs) from these premutation carriers, production of IFNγ and MCP-1 are significantly lowered compared to controls (Careaga et al., 2014). All in all, there is mounting evidence to support a role for immune abnormalities with the human FXS phenotype. However, as previously mentioned, due to high levels of mosaicism in both males and females with *FMR1* mutation, it is difficult to determine the specific immune phenotype associated with FXS. Thus, the use of genetic knockout mouse models may provide a more mechanistic depiction of the role of FMRP in immune regulation.

The Fmr1 Mouse

Behavioral phenotype. The *Fmr1* KO model of Fragile X syndrome was developed by the Dutch-Belgian Consortium in 1994 (The Dutch-Belgian Fragile et al., 1994). The murine and human genes have 99% sequence homology (Ashley, Wilkinson, Reines, & Warren, 1993). While human *FMR1* transgenic lines have also been created to model the repeat enhancement, the knockout model has high face validity for the traits of FXS: hyperactivity, decreased motor coordination, increased repetitive behaviors, diminished learning and memory, and altered sensorimotor gating (Romano, Cosentino, Laviola, & De Filippis, 2016) (Reviewed in Table 2.2). Previous work from our lab has replicated other groups' previous findings, demonstrating consistent evidence of hyperactivity, impairments in fear learning and memory and cognitive impairments in the Morris water Maze in the male *Fmr1* KO, as well as evidence of increase prepulse inhibition (Nolan et al., 2017). It should be noted that within this model, strain differences have also been a source of inherent variability within behavioral examinations of this model, both across labs and experiments as well as within the same experiment (Crawley et al., 1997). In a comprehensive review, Crawley et al. (1997) points out that the phenotype of a mutant mouse reflects the gene of interest, as well as interactions with background genes and mutations among these (Crawley et al., 1997).

Immune abnormalities. Similar to studies of individuals with FXS, as well as ASD, there is also evidence of immune abnormalities in the *Fmr1* knockout mouse. In the CA1 of the hippocampus, deficits in IL-10, CD11b and CD45 expression have been noted, as well as increases in CA3 expression of IL-1β (Pietropaolo et al., 2014).

Furthermore, evidence from our laboratory has demonstrated alteration in whole hippocampal expression of TNF-α and IL-6 (Hodges, Nolan, Taube, & Lugo, 2017). These alterations in immune signaling have been exploited as a potential therapeutic avenue, using the compound minocycline. As previously mentioned, due to the role of FMRP in activity dependent synaptic change, increased levels of small and immature dendritic spines are a hallmark of FXS and *Fmr1* mutants alike (Comery et al., 1997). Postnatal treatment with minocycline promoted the maturation of immature dendritic spines in both hippocampal cultures and in vivo (Bilousova et al., 2009).

Behaviorally, this synapse maturation related to normalization of anxiety levels in the elevated plus maze and increased exploration of the Y maze (Bilousova et al., 2009). Treatment with minocycline reversed alterations in ultrasonic vocalization production in adult male *Fmr1* mice (Rotschafer et al., 2012). Hyperactivity and increased repetitive behavior are also attenuated by long-term treatment with minocycline (Dansie et al., 2013). Overall, these data suggest that reduction in inflammatory signaling may reverse both behavioral and morphological abnormalities associated with loss of *Fmr1*.

Regarding FXS, it is possible that compounds such as DHA and EPA, are not being absorbed and incorporated as membrane phospholipids appropriately during early development. Lower levels of DHA/EPA fatty acid incorporation have been demonstrated in children with ASD (Vancassel et al., 2001). This putative failure to incorporate DHA and EPA into the membrane may increase systemic inflammation in the current model and exacerbate deficiencies during development.

Table 2.2.

Comparison of the Fmr1 KO Phenotype Across the Sexes.

Behavioral Domain	♂	♀	References
Neonatal Communication	KO↑	KO↑**	(Baker et al., 2010; Ding, Sethna, & Wang, 2014; Gauducheau et al., 2017; Nguy & Tejada-Simon, 2016; Nolan et al., 2017; Qin, Kang, & Smith, 2005; Reynolds, Nolan, Jefferson, & Lugo, 2016; Rotschafer et al., 2012; Spencer, Alekseyenko, Serysheva, Yuva-Paylor, & Paylor, 2005; Spencer et al., 2011)
Motor Coordination	KO↓	KO↑	
Sensory Gating	KO↑	KO↑	
Repetitive Behavior	KO↔	KO↑	
Social Interaction	KO↔	KO↔	
Learning and Memory	KO↓	KO↓	
Activity Levels	KO↑	KO↔	
Anxiety Levels	KO↓	KO↔	

Note. (Adapted from Romano et al, 2016). ** = Sex differences have been noted in qualitative aspects of the phenotype.

This hypothesis is supported by findings that show that emotional and social deficits in male mice with a genetic mutation in *Fmr1* gene can be attenuated by postnatal dietary supplementation with omega-3 fatty acids (Pietropaolo et al., 2014). For example, deficits in exploration of the three-chambered social task in response to novelty was ameliorated by postnatal dietary omega-3 supplementation. In social interactions with a novel female, KO mice displayed reduced levels of affiliation and this was reversed by omega-3 fatty acid supplementation. In the object recognition task, male KO mice failed to show preference for the novel object, and this was significantly attenuated by omega-3 supplementation. During this task, self-grooming in response to novelty was also shown to be increased in male KO mice, an effect that was attenuated by dietary

supplementation. There were no improvements in social recognition in the three-chambered social task or spontaneous alternation in the Y maze. These behavioral changes were associated with reversal of expression of several cytokines. In the CA1 region of the hippocampus, reduction of expression of CD11b and CD45 in CA1 of the hippocampus was reversed and TNF-α was upregulated in both WT and KO animals following exposure to DHA and EPA. In the CA3 region, overexpression of IL-1β was reversed in KO animals following exposure. In the PFC, reduced expression IL-1β was reversed in KO animals. This study also examined expression of brain derived neurotrophic factor (BDNF) in the hippocampus. Expression of BDNF in the PFC and CA3 was significantly downregulated in the KO mice, and expression of BDNF in the dentate gyrus was significantly upregulated in the KOs in response to dietary supplementation. However, there was no rescue of any BDNF expression in any of the brain areas assayed. Overall, this study provides appropriate evidence for the hypotheses and aims proposed below, yet leaves several domains open for investigation, including other behaviors and cytokines.

Aims of the Present Studies

The overall purpose of the current studies is to expand on what is known about the impact of omega-3 fatty acid dietary supplementation on the behavioral and neuroinflammatory phenotype of *Fmr1* KO mice. We hypothesize that dietary augmentation with omega-3 fatty acids will attenuate many of the behavioral and inflammatory signaling alterations in the *Fmr1* KO mouse. We will test this hypothesis using the following aims:

Aim 1

The first aim of the current proposal is to identify the impact of post-weaning supplementation with omega-3 fatty acids in *Fmr1* KO phenotype, and to measure changes in cytokine signaling in the hippocampus of these mice. First, we expect that our genotype effects will replicate those of previous studies for the effects of loss of *Fmr1* in male mice: hyperactivity, no change in anxiety, no change in social behavior, no change in repetitive behavior, deficits in amygdala-dependent fear learning and memory, enhanced prepulse inhibition and diminished startle responding. Next, the main objective of this aim is to investigate how omega-3 fatty acids will impact these same behaviors. The previous study by Pietropaolo and colleagues found promising evidence that dietary supplementation in adolescent male *Fmr1* KO mice can result in an attenuation of deficits in social behavior and repetitive self-grooming. To investigate this aim, we will replicate this study in our strain of *Fmr1* knockout mice. We also hypothesize that any impairments in social behavior and stereotypy would be ameliorated as well. However, previous investigations of the male *Fmr1* KO mouse in our lab have failed to find deficits on either of these domains. Furthermore, several key aspects of this phenotype were not included in the study by Pietropaolo and colleagues, including tests of fear learning, activity levels and sensorimotor gating. We hypothesize dietary supplementation with the omega-3 fatty acids DHA and EPA will significantly attenuate hyperactivity and deficits in sensorimotor gating in adult male *Fmr1* KO mice. With respect to memory and cognition, we expect that the robust deficits in amygdala-dependent fear memory acquisition and recall noted in the male *Fmr1* KO mouse will remain unchanged by supplementation.

The next objective of this aim is to investigate the hypothesis that a high omega-3 diet will normalize alterations in cytokine production, thereby ameliorating behavioral alterations. We will use quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) to examine expression of the following cytokines in the hippocampus: BDNF, TNF-a, IL-6, and IL-1β. We hypothesize that BDNF will be significantly decreased and IL-1β will be significantly increased in the male KO. We further expect that expression of IL-1β will be significantly rescued by dietary supplementation. While Pietropaolo and colleagues did not find an effect of DHA/EPA supplementation on BDNF, based on other evidence, we still expect that BDNF may be decreased in whole hippocampal sections and rescued by DHA/EPA supplementation. We also hypothesize that deficits in hippocampal expression of these cytokines, IL-6 and TNF-α, will be present in our *Fmr1* male KO, and expression of these will be normalized by dietary supplementation with DHA and EPA.

The final objective of this aim involves the inclusion of the standard diet control. In the previous study by Pietropaolo and colleagues, there were only two isocaloric diet groups present, allowing any comparisons to directly evaluate the impact of the omega-3 fatty acids themselves. No standard lab chow control was presented. In this study, both diets contribute more calories from fat than standard lab chows typically used by experimental labs. For example, in comparison to the diet typically used in our colony which is 13.1 % kCal from fat, the high fat diet is estimated to be 27.3% kCal. Postnatal chronic exposure high fat diet can result in impairments in prepulse inhibition (Labouesse, Stadlbauer, Langhans, & Meyer, 2013) and reduce hippocampal neurogenesis (Park et al., 2010). Furthermore, in rats, post-weaning exposure to high fat

diet alters the maturation of the hypothalamic pituitary adrenal (HPA) axis subsequent anxiety behavior (Boukouvalas, Antoniou, Papalexi, & Kitraki, 2008). Given the significant effect of a high fat diet alone, it's important to investigate the possibility that exposure to a high fat diet, irrespective of the type of fat included, can impact behavior. Thus, for the current proposal two control groups will be used: a standard diet control and diet controlling for the fat increase associated with omega-3 supplementation. We hypothesize that the omega-3 diet and high fat diet will both exhibit effects on behavior, but the high fat diet may negatively impact aspects of behavior, such as prepulse inhibition.

Aim 2

The second aim of this proposal is to investigate the impact of omega-3 fortified diet administration during prenatal and early postnatal development. It is possible that many of the structural changes, and their associated behavioral changes, resulting from loss of FMRP are irreversible at the time of post-weaning period (Krueger & Bear, 2011). Thus, an earlier time point, such as a prenatal through weaning treatment, may be considerably more effective. To investigate our hypothesis, male WT and KO will be fed with high omega-3 diet during gestation through weaning on PD21. Following weaning, mice will then be placed on the standard lab chow to determine the long-term impact of early-life high omega-3 diet supplementation. As the results of this aim can be directly compared to the results from Aim 1, overall, we expect that prenatal exposure to omega-3 fatty acids will more robustly attenuate behavioral alterations associated with loss of *Fmr1* in male mice, given that the prenatal period is more sensitive. With respect to each individual task, based on the literature reviewed here, we expect that hyperactivity and

deficits in prepulse inhibition will be ameliorated by prenatal exposure to omega-3 fatty acids. There is no evidence to suggest that alterations in anxiety behavior, fear learning, or repetitive behavior would be rescued in this model, therefore we don't anticipate seeing any effects in this paradigm. However, it has been demonstrated that maternal diet high in polyunsaturated fatty acids alters social behavior, increasing aggression (Raygada et al., 1998). Based on this, we hypothesize that in the social partition task, mice treated with DHA/EPA, independent of genotype, will have increased social behavior.

As in Aim 1, molecular methods (i.e. qRT-PCR) will be employed to assess how prenatal exposure to these different dietary changes impacts constitutive cytokine signaling in the brain. We will examine the same cytokines as before: BDNF, IL-1β, IL-6 and TNFα. Results from studies of the maternal immune activation paradigm have suggested that early life exposure to inflammatory agents can alter constitutive signaling of various cytokines and chemokines, even into adulthood. Based on this relationship as well as the evidence for the impact of deletion of *Fmr1* on expression of these cytokines reviewed in Aim 1, we expect that alterations in hippocampal expression of IL-6, TNF-α, TNF-α and IL-1β in the male *Fmr1* KO mouse will be significantly normalized by dietary treatment with omega-3 fatty acids.

The final objective of this aim is to examine how the control fat diet condition will impact behavior. As previously mentioned in Aim 1, it is possible that the high fat diet itself, regardless of the type of fat included will drive changes in behavior. Rodent studies indicate that maternal high fat diet negatively impacts several aspects of offspring behavior, including retention of spatial learning in the Morris water maze (White et al., 2009) and increases anxiety (Peleg-Raibstein, Luca, & Wolfrum, 2012). Moreover, these

changes are associated with impaired hippocampal and amygdala neuronal morphology (Janthakhin, Rincel, Costa, Darnaudery, & Ferreira, 2017). Based on these observations, we expect that the control fat diet condition will result in increased anxiety and deficits in fear learning, independent of genotype. We do not anticipate any changes to activity levels, social behaviors, repetitive behaviors, or sensorimotor gating behaviors from high fat diet alone.

CHAPTER THREE

Methods

Animals

All procedures were performed in accordance with *Baylor University Institutional Care and Use Committee* and the *Guide for the Care and Use of Laboratory Animals of the National Institutes of Health*. Male *Fmr1*$^{+/+}$ and female *Fmr1*$^{+/-}$ *FVB.129P2-Pde6b+Tyrc-ch Fmr1tm1Cgr/J* (Jackson Labs Stock No: 004624) mice originally from Jackson Labs were housed at Baylor University and bred to produce male wildtype (WT) and *Fmr1* knockout (KO) offspring. The colony was maintained on a 12-hour light/dark cycle (lights on at 7 am). On postnatal day (PD) 7, pups were separated from parents and toe clippings were collected to be sent out for genotyping and preserved in 70% ethanol (Mouse Genotype, Escondido, CA, USA). Toe clippings were made such that each mouse is identifiable based on the toes missing. Animals were housed in the Special Research Unit at the Baylor Science Building. All subjects and breeders had access to food and water *ad libitum*.

Experimental Design

Post-Weaning Paradigm

The proposed studies were broken up into two main experimental paradigms (described in Figure 3.1). In the first experimental paradigm, male offspring were maintained on standard laboratory chow until weaning on PD21. At PD21, subjects were

randomly assigned to one of three diet conditions: “Standard”, “Omega-3” and “Control Fat”. The latter two diets had identical lipid content (50g/kg). The “Omega-3” condition received a diet enriched with fish oil and dyed with red food coloring to distinguish it from the control condition (Teklad Custom Diet TD.150384). To control for potential effects due to simply increasing fat content, the Control Fat diet contained olive and palm oils with no dyes (Teklad Custom Diet TD.1500385) (See Table 3.1 for Ingredients List). For further information of the types of fatty acid chains included, see Table 3.2. Mixed-genotype littermates were housed in groups no larger than 5 animals, with each cage receiving the same diet assignment. Based on the methodologies of prior studies, subjects were maintained on this diet and tested in the behavioral paradigm started at PD90 (Pietropaolo et al., 2014). Subjects were maintained on the diet throughout the testing paradigm. The behavior tests were ordered in a way that increased in invasiveness, to minimize the effect of training history on subsequent behavioral tests (McIlwain, Merriweather, Yuva-Paylor, & Paylor, 2001).

A power analysis was conducted using G*Power 3.1.9.2 (Faul, Erdfelder, Lang, & Buchner, 2007) for the 3 (Diet: Standard, Control Fat, Omega-3) x 2 (Genotype: WT, KO) mixed-model ANOVA to determine the appropriate number of subjects for the statistical analyses planned (see Appendix A). The test family was set at F tests for an ANOVA: fixed effects, special, main effects and interactions. The input parameters were set as follows: $f = 0.40$, $\alpha = 0.05$ and power at 0.80, for 6 groups. For an 80% chance of finding a true effect, the output suggested a total sample size of 66, for a total of 11 for each of the 6 treatment combinations. From this analysis, the target sample size was 12 per group to account for potential subject loss.

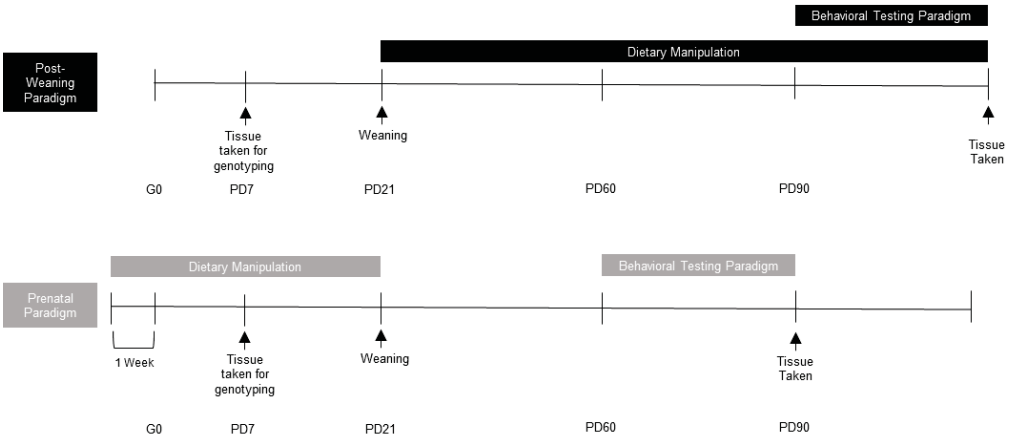

Figure 3.1. Overview of the two feeding paradigms. For the postnatal paradigm, the pups were weaned onto one of three dietary manipulations and maintained on this diet through the conclusion of the behavioral testing paradigm. For the prenatal paradigm, breeders were placed on the dietary manipulations for one week prior to pairing. Breeding pairs and litters were maintained on this diet until weaning on PD21, at which time male pups were weaned onto standard diet. All subjects in this paradigm received standard diet from PD21 through the conclusion of testing.

The final sample sizes were as follows: standard diet WT (n = 11), standard diet KO (n = 10), control fat WT (n = 17), control fat KO (n = 15), omega-3 WT (n = 19), omega-3 KO (n = 16). Due to equipment errors, some data was not available for some animals for some behaviors, and final sample sizes for each behavioral test are delineated in Table 3.3.

Prenatal Paradigm

In the second experimental paradigm, breeders were placed on one of the three experimental diets one week prior to pairing. Both parents and offspring were maintained on the assigned diet throughout pregnancy, parturition and lactation.

Table 3.1.

Diet Ingredients for Post-Weaning Paradigm.

Ingredients, g/kg	Control for EPA and DHA Diet (Control Fat)	Omega-3 EPA and DHA Diet (Omega-3 Diet)
Casein	200.0	200.0
L-Cystine	3.0	3.0
Corn Starch	347.472	347.472
Maltodextrin	132.0	132.0
Sucrose	100.0	100.0
Canola Oil	13.0	10.0
Olive Oil	65.0	20.0
Palm Oil	42.0	10.0
Fish Oil	-	80.0
Cellulose	50.0	50.0
Mineral Mix, AIN-93G	35.0	35.0
Vitamin Mix, AIN-93	10.0	10.0
Choline Bitartrate	2.5	2.5
TBHQ, antioxidant	0.028	0.028
Total	1000.0	1000.0

Note. Ingredients for the experimental diets in the post-weaning supplementation paradigm (Teklad Custom Diet TD.1500384 and TD.1500385).

Table 3.2

Fatty Acid Breakdown

Type of Fat	Fatty Acids (% of Total)	
	Control Fat Diet	Omega-3 Diet
Saturated Fatty Acids	27.9	26.8
Monounsaturated Fatty Acids	59.8	36.8
Polyunsaturated Fatty Acids	12.2	36.4

Note. Fatty acid breakdown for the experimental diets in the post-weaning supplementation paradigm (Teklad Custom Diet TD.1500384 and TD.1500385).

Table 3.3

Sample Sizes for Post-Weaning Paradigm

	Standard		Control Fat		Omega-3		
Behavioral Test/Measure	WT	KO	WT	KO	WT	KO	Total
Elevated Plus Maze	9	10	14	16	17	16	82
Sensorimotor Gating Paradigms	11	9	16	16	19	16	87
Delay Fear Conditioning	11	10	15	15	19	16	86
Social Partition	11	10	16	16	19	16	88
qRT – PCR/Western Blots	6	5	6	6	6	6	35

Many aspects of the experimental diets were the same from paradigm 1, however, there were some additional nutrients added for breeding suitability (for a list of ingredients, see Table 3.4). Moreover, to produce female KOs for the USV paradigm, female heterozygous breeders were bred with male KOs. Thus, heterozygous females may come from either breeding paradigm. Prior to testing, the toes of male and female

subjects of both genotypes were clipped on PD7 for identification and to be sent out for genotyping. On PD9, they were briefly separated from the parents and isolation-induced ultrasonic vocalizations were recorded. Male subjects were then weaned onto standard laboratory chow on PD21 and housed with mixed genotype littermates in groups of no larger than 5 mice. Male subjects were then tested in the behavioral battery beginning at PD60. The target sample size based on a similar power analysis as the postnatal paradigm, was again 12 per group. The final sample sizes were as follows: standard diet WT (n = 24), standard diet KO (n = 12), control fat WT (n = 16), control fat KO (n = 16), omega-3 WT (n = 14), omega-3 KO (n = 14). Due to equipment errors, some data was not available for all subjects some behaviors, and final sample sizes for each behavioral test are delineated in Table 3.5.

Behavioral Paradigms

Elevated Plus Maze

The elevated plus maze task was performed to evaluate changes in baseline anxiety levels (Pellow, Chopin, File, & Briley, 1985; Walf & Frye, 2007). The testing room was lit by LED dimmable lamps (30 lux in the open arm) and the background noise remained at 60 dB. The testing arena consisted of four arms (30 x 5 cm) and a center platform (5 x 5 cm) positioned approximately 40 cm above the floor. Two opposing arms were enclosed by acrylic walls. Subjects were recorded for 10 minutes and their movement was assessed via Ethovision XT video tracking software (Noldus, Netherlands). This program scored the frequency and duration of visits to the various arms and center platform. The testing apparatus was cleaned thoroughly with 30% isopropyl alcohol

solution before and after each subject was tested. Experimenters were not present during the testing window. Final sample sizes for each paradigm are delineated in Table 3.3 and Table 3.5.

Sensorimotor Gating Assessment

To determine changes in sensorimotor gating abilities in the *Fmr1* KO mice, the sensorimotor gating assessment paradigm was implemented as previously described (Frankland et al., 2004). Briefly, the apparatus consisted of an acrylic hollow constraint tube, with varying degrees of restraint availability. This apparatus was mounted on a platform equipped to transduce startle response amplitude through the SR-Lab System (San Diego Instruments, San Diego, CA, USA). This paradigm consisted of three separate testing days. During all testing sessions, background levels were maintained at 68 dB. On the first day, the animals underwent a habituation session. During this, they had a 5-minute acclimation session, followed by 80 startle stimuli delivered at a fixed interval (15 s). The startle stimulus was a 40 ms, 120 dB noise burst, with a rise/fall time of less than 1 ms.

The next day, prepulse inhibition was assessed. Following a 5-minute habituation phase, subjects received 20 presentations of a 40 ms, 120 dB stimulus. They were then presented with the prepulse phase of the trial, consisting of 90 trials. The first three trial types consisted of the 20 ms prepulse stimulus at three different decibel levels (70, 75 and 80 dB). The second three trial types then consisted of these three prepulse stimuli paired

Table 3.4.

Diet Ingredients for Prenatal Paradigm

Ingredients, g/kg	(Control Fat)	(Omega-3 Diet)
Casein	200.0	200.0
L-Cystine	3.0	3.0
Corn Starch	341.60	341.60
Maltodextrin	132.0	132.0
Sucrose	100.0	100.0
Canola Oil	13.0	10.0
Olive Oil	65.0	20.0
Palm Oil	42.0	10.0
Fish Oil	-	80.0
Cellulose	50.0	50.0
Mineral Mix AIN-93G	35.0	35.0
Vitamin Mix AIN-93	10.0	10.0
Choline Bitartrate	2.5	2.5
Calcium Phosphate, dibasic	3.1	3.1
Calcium Carbonate	1.0	1.0
Magnesium Oxide	0.154	0.154
Cupric Carbonate	0.0038	0.0038
Ferric Citrate	0.2352	0.2352
Sodium Selenite (0.0455% in sucrose)	1.25	1.25
Vitamin $K_{1,}$ phylloquinone	0.0003	0.0003
Vitamin B_{12} (0.1% in mannitol)	0.025	0.025
TBHQ, antioxidant	0.028	0.028
Total	1000.0	1000.0

Note. Ingredients for the experimental diets in the prenatal supplementation paradigm (TD.160486 and TD.160487).

with the original startle stimulus. The prepulse stimulus was then preceded by the startle stimulus by 100 ms. These trials were organized randomly and spaced by a 15 s inter-trial interval.

Table 3.5.

Sample Sizes for Prenatal Paradigm

	Standard		Control Fat		Omega-3		
Behavioral Test/Measure	WT	KO	WT	KO	WT	KO	Total
Elevated Plus Maze	22	10	17	13	14	14	90
Sensorimotor Gating Paradigms	23	9	15	16	14	13	90
Delay Fear Conditioning	24	12	16	16	14	14	96
qRT-PCR/Western Blots	6	6	6	6	6	6	36

One week following the prepulse session, the startle threshold session was conducted. Following the initial 5-minute habituation period, mice were presented with 99 trials of 11 trial types: no stimulus, and ten startle stimuli ranging from 75 – 120 dB at five dB intervals. These startle stimuli lasted 40 ms with a rise/fall of less than 1 ms. The order of these trials was pseudorandomized, such that 11 trials were presented as a block in a different order each time. Experimenters were not present during the testing window. Final sample sizes for both paradigms are delineated in Table 3.3 and Table 3.5.

Delay Fear Conditioning

The delay fear conditioning paradigm was conducted, as it has been shown to be selective for amygdala-based fear memories (Raybuck & Lattal, 2011). On the first day

of testing, the animals received 2 pairings of an 80-dB white noise stimulus (designated the CS) followed by a 0.7 mA shock stimulus (designated the US). Following the second pairing, there was a 120 second interval (ITI). The session lasted approximately 334 seconds.

On the second day, there were two testing sessions. During the first, the animal was placed in the familiar context and allowed to move freely for 300 seconds to evaluate freezing in the original context (contextual freezing behavior). Following a two-hour rest, the animal was placed in a new context for 360 seconds. The context was altered by the following manipulations: a clear acrylic square placed over the shock grid (novel tactile context), the shape of the arena altered by the insertion of an acrylic panel, shredded paper towels in the transfer cage and 1 mL of pure vanilla extract placed beneath the floor (novel olfactory context). During the first 3 minutes, we measured the freezing behavior of the mouse in a new environment. In the second 3 minutes, the CS was presented continuously for 3 minutes. During all sessions, freezing behavior was measured by an automated software (Colbourn Instruments, Allentown, PA, USA). Experimenters were not present during the testing window. Final sample sizes for this test for each experimental paradigm are delineated in Table 3.3 and Table 3.5.

Molecular Methods

Quantitative Real-time Polymerase Chain Reaction for Cytokine Expression

After behavioral testing, the brain was removed and rinsed in 1X phosphate buffer (PBS) solution. Using previously described methods (Lugo et al., 2014), hippocampi were then rapidly dissected from each hemisphere and rapidly rinsed in ice cold 1X PBS,

before being frozen on dry ice before stored in microcentrifuge tubes at -80 ºC until processing. The left hippocampus was used for all PCR assays. Total RNA was isolated from samples according to protocols from the RNeasy kit Qiagen, Hilden, Germany). Subsequently, concentration and purity of isolated samples were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, NanoDrop Products, Wilmington, DE). Using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA), extracted RNA was then reverse transcribed into complementary DNA. Using a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Carlsbad, CA), mRNA expression was then determined by quantitative real-time polymerase chain reaction (qRT-PCR) using Taq-man probe and primer chemistry. Reactions were performed in triplicate in a 384 well plate for each sample, using the endogenous control gene (β -actin) for normalization. The expression levels of each target gene, BDNF, IL-6, TNF-α and IL-1β, was calculated by normalizing the quantified mRNA amount to β –actin using the 2-ΔΔCt method of quantitation. Relative gene expression of each group was calculated in relation to the Standard Diet WT group and used to test significance between groups.

Western Blotting

Using the right hippocampus, western blotting was also conducted on tissue samples to assess proteins of interest. Similar to the PCR methods above, the brain was removed and rinsed in 1X phosphate buffer (PBS) solution. Hippocampi were then rapidly dissected from each hemisphere and rapidly rinsed in ice cold 1X PBS then frozen on dry ice before stored in microcentrifuge tubes at -80 ºC until processing. At the time of processing, hippocampi were homogenized in ice-cold homogenization buffer

consisting of 0.32 M sucrose, 5 mM Hepes and 0.32 M sucrose, as well as a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Total homogenate and crude synaptosome (P2) samples were produced as previously described (Lugo et al., 2014). Hippocampal synaptosomal samples were used for the following targets: PSD-95, GFAP, GluR1, nR2b, and MMP-9. Hippocampal homogenate samples were used for the following targets: Iba1, Caspase-3, IL-6R, phospho-s6 (s235/236 and s240/244 sites), s6 ribosomal protein, and ERK42/44. Following sample preparation, the samples were then diluted in Laemmli buffer for loading containing the following: 4X: 0.25 M Tris, pH 6.8, 6% SDS, 40% Sucrose, 0.04% Bromophenol Blue, 200 mM Dithithreitol). Samples were loaded and run through SDS – PAGE then transferred to Hybond-P polyvinyl difluoride membranes overnight (GE Healthcare, Piscataway, NJ, USA). Membranes were incubated for 1 hour at room temperature in blocking solution (5% nonfat milk diluted in 1 X Tris buffered saline (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 2.7 mM KCl) with 0.1 % Tween (1 X Tris Buffered Saline (TTBS)). Following blocking, membranes were incubated overnight at 4º C with primary antibodies diluted in the 5% milk solution. Following the overnight incubation, membranes were rinsed in 1X TTBS 3 times for 5 minutes each wash. Membranes were then incubated with horseradish peroxidase-labeled secondary antibody immunoglobulin G for 1 hour. Following the final incubation, membranes were washed in 1X TTBS 3 times for 5 minutes before being incubated with GE ECL Prime for 5 minutes (GE Healthcare). Immunoreactive bands were captured using the ProteinSimple western blot imaging system (ProteinSimple, Santa Clara, CA, USA). Information regarding primary and secondary antibodies and their concentrations, as well as the sample type used can be found in Table 3.6.

Following image capture, ProteinSimple AlphaView software was used to quantify the optical density of the bands on the blot. Measurements from all bands were normalized to each subject's actin level. All experimental data points represent a single mouse (n = 1). All groups were normalized to the average of the Standard WT group per blot.

Ultrasonic Vocalization (USV) Methods

Recording USVs

On PD9, isolation-induced ultrasonic vocalizations were recorded from pups. Briefly, male and female pups from the prenatal paradigm were brought down from the colony room in their home cage with the parents and weighed. They were then habituated to the testing room for 30 minutes prior to testing. At the time of testing, pups were separated from their parents into a clean housing pan with fresh bedding, warmed to ambient nesting temperature (~35º C) using a heating pad. During the testing window, pups were transferred into a sound attenuating chamber for 2 minutes and vocalizations between 0 and 125 kilohertz (kHz) were recorded using a condenser microphone (CM16/CMPA, Avisoft Bioacoustics, Germany). This microphone was connected to a recording interface (UltraSoundGate 116Hb, Avisoft Bioacoustics). Each pup was chosen randomly and recorded for 2 minutes, before being weighed, marked, returned to the warmed littermate cage. A live experimenter remained in the room to monitor the gain and other aspects of acquisition. Recordings were arranged such that pups were not away from the dam for longer than 30 minutes. After the last pup was tested, they were returned to the home cage with the parents.

Table 3.6.

Antibody Information

Sample Prep	Primary Antibody	Concentration	Manufacturer	Secondary Antibody	Concentration	Manufacturer	Loading Standard	Concentration
Hippocampal Synaptosome	PSD-95	1:500	NeuroMab	mouse IgG	1:20000	Cell Signaling	actin	1:1000
Hippocampal Synaptosome	GFAP	1:500	Cell Signaling	rabbit IgG	1:20000	Cell Signaling	actin	1:1000
Hippocampal Synaptosome	GluR1	1:500	NeuroMab	mouse IgG	1:20000	Cell Signaling	actin	1:1000
Hippocampal Synaptosome	nR2b	1:500	Millipore	rabbit IgG	1:20000	Cell Signaling	actin	1:1000
Hippocampal Synaptosome	MMP-9	1:500	Cell Signaling	rabbit IgG	1:20000	Cell Signaling	actin	1:1000
Hippocampal Homogenate	Iba1	1:500	Wako Chemicals	rabbit IgG	1:20000	Cell Signaling	actin	1:1000
Hippocampal Homogenate	Caspase-3	1:500	Cell Signaling	rabbit IgG	1:20000	Cell Signaling	actin	1:1000
Hippocampal Homogenate	IL6-R	1:500	Invitrogen	rat IgG	1:20000	Invitrogen	actin	1:1000
Hippocampal Homogenate	phospho-s6 (s235-236) / total s6	1:500	Cell Signaling	rabbit IgG	1:20000	Cell Signaling	actin	1:1000
Hippocampal Homogenate	phospho-s6 (s240-244) / total s6	1:500	Cell Signaling	rabbit IgG	1:20000	Cell Signaling	actin	1:1000
Hippocampal Homogenate	S6 Ribosomal Protein	1:500	Cell Signaling	rabbit IgG	1:20000	Cell Signaling	actin	1:1000
Hippocampal Homogenate	ERK42/44	1:500	Cell Signaling	mouse IgG	1:20000	Cell Signaling	mortalin	1:1000

After excluding all non-vocalizers (n = 8), the final sample size for the prenatal paradigm was as follows for males: Standard WT = 23, Standard KO = 12, Control Fat WT = 19, Control Fat KO = 14, Omega-3 WT = 14, Omega-3 KO = 13. After excluding non-vocalizers (n = 19), the final sample size for the females was as follows: Standard WT = 32, Standard HET = 29, Standard KO = 10, Control Fat WT = 15, Control Fat HET = 20, Control Fat KO = 12, Omega-3 WT = 11, Omega-3 HET = 20, Omega-3 KO = 7. A nonsignificant chi-square indicated that the proportion of non-vocalizers was similar across the sexes, $\chi^2 = 0.71$, $p = 0.41$.

USV MATLAB Data Extraction

All WAV files were also cleaned and processed using an automated analysis, freely available from: (http://jarvislab.net/research/mouse-vocal-communication/) using MathWork's MATLAB software (Chabout, Jones-Macopson, & Jarvis, 2017). Sonograms were processed using the graphical user interface according to methods described previously (Chabout et al., 2017). In the Sonogram Parameters section, we set Min Frequency to 15,000 Hz, Max Frequency to 125,000 Hz, the sampling frequency to 256 kHz, and the Threshold to 0.3. In the Whistle Options section, we set the Purity Threshold to 0.075, the Min Duration of the syllable to 3 ms, the Min Frequency sweep to 20,000 Hz, and the Filter Duration to 3 ms. For consistency, we also set the Min Note Duration to 3 ms, and the Min Note Count to 1. Following sonogram processing, densite inter-syllable interval (ISI) was determined using the accompanying song-analysis guided analysis Excel file (available at the same website). From this analysis, the following variables could be assessed: number of calls, sequence length, frequency mean,

bandwidth, amplitude, frequency variance, spectral purity and average duration of calls. For more information on how this method compares to previous cleaning methods used in our lab, see Appendix C.

Statistical Analyses

All data were analyzed using GraphPad Software 7.0 (San Diego, CA) or IBM SPSS Statistics 23 (Aramonk, NY). Results were evaluated 2 x 3 (Genotype [WT, KO] x Diet [Standard, Control Fat, Omega-3]) analysis of variance (ANOVA) on each dependent variable for the specific test. Any tests that involved repeated measures was analyzed using a within- subjects factor, by repeated measures ANOVA (specified in that results). Significant within-subjects interactions were followed up with individual one-way ANOVAs at each repeated measure. Significant interactions of genotype and diet were followed up with the use of a unique identifier for all groups (i.e. "Standard WT") and subsequent analysis. If there were multiple significant main effects, multiple comparisons were conducted using Fisher's LSD comparisons (Standard WT vs Standard KO, Standard WT vs Control Fat KO, Standard WT vs Omega-3 KO). For PCR, following a significant main effect, multiple comparisons were conducted using Tukey's multiple comparisons test (q). Correlations between cytokine expression levels and behavioral measures were conducted using Pearson's correlation statistic. For all inferential statistics, the level of significance remained at $p < 0.05$.

CHAPTER FOUR

Results

Activity Levels

Post-weaning Paradigm

Hyperactivity is a significant clinical component of FXS (Hagerman, 1997; Hagerman et al., 1986), and has been well-described in the *Fmr1* KO (Baker et al., 1998; Ding et al., 2014; Nolan et al., 2017; Pietropaolo, Guilleminot, Martin, D'Amato, & Crusio, 2011). Given the potential impact of hyperactivity on the findings of subsequent behavioral testing and the consistent appearance in the *Fmr1* KO model, we assessed the impact of high omega-3 fatty acids on activity levels in the elevated plus maze. For the post-weaning paradigm, in the elevated plus maze, loss of *Fmr1* did not influence distance moved, $F_{\text{genotype}}(1, 76) = 1.57$, $p = 0.21$ (Figure 4.1A). Diet also did not influence distance moved, $F_{\text{diet}}(2, 76) = 0.32$, $p = 0.74$. However, there was a trending interaction of genotype and diet, $F_{\text{genotype x diet}}(2, 76) = 2.75$, $p = 0.07$. Preplanned comparisons were then conducted as discussed in the Statistical Analyses portion of the Methods. Indeed, exposure to omega-3 fatty acids in the *Fmr1* KO mouse [(a) Standard WT vs (a) Omega-3 KO, $p = 0.59$] attenuated hyperactivity seen between the standard WT and standard KO [(a) Standard WT vs (b) Standard KO, $p = 0.02$]. The control fat diet did not significantly influence this hyperactivity [Control Fat KO vs Standard KO, $p = 0.16$; Control Fat KO vs Standard WT, $p = 0.24$].

A similar pattern was detected for velocity. Alone, loss of *Fmr1* did not significantly increase velocity, $F_{genotype}(1, 76) = 0.88$, $p = 0.35$ (Figure 4.1B). However, diet did not significantly influence velocity on its own, $F_{diet}(2, 76) = 0.99$, $p = 0.38$. There was a trending interaction between genotype and diet, $F_{genotype\ x\ diet}(2, 76) = 2.96$, $p = 0.06$. Similar to the results for distance moved, exposure to omega-3 fatty acids in the *Fmr1* KO mouse [(a) Standard WT vs (a) Omega-3 KO, $p = 0.91$] attenuated hyperactivity seen following the loss of *Fmr1* in the standard diet condition [(a) Standard WT vs (b) Standard KO, $p = 0.03$].

Overall, these results suggest that postnatal dietary supplementation with omega-3 fatty acids, but not a diet high in other types of fatty acids, is sufficient to reduce hyperactivity in the *Fmr1* KO mouse in the elevated plus maze. However, exposure to a typical Western diet had little effect on, or may have actually exacerbated, hyperactivity in the *Fmr1* KO.

Prenatal Paradigm

For the prenatal paradigm, in the elevated plus maze, loss of *Fmr1* resulted in hyperactivity when measuring distance moved, $F_{genotype}(1, 84) = 21.04$, $p = 0.001$ (Figure 4.1C). Diet did significantly influence distance moved, $F_{diet}(2, 84) = 4.26$, $p = 0.02$. Post-hoc multiple comparisons with LSD indicated that subjects, across genotypes, receiving omega-3 fatty acids had reduced distance moved and velocity when compared to both standard and control fat conditions, $p < 0.05$ (Figure 4.1C) A significant interaction was detected for distance moved, $F_{genotype\ x\ diet}(2, 84) = 3.35$, $p = 0.04$, and a follow-up analysis with unique group identifiers (i.e. Standard Diet WT) was conducted. Interestingly, the control fat diet significantly exacerbated hyperactivity in the KO

(Control Fat KO vs Standard WT, $p = 0.02$). However, the control fat diet reduced hyperactivity in the wildtypes (Control Fat WT vs Standard WT, $p = 0.004$). This was similarly true for the omega-3 WT diet (Omega-3 WT vs Standard WT, $p = 0.01$). However, the omega-3 KO group expressed levels of activity similar to the standard WT group, $p = 0.62$ (Figure 4.1C).

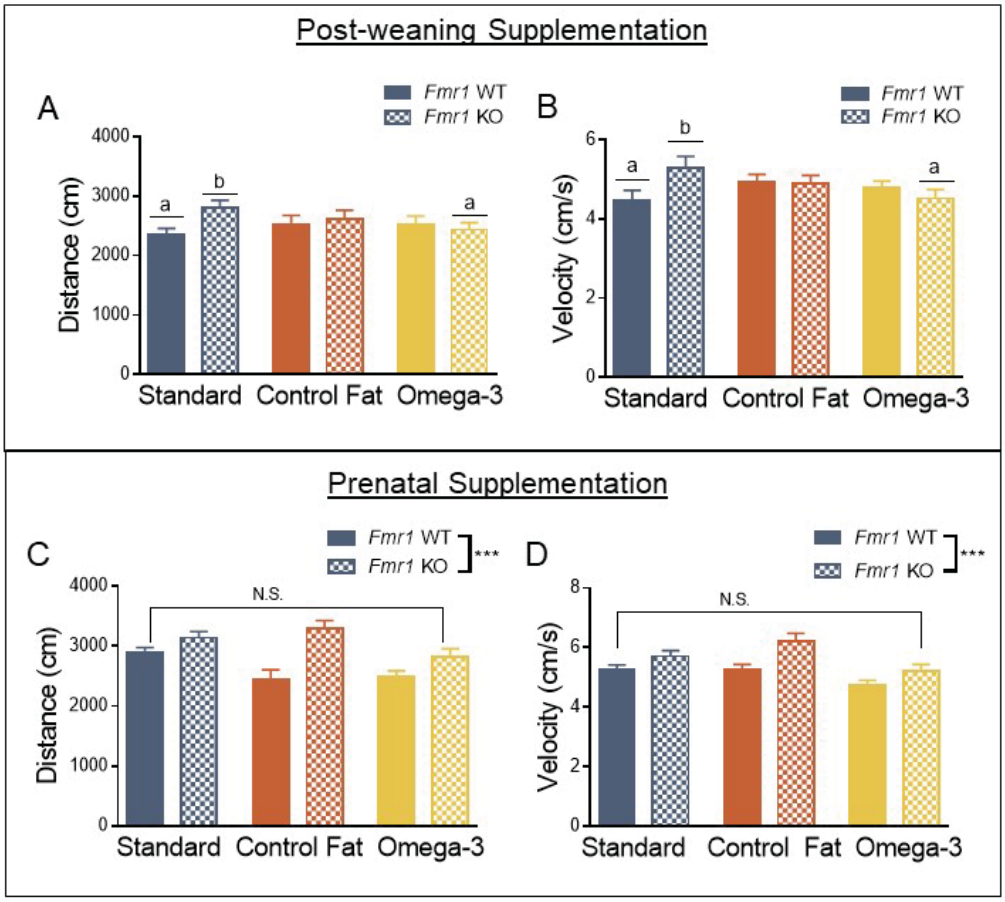

Figure 4.1. Prenatal and post-weaning supplementation with a high omega-3 diet attenuates hyperactivity in the elevated plus maze in the Fmr1 knockout mouse. A. Exposure to omega-3 fatty acids reduced hyperactivity as measured by distance moved in the elevated plus maze. B. A similar pattern was detected for velocity in the elevated plus maze. C. Prenatal exposure to omega-3 fatty acids reversed Fmr1 knockout induced hyperactivity in the elevated plus maze, as measured by distance moved. D. Prenatal exposure to omega-3s also reversed hyperactivity as measured by velocity in the elevated plus maze. Data are expressed as mean ± SEM. *** = $P < 0.001$. A designation of "b" indicates that this group differed from the "a" comparison group at the level of $p < 0.05$.

The results for velocity were very similar. Overall, loss of *Fmr1* increased velocity, $F_{genotype}(1, 84) = 14.41$, $p = 0.001$ (Figure 4.1D). While no interaction was detected, $F_{genotype\ x\ diet}(2, 84) = 1.21$, $p = 0.30$, exposure to the different dietary manipulations did indeed affect velocity, $F_{diet}(2, 84) = 7.52$, $p = 0.001$. Pre-planned comparisons indicated that exposure to the omega-3 diet ameliorated hyperactivity following loss of *Fmr1* (Standard WT vs Omega-3 KO, $p = 0.62$), while exposure to the control fat diet increased activity levels in the KO (Standard WT vs Control Fat KO, $p = 0.02$).

Overall, similar to the post-weaning paradigm, exposure to omega-3 fatty acids, but not other types of fatty acids, attenuates hyperactivity in the adult *Fmr1* KO measured via the elevated plus maze.

Sensorimotor Gating Behavior

Post-weaning Paradigm

To examine the potential therapeutic efficacy of omega-3 fatty acids, we examined this behavior in a three-day paradigm similar to previously published methodology (Frankland et al., 2004). For the post-weaning paradigm, results for habituation to the startle stimulus (data not shown) indicated no impact of loss of *Fmr1* on the ability to habituate to the chamber, $F_{genotype}(1, 81) = 0.001$, $p = 0.97$, and this did not vary across the testing window, $F_{genotype\ x\ time}(7, 567) = 0.76$, $p = 0.62$. Diet also did not impact habituation, $F_{diet}(2, 81) = 0.72$, $p = 0.49$, and this effect did not change over time, $F_{diet\ x\ time}(14, 567) = 0.89$, $p = 0.57$. The interaction of the different levels of genotype and diet were not significant, both overall, $F_{genotype\ x\ diet}(2, 81) = 1.91$, $p = 0.16$,

or across time, $F_{\text{genotype x diet x time}}(14, 567) = 0.52$, $p = 0.92$. Overall, this indicated that neither diet nor genotype had a significant impact on habituation to the startle stimulus.

As expected, loss of *Fmr1* resulted in overall exaggerated levels of prepulse inhibition (measured on Day 2), $F_{\text{genotype}}(1, 81) = 6.15$, $p = 0.02$ (Figure 4.2A). This effect was consistent across decibel levels, $F_{\text{genotype x decibel}}(2, 162) = 2.57$, $p = 0.08$. Because this effect was consistent across the testing window, data is shown in the figure summarized across decibel level. Diet did not significantly impact percent inhibition alone, $F_{\text{diet}}(2, 81) = 0.05$, $p = 0.95$, or across the levels of decibel of prepulse stimulus, $F_{\text{diet x decibel}}(4, 162) = 0.15$, $p = 0.97$. Similarly, the combination of genotype and diet was not statistically significant depending on the levels of the stimulus, $F_{\text{genotype x diet x decibel}}(4, 162) = 0.15$, $p = 0.96$. However, overall, the combination of these two factors differentially impacted percent inhibition, $F_{\text{genotype x diet}}(2, 81) = 3.70$, $p = 0.03$. Post-hoc analyses using LSD testing indicated that in standard diet condition, loss of *Fmr1* exaggerated PPI [(a) Standard WT vs (b) Standard KO, $p = 0.002$]. In addition, exposure to both the omega-3 [(a) Standard WT vs (b) Omega-3 KO, $p = 0.04$] and the control fat diet [(a) Standard WT vs (b) Control Fat KO, $p = 0.05$] did not differentially impact the *Fmr1* KO PPI phenotype (Figure 4.2A). Altogether, these results support previous findings that loss of *Fmr1* results in exaggerated prepulse inhibition, though this was unaffected by the two post-weaning dietary manipulations.

Finally, startle threshold was assessed one week later. As expected, loss of *Fmr1* did not impact the startle response overall, $F_{\text{genotype}}(1, 81) = 1.72$, $p = 0.19$ (Figure 4.2B). However, there was a significant interaction of genotype and decibel levels, $F_{\text{genotype x decibel}}(10, 810) = 2.88$, $p = 0.002$. Further investigation indicated that *Fmr1* KOs had

reduced startle responding during this testing window at higher stimulus levels (115 and 120 dB). Diet exposure did not impact startle responding overall, F_{diet} (2, 81) = 0.42, p = 0.66, and the slope of the startle threshold curve was not shifted, $F_{\text{diet x decibel}}$ (20, 810) = 0.98, p = 0.38. The overall combination of genotype and diet also failed to reach significance, $F_{\text{genotype x diet}}$(2, 81) = 0.65, p = 0.53. However, these interaction of these levels of diet and genotype did significantly impact startle responding, $F_{\text{genotype x diet x decibel}}$(20, 810) = 1.87, p = 0.01. Post-hoc analyses indicated that at 115 dB and 120 dB, Standard KO demonstrated significantly reduced startle responding compared to Standard WT, p = 0.05. This was ameliorated by post-weaning exposure to both experimental diets at both 115 dB (Control Fat KO vs Standard WT, p = 0.97; Omega-3 KO vs Standard WT, p = 0.43) and 120 dB (Control Fat KO vs Standard WT, p = 0.94; Omega-3 KO vs Standard WT, p = 0.37) (Figure 4.2B). Together, these results suggest that increasing the fat content of the post-weaning diet can potentially improve reduced startle responding at higher decibels in the *Fmr1* KO.

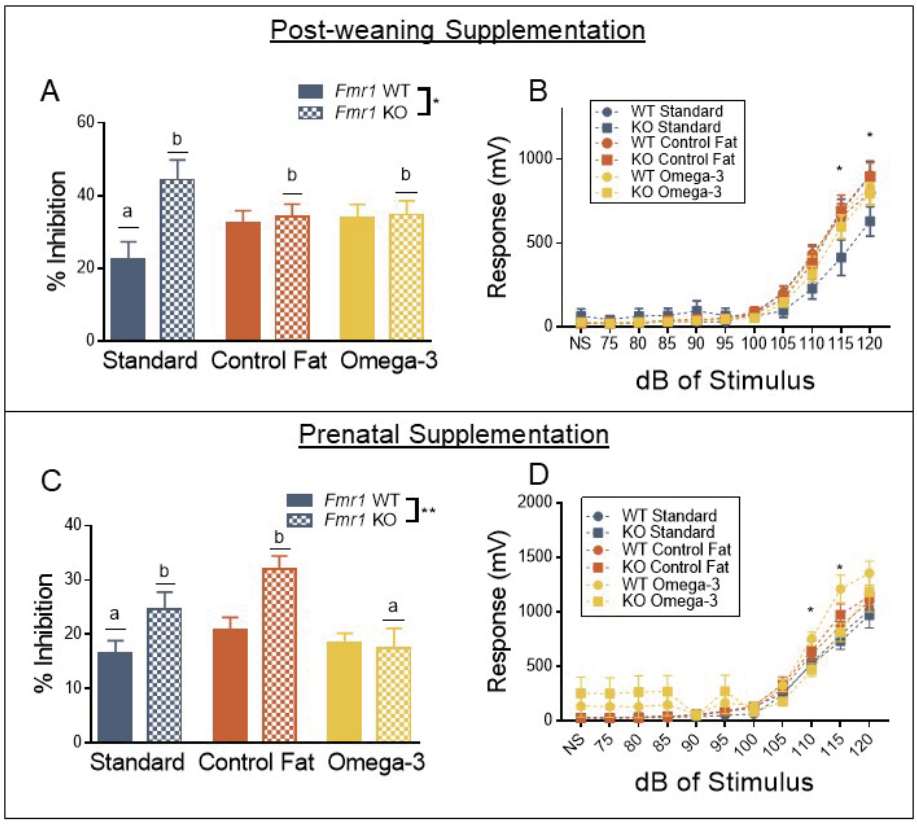

Figure 4.2. Post-weaning exposure to high fat diets reverses startle threshold, but not PPI, deficits, while prenatal exposure to omega-3 fatty acids reverses PPI deficits, but not startle threshold deficits. A. Exposure to both experimental diets did not reverse exaggerated PPI in the *Fmr1* knockout. B. Startle threshold was diminished in the *Fmr1* knockout, and this was reversed by exposure to the two experimental diets. C. Prenatal exposure to omega-3 fatty acids, but not the control fat diet, reversed PPI deficits. D. Prenatal exposure to the experimental diets did not impact the startle threshold deficits seen in the *Fmr1* knockout. Data are expressed as mean ± SEM. * = $P < 0.05$, ** = $P < 0.01$. A designation of "b" indicates that this group differed from the "a" comparison group at the level of $p < 0.05$.

Prenatal Paradigm

For the prenatal paradigm, loss of *Fmr1* reduced responding to the startle stimulus overall, $F_{genotype}(1, 84) = 5.73$, $p = 0.02$, during the habituation session (data not shown for this phase) and this effect become more significant over the testing window, $F_{genotype \times time}(7, 588) = 2.01$, $p = 0.05$. Moreover, diet did significantly impact overall responding to

the startle stimulus, $F_{diet}(2, 84) = 3.49$, $p = 0.04$. Further post-hoc LSD testing indicated that the omega-3 fatty acid diet increased startle responding, compared to the standard diet, at the level of $p < 0.05$, regardless of genotype. This effect was consistent across time, $F_{diet\ x\ time}(14, 588) = 1.59$, $p = 0.08$. The control fat diet was not significantly different from either the standard or omega-3 diet. The unique combination of genotype and diet did not significantly impact startle responding during the habituation task, both overall, $F_{genotype\ x\ diet}(2, 84) = 0.35$, $p = 0.70$, or across time, $F_{genotype\ x\ diet\ x\ time}(14, 588) = 0.54$, $p = 0.91$.

When looking at the prepulse inhibition phase, loss of *Fmr1* significantly increased percent inhibition overall, $F_{genotype}(1, 84) = 7.74$, $p = 0.01$ (Figure 4.2C). This sensitivity was also greater with increasing decibel levels, $F_{genotype\ x\ decibel}(2, 168) = 5.32$, $p = 0.01$. Results also indicated that diet significantly altered percent inhibition, regardless of genotype, $F_{diet}(2, 84) = 5.55$, $p = 0.01$. Post-hoc analyses indicated that the control fat diet significant increased PPI compared to the standard diet and omega-3 diet, while the omega-3 diet was not significantly different from the standard diet, at the level of $p < 0.05$. This effect was not dependent on decibel levels, $F_{diet\ x\ decibel}(4, 168) = 1.93$, $p = 0.11$. The combination of genotype and diet did not impact this behavior, both overall, $F_{genotype\ x\ diet}(2, 84) = 2.89$, $p = 0.06$, and across decibel levels, $F_{genotype\ x\ diet\ x\ decibel}(4, 168) = 1.89$, $p = 0.11$. Given the trending nature of the interaction and the significant main effect of diet, a follow-up analysis was conducted for total prepulse inhibition as was specified in the a priori hypothesis. In the standard diet, the KO had exaggerated prepulse inhibition [(a) Standard WT vs (b) Standard KO, $p = 0.04$] (Figure 4.2C). This effect was rescued by exposure to omega-3 fatty acids [(a) Standard WT vs (a) Omega-3 KO, $p =$

0.82]. However, this effect was specific to the omega-3 condition [(a) Standard WT vs (b) Control Fat KO, $p = 0.001$]. These results suggest that, as expected, something specific about the omega-3 dietary supplementation during the prenatal period prevents the development of exaggerated prepulse inhibition in the *Fmr1* KO. This may occur through a number of different mechanisms, including normalizing altered neuroinflammatory signaling shown previously in this model (Krasovska & Doering, 2018).

For the startle threshold test, loss of *Fmr1* reduced startle responding at higher decibel levels, $F_{\text{genotype x decibel}}(10, 840) = 2.14$, $p = 0.02$ (Figure 4.2D). This was not indicative of overall lowered startle responding in this task, $F_{\text{genotype}}(1, 84) = 0.02$, $p = 0.88$. Diet also affected the startle threshold curve, both overall, $F_{\text{diet}}(2, 84) = 4.01$, $p = 0.02$, and across the different levels, $F_{\text{diet x decibel}}(20, 840) = 2.89$, $p = 0.001$. Overall, the combination of diet and genotype did not impact startle responding, $F_{\text{genotype x diet}}(2, 84) = 0.28$, $p = 0.76$. However, it did alter the threshold of startle responding, $F_{\text{genotype x diet x decibel}}(20, 8840) = 2.78$, $p = 0.0001$. Subsequent analyses indicated the interaction between genotype and diet was only significant at the level of 110 and 115 decibels. Further testing indicated in WT animals, the omega-3 diet increased startle responding, [Omega-3 WT vs Standard WT, $p < 0.05$; Omega-3 WT vs Standard KO, $p < 0.05$; Omega-3 KO vs Standard WT, $p < 0.05$] (Figure 4.2D). Moreover, prenatal control fat diet shifted both the WT and KO animals to an intermediate position that was not statistically different between either group. Together these findings suggest that neither experimental diet improved reduced startle responding following loss of *Fmr1*.

Anxiety Levels

Post-weaning Paradigm

Previous studies conducted in this model have indicated that nonsocial anxiety is significantly reduced following loss of *Fmr1* (Liu & Smith, 2009). To examine the influence of these dietary paradigms on anxiety, we assessed animals in the elevated plus maze, and recorded the percentage of time spent in open arms of the maze. For the post-weaning paradigm, loss of *Fmr1* did not impact anxiety in this task, $F_{\text{genotype}}(1, 76) = 0.003$, $p = 0.96$, and diet was also not a significant factor, $F_{\text{diet}}(2, 76) = 2.03$, $p = 0.14$ (Figure 4.3A). These variables also did not significantly interact, $F_{\text{genotype x diet}}(2, 76) = 1.54$, $p = 0.22$. The results suggest that the reduced anxiety characteristic of the *Fmr1* KO is not present when tested around PD90 in this model, unlike previous studies conducted at an earlier time points (Nolan et al., 2017). Furthermore, neither dietary fatty acid manipulation affected the anxiety phenotype of either WT or KO animals.

Prenatal Paradigm

For the prenatal paradigm, loss of *Fmr1* resulted in decreased anxiety, $F_{\text{genotype}}(1, 84) = 7.91$, $p = 0.01$, as suggested by increased percentage of time spent in the open arms (Figure 4.3B). Increased time spent in the open arm in the *Fmr1* KO was significantly exaggerated by exposure to the control fat diet, $F_{\text{diet}}(2, 86) = 8.01$, $p = 0.001$. Preplanned follow-up analyses indicated that the control fat diet ("b") significantly increased the proportion of time spent in the open arms compared to both standard ("a") and omega-3 diets ("a"), at the level of $p < 0.05$. However, the standard diet and omega-3 conditions produced similar effects (Figure 4.3B). Genotype and diet also did not significantly interact, $F_{\text{genotype x diet}}(2, 84) = 2.10$, $p = 0.13$. These results suggest that the omega-3 fatty

acid diet did not to improve altered anxiety characteristic of the *Fmr1* model. Moreover, the control fat diet, similar to a typical Western diet, further exacerbated this reduced anxiety phenotype.

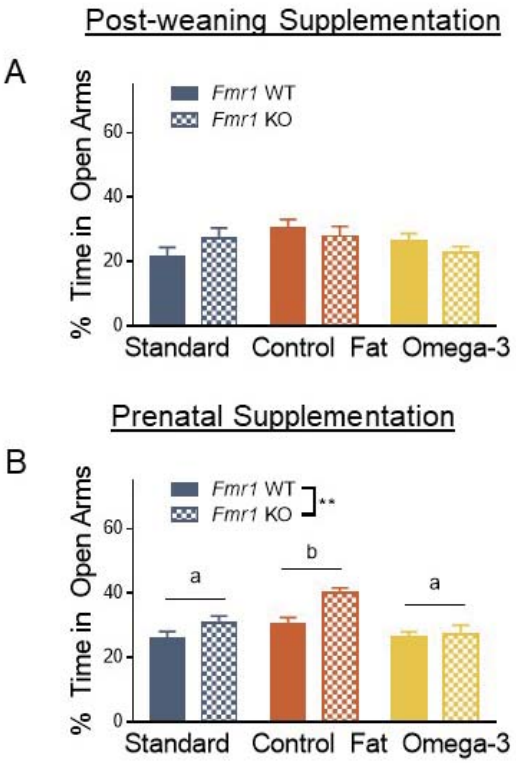

Figure 4.3. Neither post-weaning nor prenatal omega-3 fatty acids influence anxiety in the elevated plus maze, while prenatal monounsaturated fatty acids exacerbate anxiety changes seen in the *Fmr1* knockout. A. No effect of the experimental diets was seen in the post-weaning paradigm. B. Loss of *Fmr1* was associated with diminished anxiety in the elevated plus maze, though this effect was not ameliorated by exposure to omega-3 fatty acids. Rather, exposure to the control fat diet exacerbated this genotype effect. Data are expressed as mean ± SEM. ** = $P < 0.01$. A designation of "b" indicates that this group differed from the "a" comparison group at the level of $p < 0.05$.

Cognitive Behavior

Post-Weaning Paradigm

In previous studies of the *Fmr1* KO, the delay fear conditioning paradigm demonstrates impaired acquisition of a fear response and impairments in hippocampal-dependent cued recall (Nolan et al., 2017). Thus, we chose this paradigm to assess the ability of our treatment to improve this cognitive dysfunction. For the post-weaning paradigm, results for acquisition indicated no effect of loss of *Fmr1* on freezing overall during the acquisition of a fear memory, $F_{\text{genotype}}(1, 80) = 1.06$, $p = 0.31$ (Figure 4.4A). This effect was consistent across time, $F_{\text{genotype x time}}(4, 320) = 1.05$, $p = 0.38$. Assignment to either one of the two experimental diets did, however, reduce freezing behavior during acquisition, $F_{\text{diet}}(2, 80) = 11.38$, $p = 0.0001$. Moreover, the magnitude of this effect was different across the testing window, $F_{\text{diet x time}}(8, 320) = 5.99$, $p = 0.0001$. In fact, beginning at tone 1, both omega-3 and control fat diets, across genotypes, displayed reduced levels of freezing, compared to standard diet controls, at the level of $p < 0.05$, and the magnitude of the difference grew over time. However, this effect was also dependent on genotype, as the interaction term was significant, both overall, $F_{\text{genotype x diet}}(2, 80) = 3.18$, $p = 0.05$, and across time, $F_{\text{genotype x diet x time}}(8, 320) = 2.15$, $p = 0.03$. Subsequent analyses indicated that this interaction reached significance only at the first inter-trial interval, the second presentation of the tone, and the second inter-trial interval at the level of $p < 0.05$. Analysis using a unique grouping variable indicated that at ITI 1, tone 2 and ITI 2, all other groups displayed diminished freezing compared to the Standard WT group, at the level of $p < 0.05$ (Figure 4.4A). Altogether, these results

suggest that post-weaning exposure to both experimental diets reduced short-term learning of the association between the tone (CS) and shock (US) in this paradigm.

The next day, conditioning to the context was measured via freezing across the 5 minute window. As expected, loss of *Fmr1* did not impact freezing behavior overall, $F_{\text{genotype}}(1, 80) = 1.22$, $p = 0.27$ (Figure 4.4B). This effect was consistent across the testing window, $F_{\text{genotype x time}}(4, 320) = 1.61$, $p = 0.17$. Diet did significantly impact freezing behavior to the conditioned context, $F_{\text{diet}}(2, 80) = 11.07$, $p = 0.0001$. Subsequent analyses with LSD post-hoc multiple comparisons indicated that subjects exposed to both omega-3 ("b") and control fat diets ("b") displayed significantly reduced freezing behavior in the conditioned context compared to the standard diet ("a"), $p < 0.05$ (Figure 4.4B). This trend was consistent across the testing window, $F_{\text{diet x time}}(8, 320) = 0.19$, $p = 0.83$. The combination of genotype and diet did not significantly impact freezing behavior, both overall, $F_{\text{genotype x diet}}(2, 80) = 1.63$, $p = 0.20$, and across time, $F_{\text{genotype x diet x time}}(8, 320) = 1.37$, $p = 0.21$. These results suggest that post-weaning exposure to the high-fat experimental diets reduces contextual fear learning

Following a two-hour rest, animals were placed in a novel context and the level of conditioning to the stimulus was measured via freezing to the tone expression. Loss of *Fmr1* did not alter freezing behavior across the testing window, $F_{\text{genotype}}(1, 80) = 1.0$, $p = 0.76$ (Figure 4.4C). This effect was consistent across time, $F_{\text{genotype x time}}(1, 80) = 1.46$, $p = 0.23$. However, diet significantly impacted freezing overall during this window, $F_{\text{diet}}(2, 80) = 9.88$, $p = 0.0001$. Further multiple-comparisons with LSD indicated that exposure to both omega-3 ("b") and control fat diets ("b") significantly reduce freezing behavior overall compared to the standard diet condition ("a") (Figure 4.4C). This effect was

consistent across the testing window, $F_{\text{diet x time}}(2, 80) = 1.46$, $p = 0.24$. Because the effect on freezing was the same across the testing window, only freezing in response to the cue is shown in the figure. The combination of genotype and diet did not significantly impact freezing behavior, both overall, $F_{\text{genotype x diet}}(2, 80) = 0.39$, $p = 0.68$, and across time, $F_{\text{genotype x diet x time}}(2, 80) = 2.04$, $p = 0.14$. These results suggest that post-weaning exposure to both experimental diets reduces cued recall of the fear memory.

Prenatal Paradigm

For the prenatal paradigm, the results showed a different pattern. During the acquisition phase, loss of *Fmr1* resulted in no change overall to freezing levels across the testing window, $F_{\text{genotype}}(1, 90) = 3.40$, $p = 0.07$ (Figure 4.4D). However, the interaction of genotype and time did significantly impact freezing behavior, $F_{\text{genotype x time}}(4, 360) = 6.27$, $p = 0.001$ (Figure 4.4D).

As expected, post-hoc multiple comparisons indicated that loss of *Fmr1* resulted in diminished freezing starting at the second iteration of the tone, at the level of $p < 0.05$.Diet significantly impacted freezing overall during this acquisition phase, $F_{\text{diet}}(2, 90) = 5.08$, $p = 0.01$, as well as across time, $F_{\text{diet x time}}(8, 360) = 3.34$, $p = 0.001$. Further multiple comparisons testing indicated that both omega-3 fatty acid (“b”) and control fat (“b”) diets significantly increased freezing behavior across the testing window, at the level of $p < 0.05$, and the magnitude of this effect increased over time (Figure 4.4D). There was also a trending interaction of genotype and diet, $F_{\text{diet x genotype}}(2, 90) = 2.77$, $p = 0.07$. Due to the significant main effects and interactions, follow-up analyses were conducted for the second presentation of the tone and the second ITI.

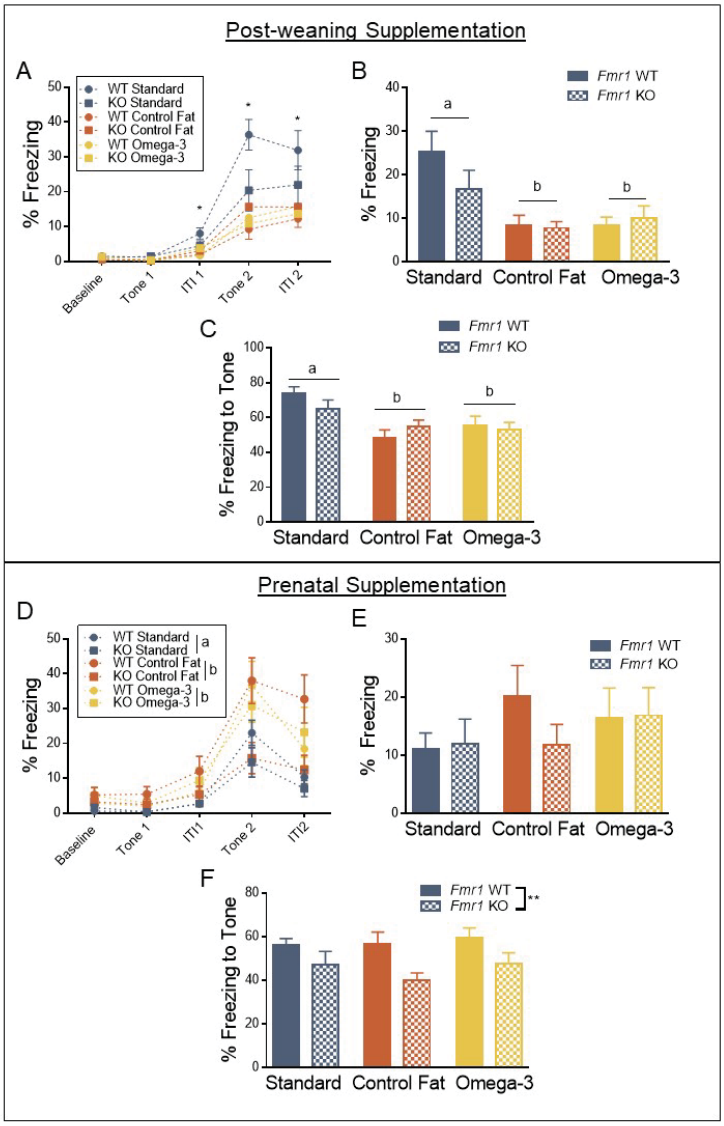

Figure 4.4. Post-weaning supplementation with high fat diets impairs training, contextual and cued recall, while prenatal supplementation improves training in the delay fear conditioning task. A. Acquisition of a fear memory was impaired by exposure to both experimental diets in wildtype animals, and this effect was not additive for the *Fmr1* knockout. B. Contextual memory was impaired in both wildtype and knockout animals in the experimental diet conditions. C. Cued recall was impaired in wildtype and knockout animals, and this effect was not additive in the *Fmr1* knockout. D. Prenatal omega-3 fatty acids improve training, while the control fat diet exhibits no effect. E. No effect of either diet or genotype was detected for contextual memory. F. No effect of either diet or genotype was detected for cued recall. Data are expressed as mean ± SEM. * = $P < 0.05$, ** = $P < 0.01$. A designation of "b" indicates that this group differed from the "a" comparison group at the level of $p < 0.05$.

Results indicated that genotypic decreases in freezing were improved in KO Omega-3 groups (Standard WT vs Omega-3 KO, $p = 0.25$) at the second presentation of the tone. When compared to the standard KO group, the Omega-3 KO demonstrated increased freezing ($p = 0.04$). This overall pattern continued at the second ITI (Standard WT vs Omega-3 KO, $p = 0.05$; Standard KO vs Omega-3 KO, $p = 0.003$). At the second tone presentation, the Control Fat KO group expressed no change in freezing behavior compared to the Standard WT ($p = 0.28$) and the Standard KO ($p = 0.88$). A similar pattern was detected for the second ITI (Standard WT vs Control Fat KO, $p = 0.74$; Standard KO vs Control Fat KO, $p = 0.46$). No significant three-way interaction was detected, $F_{\text{genotype x diet x time}}(8, 360) = 1.65$, $p = 0.11$. Overall, these results suggested that prenatal exposure to both experimental diets improved acquisition of a fear response in the *Fmr1* KO.

Results for contextual fear conditioning, averaged across the 5 minute testing window, indicated no overall effect of genotype, $F_{\text{genotype}}(1, 90) = 0.47$, $p = 0.50$ (Figure 4.4E). No overall effect of diet was detected, $F_{\text{diet}}(2, 90) = 0.87$, $p = 0.42$. Moreover, the different diet manipulations did not interact with genotype, $F_{\text{genotype x diet}}(2, 90) = 0.77$, $p = 0.47$.

Results for examining memory for the conditioned tone indicated firstly that loss of *Fmr1* resulted in decreased freezing behavior across the test window, $F_{\text{genotype}}(1, 90) = 10.30$, $p = 0.002$ (Figure 4.4F). This reduced freezing was significantly greater in response to the tone, $F_{\text{genotype x time}}(1, 90) = 11.63$, $p = 0.001$ (Figure 4.4F). This decrement in freezing behavior was not, however, impacted by dietary manipulations, both overall, $F_{\text{diet}}(2, 90) = 0.66$, $p = 0.52$, or across time, $F_{\text{diet x time}}(2, 90) = 0.62$, $p = 0.54$. There was

no significant between-subjects interaction of genotype and diet, $F_{\text{genotype x diet}}(2, 90) = 0.60$, $p = 0.52$. No significant three-way interaction was detected, $F_{\text{genotype x diet x time}}(2, 90) = 0.13$, $p = 0.55$. Overall, this suggests that the improvement in acquisition following prenatal exposure to these diets did not translate to cued recall the following day.

Cytokine Expression

Post-weaning Paradigm

Among the potential mechanisms potentially in play here, omega-3 fatty acids are known to normalize expression of cytokine signaling markers (Kang & Weylandt, 2008; Pietropaolo et al., 2014). Following the conclusion of behavioral testing, expression of various cytokines in whole hippocampal samples was assayed. For the post-weaning paradigm, no effect of genotype or diet was detected on: BDNF [$F_{\text{genotype}}(1, 29) = 0.04$, $p = 0.84$; $F_{\text{diet}}(2, 29) = 0.39$, $p = 0.68$; $F_{\text{diet x genotype}}(2, 29) = 1.07$, $p = 0.36$] (Figure 4.5A) or IL-1β [$F_{\text{genotype}}(1, 29) = 0.95$, $p = 0.34$; $F_{\text{diet}}(2, 29) = 1.54$, $p = 0.23$; $F_{\text{diet x genotype}}(2, 29) = 0.14$, $p = 0.87$] (Figure 4.5B). However, similar to previously published studies in our lab, IL-6 [$F_{\text{genotype}}(1, 29) = 4.57$, $p = 0.04$] (Figure 4.5C) and TNF-α [$F_{\text{genotype}}(1, 29) = 7.91$, $p = 0.01$] (Figure 4.5D) were significantly reduced following loss of *Fmr1* (Hodges et al., 2017). TNF-α was not significantly impacted by post-weaning exposure to any of the experimental diets, either overall, $F_{\text{diet}}(2, 29) = 0.40$, $p = 0.67$, or according to genotype, $F_{\text{diet x genotype}}(2, 29) = 0.67$, $p = 0.52$. However, dietary exposure to high levels of omega-3 fatty acids further reduced IL-6 expression, $F_{\text{diet}}(2, 29) = 4.29$, $p = 0.02$. Post-hoc Tukey's multiple comparison supported this conclusion (Standard Diet vs Omega-3,

q = 4.19, $p = 0.02$). Moreover, this effect was consistent across genotypes, $F_{\text{diet x genotype}}(2, 29) = 0.46$, $p = 0.63$.

Prenatal Paradigm

For the prenatal paradigm, a total of 36 samples (n = 6 per group) were used to assess hippocampal expression of proinflammatory cytokines and BDNF. No effect of genotype was detected for BDNF, $F_{\text{genotype}}(1, 30) = 0.007$, $p = 0.93$ (Figure 4.5E). However, exposure to both high fat diets reduced hippocampal expression of BDNF, $F_{\text{diet}}(2, 30) = 7.38$, $p = 0.003$. Post-hoc Tukey's multiple comparisons indicated that both high fat diets reduced hippocampal BDNF expression [(a) Standard Diet vs (b) Omega-3, q = 4.97, $p = 0.004$; (a) Standard Diet vs (b) Control Fat, q = 4.39, $p = 0.01$] (Figure 4.5E). This effect was consistent across the levels of genotype, $F_{\text{diet x genotype}}(2, 29) = 0.33$, $p = 0.72$. A similar pattern was detected for IL-1β. No effect of genotype was detected, $F_{\text{genotype}}(1, 30) = 0.42$, $p = 0.52$ (Figure 4.5F). However, exposure to the two dietary manipulations reduced expression of IL-1β, $F_{\text{diet}}(2, 30) = 8.03$, $p = 0.002$. Post-hoc Tukey's multiple comparison supported this [(a) Standard Diet vs (b) Omega-3 Diet, q = 3.59, $p = 0.004$; (a) Standard Diet vs (b) Control Fat Diet, q = 3.34, $p = 0.007$] (Figure 4.5F). This effect was consistent across genotypes as well, $F_{\text{diet x genotype}}(2, 29) = 0.16$, $p = 0.85$. Unlike the post-weaning paradigm, no effects were seen for either IL-6 [$F_{\text{genotype}}(1, 30) = 0.04$, $p = 0.84$; $F_{\text{diet}}(2, 30) = 2.62$, $p = 0.09$; $F_{\text{diet x genotype}}(2, 29) = 0.02$, $p = 0.98$] (Figure 4.5G) or TNF-α [$F_{\text{genotype}}(1, 30) = 0.38$, $p = 0.54$; $F_{\text{diet}}(2, 30) = 2.16$, $p = 0.13$; $F_{\text{diet x genotype}}(2, 29) = 0.11$, $p = 0.89$] (Figure 4.5H). Overall, these results suggest that prenatal exposure to both dietary manipulations reduced hippocampal expression of BDNF and IL-1β regardless of expression of *Fmr1*.

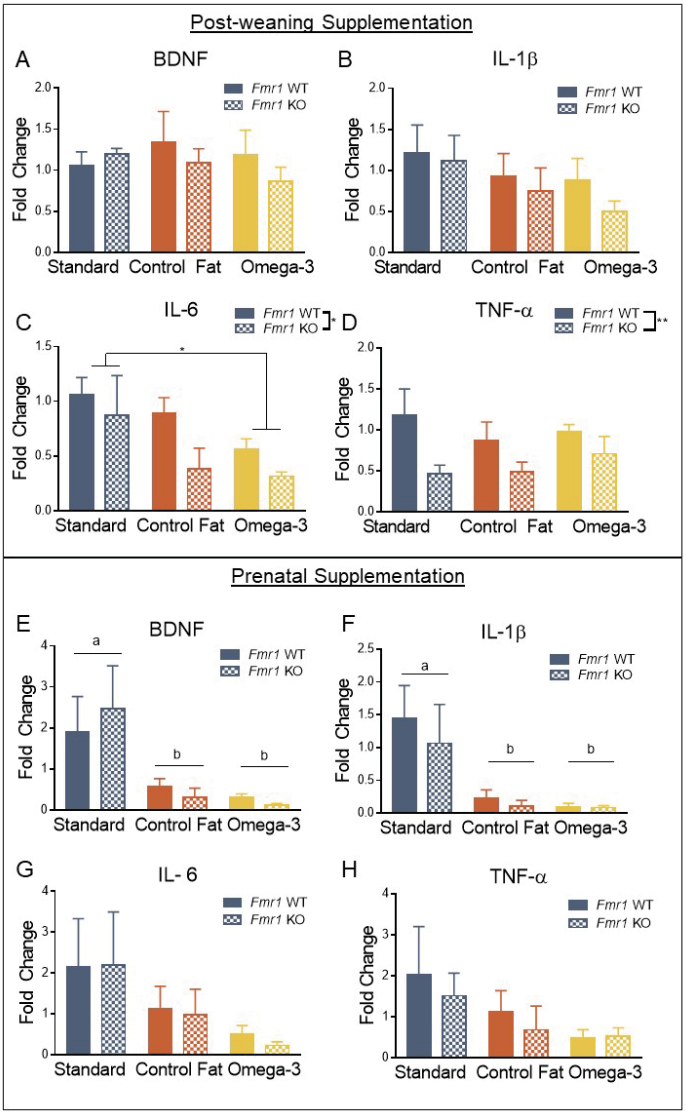

Figure 4.5. Prenatal and post-weaning exposure to high fat diets differentially impacts hippocampal proinflammatory cytokine and BDNF expression. A. Hippocampal expression of BDNF was not impacted by loss of *Fmr1* or exposure to experimental diets. B. Hippocampal expression IL-1β was not impacted by loss of *Fmr1* or exposure to experimental diets. C. Expression of IL-6 in the hippocampus was diminished in the *Fmr1* knockout, and exposure to omega-3 fatty acids also reduced its expression. The control fat diet had no effect on IL-6 expression. D. TNF-α expression was also reduced in the *Fmr1* knockout with no effect of diet. E. BDNF expression was reduced by both experimental diets. F. Similar to BDNF, IL-1β was reduced in the hippocampus of animals exposed to both experimental diets. G. Unlike the postnatal paradigm, no effect was detected for IL-6. H. No effects were detected for TNF-α. Data are expressed as mean ± SEM. * = $P < 0.05$, ** = $P < 0.01$. A designation of "b" indicates that this group differed from the "a" comparison group at the level of $p < 0.05$.

Cytokine Associations with Behavior

Post-weaning Paradigm

Given the nature of the experimental design used, it is difficult to determine if these changes in cytokine expression level are related to the changes we saw in behavior. Thus, we next aimed to determine if these changes were statistically associated with the behavioral phenotypes shown in the current study. These results are summarized in Table 4.1.

Given the decrements in acquisition seen following post-weaning administration of omega-3 fatty acids, we were most interested in whether the expression of IL-6 was associated with freezing behavior during the fear conditioning task. As expected, reductions in IL-6 expression were significantly associated with reductions in freezing behavior to the second presentation of the tone during the acquisition phase, $r(35) = 0.40$, $p = 0.02$ (Figure 4.6A). Reductions in IL-6 expression were also significantly associated with freezing behavior during the contextual phase of the paradigm, $r(35) = 0.43$, $p = 0.01$ (Figure 4.6B). However, this same pattern did not hold up for cued learning the second day, $r(35) = 0.12$, $p = 0.49$ (Figure 4.6C). No other cytokines measured were significantly correlated with these measures, suggesting this is specific to IL-6. These results are in line with the understanding that cued learning is considered amygdala-dependent, while contextual conditioning is hippocampal dependent (Raybuck & Lattal, 2011).

Given the studies showing that cytokine signaling predicts hyperactivity, we also wanted to determine if these reductions in IL-6 or other individual differences in expression levels were significantly associated with our measures of activity levels in the

elevated plus maze (Han et al., 2017). The only significant association was between hippocampal expression of TNFα and velocity during the task, $r(35) = -.40$, $p < 0.05$.

Prenatal Paradigm

Similar to the post-weaning paradigm, correlations were conducted to ascertain if changes in cytokine expressions were related to any changes in behavior (summarized in Table 4.1). Given the improvements in fear learning acquisition seen in the preweaning paradigm concurrent with reductions in BDNF and IL-1β expression, we also assessed if the expression of BDNF and IL-1β was similarly associated with freezing behavior. Indeed, results of the Pearson correlation indicated that there was a significant negative association between hippocampal BDNF expression and freezing to the second tone during the acquisition phase, $r(35) = -.34$, $p = 0.05$ (Figure 4.6D). Similar results were demonstrated for IL-1β expression in the hippocampus, $r(35) = -.35$, $p = 0.04$ (Figure 4.6E). However, these cytokine levels were not significantly related to any other time point during the fear conditioning protocol.

Similar to the post-weaning paradigm, we found it pertinent to examine whether these reductions in cytokine signaling in the hippocampus were related to changes in activity levels. However, results suggested that no cytokines measured were significantly related to either velocity or distance moved in the elevated plus maze.

Table 4.1.

Cytokine Associations with Behavior for Both Paradigms

Cytokine	Post-Weaning Paradigm Measures					Prenatal Paradigm Measures				
	1	2	3	4	5	1	2	3	4	5
BDNF	-.13	-.11	.13	-.06	-.04	**-.34***	-.25	-.10	.03	-.07
IL-1β	.20	.23	.09	.15	.17	**-.35***	-.27	-.07	.11	.04
IL-6	**.40***	**.43****	.12	-.24	-.22	-.27	-.09	-.07	.08	.10
TNFα	.04	.32	.04	-.33	**-.40***	-.20	-.17	-.04	-.008	-.12

Note. Variables Measured: 1) Acquisition Phase Response to Tone; 2) Freezing during Contextual Phase; 3) Freezing to Tone During Cued Recall Phase; 4) Distance Moved in EPM; 5) Velocity during EPM. * = $p < 0.05$; ** = $p < 0.01$

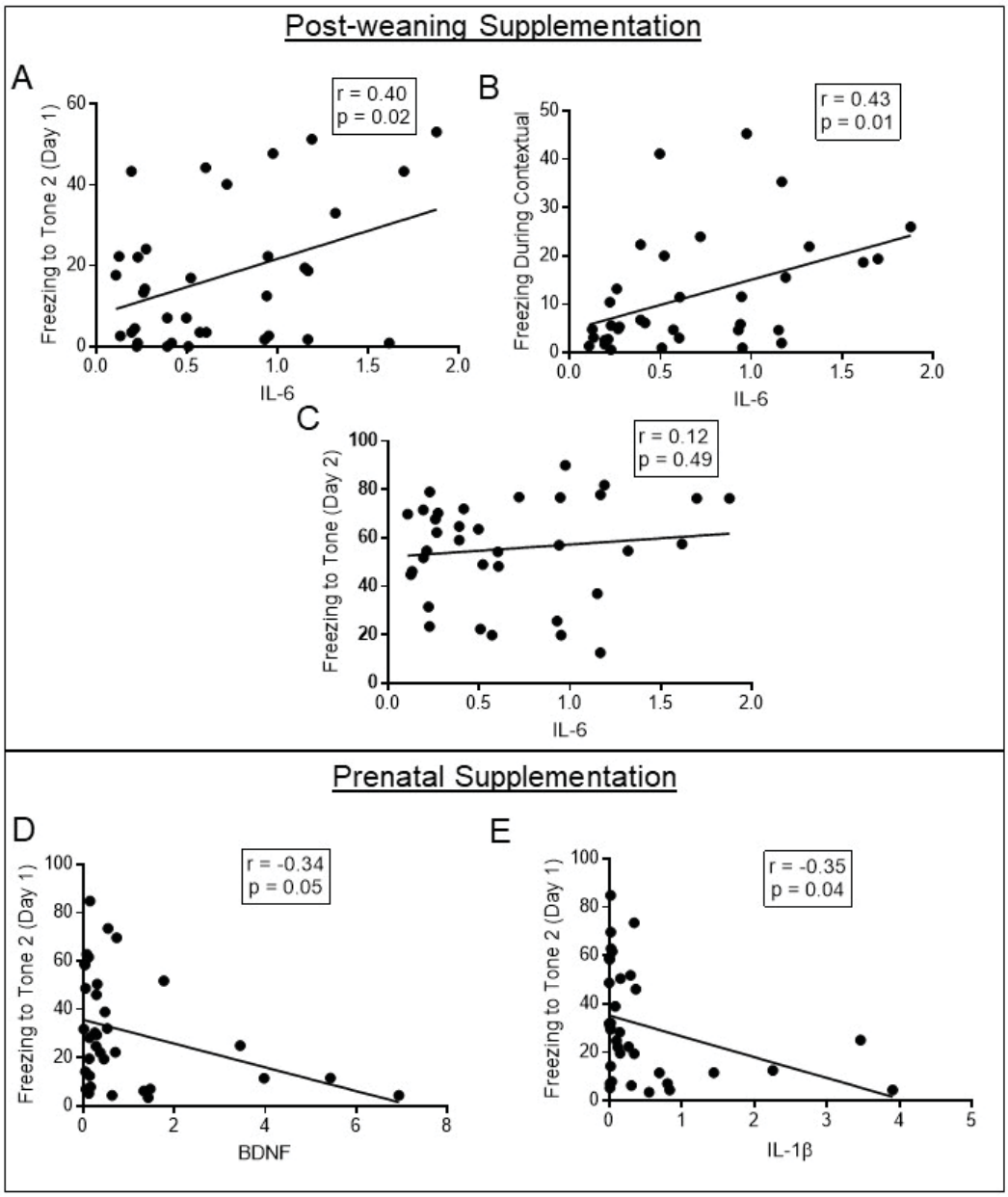

Figure 4.6. Reductions in proinflammatory cytokine and BDNF signaling are associated with fear conditioning performance. A. Reductions in hippocampal IL-6 expression were significantly associated with the reduced freezing behavior seen during the acquisition of delay fear conditioning, specifically during the second presentation of the tone. B. Similarly, reduced freezing behavior during the contextual phase was also significantly associated with IL-6 expression. C. The association between IL-6 and freezing during the cued recall phase was not significant. D. Concurrent with improvements seen in acquisition of a fear memory, BDNF is significantly negatively associated with freezing during the second presentation of the tone. E. Similar results were found for IL-1β expression. Data points represents individual subject scores.

Protein Levels

Post-weaning Paradigm

The results from the western blots for the post-weaning and the prenatal paradigms are summarized in Tables 4.2 and 4.3, respectively. Briefly, for the post-weaning paradigm, exposure to omega-3 fatty acids ("b") during this time period upregulated protein expression of the GluR1 receptor, compared to both the control fat ("a") and standard diet conditions ("a") (Figure 4.7A).

Prenatal Paradigm

For the prenatal paradigm, an effect similar to that seen in the post-weaning paradigm was detected for GluR1. However, GluR1 was only upregulated in the *Fmr1* KO ("b") in this condition (Figure 4.7B). Additionally, prenatal exposure to omega-3 fatty acids ("b) upregulated protein expression of MMP-9, regardless of genotypes, compared to both the control fat ("a") and omega-3 ("a") diets (Figure 4.7C).

PD9 Ultrasonic Vocalizations

To further assess the potential therapeutic potential of these experimental manipulations, we also assessed ultrasonic vocalization production on postnatal day 9 for mice from the prenatal paradigm. Furthermore, we were further able to include females in this paradigm to assess potential sex differences in response to these diets.

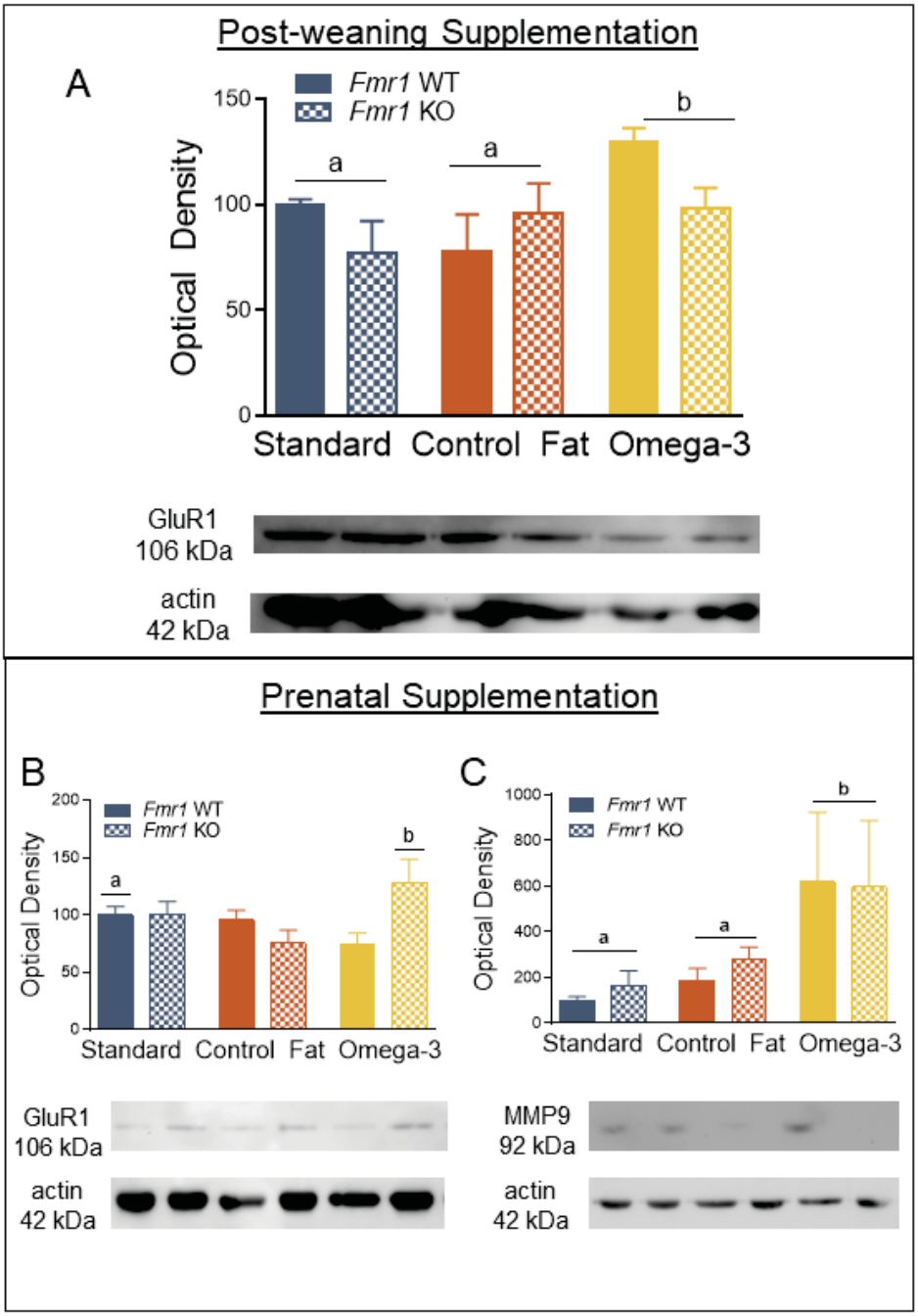

Figure 4.7. Both post-weaning and prenatal exposure to omega-3 fatty acids upregulated protein levels of GluR1. A. Post-weaning exposure to omega-3 fatty acids upregulated hippocampal protein levels of GluR1, regardless of genotype. B. Similarly, prenatal exposure to omega-3 fatty acids upregulated hippocampal levels of GluR1, in the *Fmr1* knockout only. C. Additionally, MMP9 was upregulated in response to prenatal exposure to omega-3 fatty acids, regardless of genotype. Data are expressed as mean ± SEM. A designation of "b" indicates that this group differed from the "a" comparison group at the level of $p < 0.05$.

Table 4.2.

Post-Weaning Western Results

Sample Prep	Primary Antibody	ANOVA Results			Post-hoc Analyses
		Genotype	Diet	Interaction	
Hippocampal Synaptosome	PSD-95	F(1, 29) = 1.11, p = 0.30	F(2, 29) = 0.96, p = 0.40	F(2, 29) = 0.96, p = 0.40	-
	GFAP	F(1, 29) = 0.71, p = 0.41	F(2, 29) = 0.46, p = 0.46	F(2, 29) = 0.56, p = 0.56	-
	GluR1	F(1, 29) = 1.49, p = 0.22	F(2, 29) = 3.23, p = 0.05	F(2, 29) = 2.45, p = 0.10	Diet: Standard vs Omega-3, p = 0.05; Control Fat vs Omega-3, p = 0.03
	nR2b	F(1, 29) = 0.11, p = 0.75	F(2, 29) = 1.96, p = 0.16	F(2, 29) = 0.06, p = 0.94	-
	MMP-9	F(1, 27) = 0.29, p = 0.59	F(2, 27) = 1.09, p = 0.35	F(2, 27) = 0.57, p = 57	-
Hippocampal Homogenate	Iba1	F(1, 29) = 0.07, p = 0.80	F(2, 29) = 0.65, p = 0.53	F(2, 29) = 0.93, p = 0.41	-
	Caspase-3	F(1, 29) = 0.0005, p = 0.98	F(2, 29) = 0.26, p = 0.77	F(2, 29) = 0.52, p = 0.60	-
	IL6-R	F(1, 29) = 0.31, p = 0.58	F(2, 29) = 1.97, p = 0.16	F(2, 29) = 0.18, p = 0.84	-
	% phospho-s6 (s235-236) / total s6	F(1, 29) = 0.68, p = 0.51	F(2, 29) = 0.06, p = 0.94	F(2, 29) = 0.68, p = 0.51	-
	% phospho-s6 (s240-244) / total s6	F(1, 29) = 1.40, p = 0.25	F(2, 29) = 0.60, p = 0.55	F(2, 29) = 0.08, p = 0.92	-
	S6 Ribosomal Protein	F(1, 29) = 0.12, p = 0.73	F(2, 29) = 0.53, p = 0.60	F(2, 29) = 0.12, p = 0.90	-
	ERK 42	F(1, 27) = 0.45, p = 0.51	F(2, 27) = 2.72, p = 0.08	F(2, 27) = 0.85, p = 0.44	-
	ERK 44	F(1, 27) = 0.19, p = 0.67	F(2, 27) = 3.05, p = 0.06	F(2, 27) = 1.09, p = 0.35	-

Table 4.3

Prenatal Western Results.

Sample Prep	Primary Antibody	ANOVA Results			Post-hoc Analyses
		Genotype	Diet	Interaction	
Hippocampal Synaptosome	PSD-95	F(1, 30) = 0.03, p = 0.87	F(2, 30) = 0.22, p = 0.80	F(2, 30) = 2.19, p = 0.13	-
	GFAP	F(1, 30) = 0.15, p = 0.70	F(2, 30) = 2.13, p = 0.14	F(2, 30) = 0.22, p = 0.80	-
	GluR1	F(1, 30) = 1.29, p = 0.26	F(2, 30) = 0.22, p = 0.80	F(2, 30) = 4.98, p = 0.01	KO Omega-3 displayed upregulated levels of GluR1
	nR2b	F(1, 30) = 0.14, p = 0.70	F(2, 30) = 0.90, p = 0.42	F(2, 30) = 0.04, p = 0.96	-
	MMP-9	F(1, 30) = 0.10, p = 0.76	F(2, 30) = 4.01, p = 0.03	F(2, 30) = 0.06, p = 0.94	Standard Diet vs Omega-3 Diet, $p < 0.05$
Hippocampal Homogenate	Iba1	F(1, 30) = 1.52, p = 0.23	F(2, 30) = 1.00, p = 0.38	F(2, 30) = 0.54, p = 0.59	-
	Caspase-3	F(1, 30) = 1.45, p = 0.24	F(2, 30) = 1.33, p = 0.28	F(2, 30) = 0.01, p = 0.99	-
	IL6-R	F(1, 30) = 0.27, p = 0.61	F(2, 30) = 0.22, p = 0.81	F(2, 30) = 0.17, p = 0.85	-
	% phospho-s6 (s235-236) / total s6	F(1, 30) = 2.90, p = 0.10	F(2, 30) = 2.28, p = 0.12	F(2, 30) = 1.62, p = 0.21	-
	% phospho-s6 (s240-244) / total s6	F(1, 30) = 0.008, p = 0.93	F(2, 30) = 0.76, p = 0.48	F(2, 30) = 0.89, p = 0.42	-
	S6 Ribosomal	F(1, 30) = 1.31, p = 0.26	F(2, 30) = 0.22, p = 0.81	F(2, 30) = 1.93, p = 0.16	-
	ERK 42	F(1, 30) = 1.22, p = 0.28	F(2, 30) = 0.08, p = 0.92	F(2, 30) = 1.20, p = 0.31	-
	ERK 44	F(1, 30) = 1.09, p = 0.31	F(2, 30) = 0.10, p = 0.91	F(2, 30) = 1.09, p = 0.35	-

Males

The analyses for males and females were conducted separately due to the addition of a heterozygous group for females. For males, the analyses were conducted as they were for the adult paradigm (2 x 3 ANOVA). No effect of genotype was detected for any of the variables measured: number of calls, $F_{genotype}(1, 89) = 0.19$, $p = 0.66$ (Figure 4.8A), average duration, $F_{genotype}(1, 89) = 3.60$, $p = 0.06$ (Figure 4.8B), fundamental frequency, $F_{genotype}(1, 89) = 0.96$, $p = 0.33$ (Figure 4.8C), or spectral purity, $F_{genotype}(1, 89) = 2.38$, $p = 0.13$ (Figure 4.8D). No effect of diet was detected for any of the following variables: number of calls, $F_{diet}(2, 89) = 1.54$, $p = 0.22$, average duration, $F_{diet}(2, 89) = 1.52$, $p = 0.23$, or spectral purity, $F_{diet}(2, 89) = 0.94$, $p = 0.40$. There were, however, some effects of significance. Fundamental frequency was significantly altered by diet, $F_{diet}(2, 89) = 4.44$, $p = 0.02$. Post-hoc analyses with LSD indicated that the omega-3 diet ("b") increased the fundamental frequency, compared to both standard ("a") and control fat ("b")conditions. No significant interactions of genotype and diet for any variable measured were detected: number of calls, $F_{diet\ x\ genotype}(2, 89) = 0.28$, $p = 0.76$, average duration, $F_{diet\ x\ genotype}(2, 89) = 0.52$, $p = 0.60$, fundamental frequency, $F_{diet\ x\ genotype}(2, 89) = 0.78$, $p = 0.46$, or spectral purity, $F_{diet\ x\ genotype}(2, 89) = 0.14$, $p = 0.87$. Overall, the only significant finding in males demonstrated that fundamental frequency was significantly increased in pups receiving the omega-3 fatty acid diet.

Females

For females, analyses indicated more areas of significance compared to males. Firstly, no main effect of genotype was detected on the number of calls, $F_{genotype}(2, 147) = 0.91$, $p = 0.40$ (Figure 4.8E). No main effect of diet was detected for number of calls,

$F_{diet}(2, 147) = 2.77$, p = 0.07. However, a significant interaction was detected, $F_{diet\ x\ genotype}(2, 147) = 6.52$, p = 0.001, and was followed up with the creation of the unique "group" identifier for all 9 groups (e.g. "Standard WT" or "Standard Het"). Post-hoc LSD analyses indicated a few comparisons of interest (Figure 4.8H). First, in the standard diet condition, heterozygous deletion of *Fmr1* in the two experimental dietary conditions resulted in a decrease in the number of calls produced [(a) Standard HET vs (b) Standard WT, p = 0.001]. While the control fat diet had no effect on this [(b) Control Fat HET vs (a) Standard WT, p = 0.001], and the decrease in calls in the HET mice was blocked by exposure to the omega-3 fatty diet, [(a) Standard WT vs (a) Omega-3 HET, p = 0.11]. Second, exposure to the control fat condition decreased the number of vocalizations produced in the wildtypes [(a) Standard WT vs (b) Control Fat WT, p = 0.001], but had no impact on the *Fmr1* KO [(a) Standard WT vs (a) Control Fat KO, p = 0.67). Third, exposure to the omega-3 condition decreased the number of vocalizations in the *Fmr1* KO [(a) Standard WT vs (b) Omega-3 KO, p = 0.001).

No main effect of genotype was detected average duration, $F_{genotype}(2, 147) = 0.63$, p = 0.53 (Figure 4.8F), nor was there a main effect of diet on average duration of calls produced, $F_{diet}(2, 147) = 0.004$, p = 0.996. The interaction between genotype and diet was not significant for average duration, $F_{diet\ x\ genotype}(2, 147) = 1.73$, p = 0.15 (Figure 4.8F).

No main effect of genotype was detected for fundamental frequency, $F_{genotype}(2, 147) = 0.09$, p = 0.92 (Figure 4.8G). Diet did, however, affect fundamental frequency, $F_{diet}(2, 147) = 4.17$, p = 0.02. Post-hoc analyses with LSD indicated that exposure to the control fat diet lowered the fundamental frequency, compared to the standard diet control,

at the level of $p < 0.05$. This was not the case for the omega-3 diet condition ($p = 0.10$). A significant interaction of genotype and diet, $F_{\text{diet x genotype}}(2, 147) = 2.91$, $p = 0.02$, was followed up LSD analyses (Figure 4.8G). First, in the standard diet condition, heterozygous loss of *Fmr1* decreased fundamental frequency [(a) Standard WT vs (b) Standard Het, $p = 0.003$). This was only true in the heterozygous condition but not homozygous deletion [(a) Standard WT vs (b) Standard KO, $p = 0.09$]. In the control fat condition, all three genotypes displayed diminished fundamental frequency ("a" vs "b" $p < 0.01$) compared to the standard WT. It should be noted that the magnitude of this difference was not different between the HET and KOs in this dietary group ($p > 0.05$). Similarly, exposure to the omega-3 condition reduced fundamental frequency in the wildtype animals [(a) Standard WT vs (b) Omega-3 WT, $p = 0.006$]. The reduction in fundamental frequency shown by the standard diet HETs effect was similar for heterozygous deletion in the omega-3 condition, [(a) Standard WT vs (b) Omega-3 HET $p = 0.05$].

Results also indicate that loss of *Fmr1* resulted in diminished spectral purity, $F_{\text{genotype}}(2, 147) = 3.33$, $p = 0.04$ (Figure 4.8H). Similar to fundamental frequency, there was a main effect of diet on spectral purity, $F_{\text{diet}}(2, 147) = 4.17$, $p = 0.02$. Post-hoc LSD analyses indicated no significant effects however, which may be due to the significant two-way interaction. The significant interaction, $F_{\text{diet x genotype}}(2, 147) = 7.01$, $p = 0.001$, was followed up with post-hoc LSD analyses using a unique group identifier (i.e. Standard WT) (Figure 4.8H). First, results indicated that homozygous loss of *Fmr1* resulted in reduced spectral purity in the standard diet condition [(a) Standard WT vs (b) Standard KO, $p = 0.05$). This effect was ameliorated by exposure to both the control fat

[(a) Standard WT vs (a) Control Fat KO, $p = 0.79$) and omega-3 diets [(a) Standard WT vs (a) Omega-3 KO, $p = 0.86$].

Overall, these results suggest that communication behavior in females may be more sensitive to dietary manipulations during the prenatal and early neonatal period. However, these results do not conclusively point to one diet as optimal for the development of this behavior and thus, further investigations are necessary.

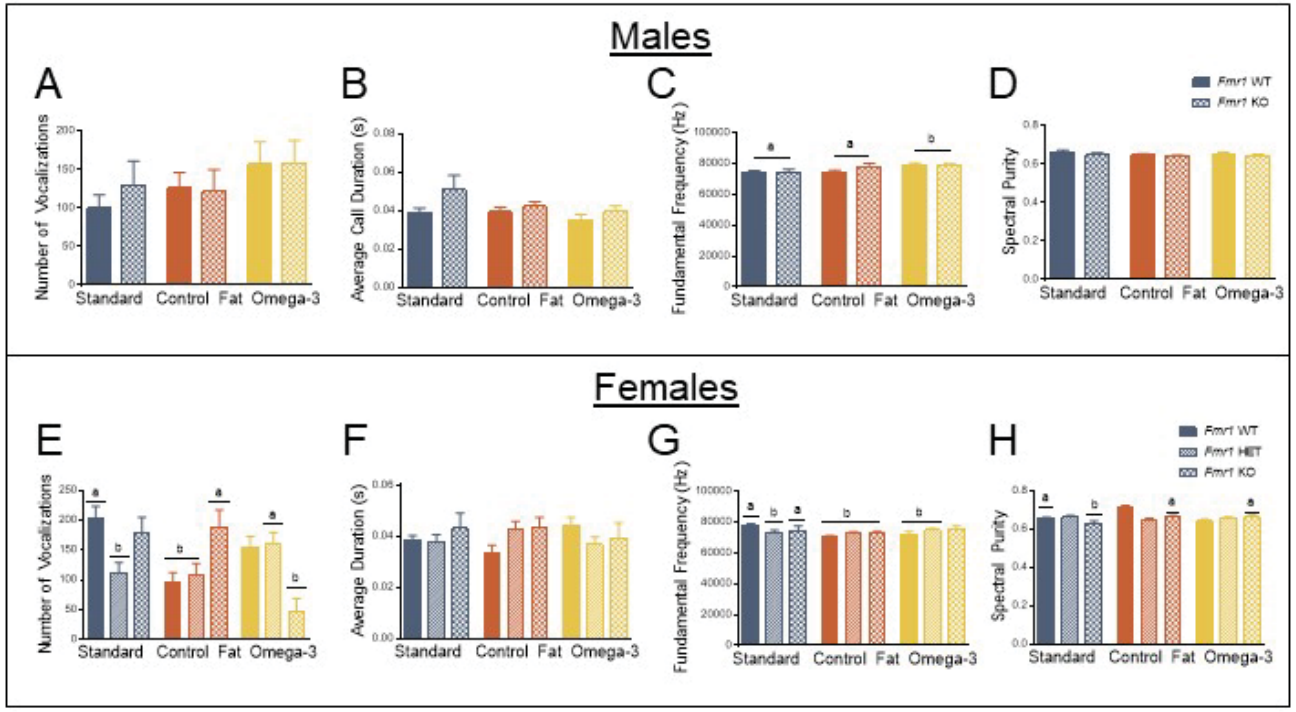

Figure 4.8. Ultrasonic vocalizations on PD9 are differentially affected by diet across males and females. A. Number of vocalizations was unaffected by diet and genotype in males. B. Average duration of vocalizations was unaffected by diet and genotype in males. C. In males, fundamental frequency was significantly increased in subjects receiving the prenatal omega-3 diet, regardless of genotype. D. Spectral purity was unaffected by diet or genotype in males. E. Compared to the female Standard WT, both the female Standard Het and the female Control Fat Het showed reduced number of calls, and this was protected against by exposure to the omega-3 fatty acid diet. F. Average duration was unaffected by both genotype and diet in females. G. In females, fundamental frequency was reduced by heterozygous loss of *Fmr1* and unaffected by the dietary condition. Moreover, the female WTs receiving the two experimental diets showed similar reductions in fundamental frequency. H. In the Standard Diet condition, homozygous loss of *Fmr1* resulted in diminished spectral purity, however, exposure to the two experimental diet protected against this effect. Data are expressed as mean ± SEM. A designation of "b" indicates that this group differed from the "a" comparison group at the level of $p < 0.05$.

CHAPTER FIVE

Discussion

Hyperactivity is a significant clinical component of the FXS phenotype (Baumgardner, Reiss, Freund, & Abrams, 1995; Fryns, Jacobs, Kleczkowska, & van den Berghe, 1984; Hagerman, 1987; Hatton et al., 2002). The hyperactivity phenotype has also been replicated in several studies using the *Fmr1* knockout mice (Baker et al., 2010; Ding et al., 2014; Pietropaolo et al., 2011). Previous clinical studies demonstrated that treatment with omega-3 during a similar window reduced hyperactivity in individuals with ASD (Amminger et al., 2007). With respect to the *Fmr1* mouse model, previous animal studies have indicated that treatment with other anti-inflammatory agents also reduced hyperactivity (Dansie et al., 2013). Based on these findings, we hypothesized that dietary supplementation would ameliorate hyperactivity. Indeed, the results of the current study demonstrated that both post-weaning and prenatal exposure to omega-3 fatty acids attenuated hyperactivity shown in the standard diet KO subjects. This effect was specific to the type of fatty acid, as the control fat diet had no impact on activity. Moreover, in clinical studies, levels of circulating cytokines, such as those affected by omega-3s, are directly associated with hyperactivity in children with ASD (Ashwood et al., 2008). Along these lines, we found that velocity in the EPM was negatively associated with hippocampal expression of TNFα, suggesting this relationship may be similarly relevant to the current model. Overall, the results of the present study find that

supplementation with omega-3 fatty acids across the lifespan is sufficient to attenuate hyperactivity in the *Fmr1* knockout.

Similarly, the results of the current study indicated that prenatal dietary manipulation with omega-3 fatty acids can improve pre-pulse inhibition in the *Fmr1* knockout. The acoustic startle response (ASR) and its inhibition is a highly conserved evolutionary process. The circuitry surrounding this phenotype is amenable to a variety of inputs, such as magnitude of the stimulus, background noise, and perceptual/emotional states of the organism (Koch, 1999). Alterations in these sensorimotor gating behaviors are a significant clinical component for children with FXS (Hagerman, 1997; Hagerman et al., 1986). However, no studies have examined the effect of prenatal omega-3 on prepulse inhibition in the *Fmr1* knockout. For the prenatal paradigm, loss of *Fmr1* resulted in exaggerated prepulse inhibition that was reversed by exposure to the omega-3 diet. In support of our findings, in the ketamine model of schizophrenia, changes in prepulse inhibition phenotypes are amenable to omega-3 fatty acids (Zugno et al., 2014). Moreover, other manipulations of dietary fat, such as the ketogenic diet, support the role of fatty acid signaling in the sensorimotor phenotype, showing that a diet consisting of 77.6% calories from fat improves deficits in PPI in a rodent model of schizophrenia (Kraeuter, van den Buuse, & Sarnyai, 2018). Broadly, this phenotype can be measured similarly between rodents and humans, with both showing a reliable and clinically significant phenotype (Frankland et al., 2004). Thus, the results of the current study provide support that a prenatal diet high in omega-3 fatty acids may hold promise as a potential therapeutic in patients with FXS.

The results of the current study also demonstrated that the timing of the dietary manipulation is important for the effect on the sensorimotor gating phenotype. Converse to the results of the prenatal paradigm, results from the post-weaning paradigm indicated that the exaggerated PPI shown in the KO mouse was not affected by exposure to either experimental diet during the post-weaning period. However, reduced startle threshold was ameliorated by exposure to both omega-3 and control fat diets during the post-weaning period in the KO mice, and this wasn't seen in the post-weaning paradigm. Previous work has demonstrated that startle responding and inhibition of the startle response with a pre-pulse stimulus rely on separate circuitry and mechanisms (Azzopardi, Louttit, DeOliveira, Laviolette, & Schmid, 2018; Ison, Taylor, Bowen, & Schwarzkopf, 1997). Thus, this discrepancy in findings between the two paradigms could be related differences in circuit susceptibility across time. While no previous studies have examined this effect of omega-3 fatty acids on sensorimotor gating behaviors in this model, our results are supported by dietary manipulation in a similar timeframe attenuated deficits in prepulse inhibition following prenatal exposure to polyI:C (Li et al., 2015). The results of the present study suggest that this relationship may be more complex than previously hypothesized.

The current study finds that the timing of the intervention also mattered for the impact on fear learning and memory. Clinical data demonstrates mixed findings regarding the efficacy of omega-3 fatty acids on aspects of cognition functioning. First, clinical studies have shown no significant impact of omega-3 fatty acids on cognition in neurotypical adults (Jackson et al., 2011; van de Rest et al., 2008). However, high omega-3 fatty acids may prove beneficial for treatment of patients with mild cognitive

impairment (Lee et al., 2013; Mazereeuw et al., 2012). In animals, a high omega-3 diet similar to our post-weaning paradigm attenuated deficits in hippocampal-dependent novel object recognition in the *Fmr1* knockout (Pietropaolo et al., 2014). Thus, we expected that other aspects of nonspatial hippocampal-dependent memory might be similarly improved by this treatment. In the present study, post-weaning exposure to the two experimental diets diminished freezing behavior during acquisition, thus inhibiting learning of the acquired response. When tested 24 hours later, post-weaning exposure to the experimental diets also reduced contextual fear conditioning and cued recall, regardless of genotype. While many methodological factors could be involved, the discrepancy between our findings and the previous study may be due to the inclusion of the standard diet control group. Specifically, the two experimental diets included in the previous study mirror our omega-3 and control fat groups, and for our study we included a standard dietary control. In the present study, the control fat diet mirrors a typical Western diet, which has been demonstrated to have its own effects on behavior (Peleg-Raibstein et al., 2012; Williams, Seki, Vuguin, & Charron, 2014). Thus, the inclusion of the standard diet control renders direct comparisons between the two studies difficult. Previous studies have signaled that potential therapeutic benefit of omega-3 is effectively modulated by the timing of the manipulation as well as the timing of testing (Carrie et al., 2000). Converse to the findings of the post-weaning paradigm, the prenatal paradigm resulted in improved acquisition of a fear response for both diets. We were surprised that both diets influenced this behavior, and believe that this may reflect the hypothesis that manipulation of fat content can induce large changes in developing an appropriate fear response (Owada et al., 2006; Peleg-Raibstein et al., 2012; Sasaki, de Vega, Sivanathan,

St-Cyr, & McGowan, 2014; Sullivan, Riper, Lockard, & Valleau, 2015). Overall, the present study adds to the knowledge that manipulation of fat content across the lifespan can significantly impact fear learning and memory.

The results for vocalization behavior in the current study also provide support for the impact of these diets on behavior, particularly early in development. Specifically, experimental manipulation of prenatal dietary fat influenced various aspects of vocalization behavior on PD9, including the spectral purity. Spectral purity is calculated as "the instantaneous maximum power at the peak frequency normalized by the instantaneous total power in the spectrum, averaged across the entire syllabus" (Arriaga, Zhou, & Jarvis, 2012). Of biological relevance, congenitally deaf mice have a lower spectral purity than their hearing-intact counterparts, suggesting that auditory feedback is integral to the development of these vocalization (Arriaga & Jarvis, 2013). This postulation is considered somewhat contentious, as many support that vocal learning as a construct is unique to a few species of birds, humans, cetaceans, bats, elephants and pinnipeds (Janik & Slater, 2000; Jarvis, 2004). According to this definition, vocalizations produced in mice are considered innate. Indeed, it should be noted that number, structure and usage of vocalizations has been shown not to differ in congenitally deaf mouse pups (Hammerschmidt et al., 2012). Moreover, normative production of most aspects of vocalizations occurs without the presence of a normally developed cerebral cortex, suggesting this is a fundamental and reflexive behavior (Hammerschmidt, Whelan, Eichele, & Fischer, 2015). The current study presents novel evidence that homozygous deletion of *Fmr1* in female pups reduced spectral purity, which may reflect disruption of auditory brainstem networks shown previously in this model (Ruby, Falvey, & Kulesza,

2015). With relevance to the aim of the present study, we found that this diminished spectral purity could be rescued by exposure to either the high fat or high omega-3 diets. Prenatal DHA levels have been shown to significantly influence the development of the auditory brainstem in rodent models (Haubner et al., 2002; Stockard et al., 2000). Thus, it is possible that improvements in spectral purity due to increased fatty acid consumption during this early period reflect an effect on previously unknown pathology in the *Fmr1* model. This is further evidenced by the lack of effect in WT animals. However, impaired fatty acid metabolism has not been yet demonstrated in clinical samples of FXS, though previous studies have indicated dysregulation of fatty acid metabolism markers in individuals with ASD (El-Ansary, Bacha, & Al-Ayahdi, 2011; Mostafa & Al-Ayadhi, 2015; Mostafa, El-Khashab, & AL-Ayadhi, 2015; Sun et al., 2013). Further studies are needed to elucidate the role of fatty acid metabolism during early development in both the *Fmr1* knockout and in clinical FXS samples.

The current study also sought to examine the possibility that this treatment may differentially affect the two sexes by examining ultrasonic vocalizations early in development. In the present study, in addition to the changes in spectral purity, both the number of vocalizations and fundamental frequency were vulnerable to manipulations of fat content in females, but not in males. It's possible that the results of the current study reflect a combination of the susceptibility to the behavioral effects of loss of *Fmr1* and the sex-specific nature of biological processes, like the trajectory of neurodevelopment or fatty acid metabolism (Decsi & Kennedy, 2011). In support of the latter, clinical findings have demonstrated that female children are born with higher levels of omega-3 fatty acids, suggesting a disparity in fetal metabolism (Colquhoun & Bunday, 1981). In

addition to the sex-specificity of fatty acid metabolism, previous studies have also supported the sex-specific trajectory of vocalization production early in development (Reynolds et al., 2016). Few studies have examined the sex-specific nature of the *Fmr1* phenotype and even fewer have investigated differences early in development (Baker et al., 2010; Ding et al., 2014; Nolan et al., 2017; Qin et al., 2005). However, the exact combination of these factors at play in the present study is unclear, and future studies should elaborate on this question.

A fundamental hypothesis of this study was that changes in behavior resulting from these dietary manipulations could be related to changes in inflammatory cytokine signaling, and the results of this study broadly supported this. We first sought to replicate original findings from our lab, where we demonstrated reduced expression of IL-6 and TNF-α following loss of *Fmr1* in animals aged over PD90 (Hodges et al., 2017). Next, we found that post-weaning treatment with omega-3 fatty acids further reduced IL-6 expression levels. Prior to conducting the study, we had anticipated that a normalization of hippocampal IL-6 expression would coincide with improvements in hippocampal dependent behaviors; however, the current study indicated that in conjunction with reduced IL-6 expression, post-weaning exposure to omega-3s was detrimental to fear conditioning performance. The IL-6 signaling molecule is important for many functions across the brain, including promotion of neuronal survival, protection against damage and modulation of neurotransmitter synthesis (Gadient & Otten, 1997). In knockout studies, IL-6 has been demonstrated to be necessary for adequate performance in the novel object recognition task, as well as water maze performance, further suggesting its importance in normative hippocampal functioning (Baier, May, Scheller, Rose-John, & Schiffelholz,

2009). In support of this role, we found that this further reduction in IL-6 by omega-3 fatty acids was significantly associated with impairments in both acquisition of the fear response and contextual fear conditioning, which are both known to be hippocampal dependent. Conversely, levels of IL-6 signaling were not significantly associated with the decrements seen in the amygdala-dependent portion of our paradigm. While previous studies across many fields have maligned IL-6 for its role in health and disease, new hypotheses suggest that IL-6 may only take on a negative role in the presence of other proinflammatory cytokines (Raison, Knight, & Pariante, 2018). Taken as a whole, these results support that constitutive expression of IL-6, rather than overall reduction, is necessary for proper hippocampal functioning in the *Fmr1* model.

Similarly, reduction of hippocampal levels of IL-1β and BDNF also was associated with significant effects on behavior. Following exposure to the prenatal paradigm, we found that IL-1β expression levels were reduced by exposure to both experimental diets, compared to the standard diet control. Additionally, these changes were significantly associated with improvements in acquisition of a fear response. These findings are congruent with previous findings have shown that reduction of IL-1β in this model is associated with improvements in hippocampal dependent tasks (Pietropaolo et al., 2014). Broadly, IL-1β is associated with neurodegeneration and cell death in the hippocampus (Stojakovic et al., 2017). Thus, we posit that IL-1β is detrimental to normal hippocampal functioning in this model. Similarly, reduction of hippocampal BDNF expression was associated with improvements in acquisition of a fear memory. Previous studies have supported a beneficial effect for the reduction of BDNF for hyperactivity and sensorimotor gating behavior in the *Fm1* knockout (Uutela et al., 2012). While BDNF is typically

considered a beneficial neurotrophic factor that promotes neuronal survival, in the *Fmr1*-null brain, BDNF-TrkB signaling is upregulated (Louhivuori et al., 2011). The results of the current study support the finding that downregulation of BDNF improves aspects of the *Fmr1* knockout phenotype. Altogether, the results broadly suggest that IL-1β and BDNF may significantly impair the acquisition of a fear response.

One possible limitation of the current study was the absence of multiple timepoints and brain areas for assessment of cytokine and protein levels, as tissue was taken only after the conclusion of all behavioral testing. Recent research has indicated that contrary to our lab's adulthood findings, IL-6 expression is increased in whole cortical samples of the *Fmr1* knockout on PD7, 14, and 21 in the same background strain (Krasovska & Doering, 2018). This finding raises several questions for the present study, such as the impact of these diets on the signaling markers assessed in the present study during the early postnatal period. Our behavioral characterization conducted during this early period indicated significant effects of both dietary manipulations on ultrasonic vocalization behavior. Previous studies have indicated a relationship between circulating cytokines and changes in USV behavior. For example, in the maternal immune activation model, early postnatal development is characterized by elevated proinflammatory cytokines (Garay et al., 2013). Concurrent with these elevations in proinflammatory cytokines, recordings of USVs show many changes compared to control groups (Malkova et al., 2012; Schwartzer et al., 2013). In addition to not assessing during early development, we only assessed cytokine expression in the hippocampus. Limited research has investigated cytokine expression across the various brain regions in the *Fmr1* model, so it is unclear if different brain areas would show distinctive patterns at

baseline. However, in support of the likelihood that changes in the current study in the hippocampus mimic other brain structures, Garay et al., 2013 found that the frontal cortex, cingulate cortex and hippocampus showed similar patterns of elevated cytokine expression following maternal immune activation, with few exceptions (Garay et al., 2013). Future studies investigate these possible regional and age-related specificities.

Unexpectedly, in our study we found no significant effect of loss of *Fmr1* on hippocampal expression of matrix metallopeptidase 9 (MMP-9) in either paradigm, as well as decreased anxiety as a result of *Fmr1* loss. To the contrary of the current study, previous studies have demonstrated that attenuation of increased MMP-9, characteristic of the *Fmr1* knockout hippocampus, by perinatal treatment with minocycline is concurrent with reduction of increased anxiety (Bilousova et al., 2009; Dziembowska et al., 2013). One reason for the lack of difference in MMP-9 in our study may be due to strain differences. Strain differences are a common source of variation of the *Fmr1* model (Crawley et al., 1997; Dobkin et al., 2000; Paradee et al., 1999). Specifically, the earlier studies were conducted using the C57BL/6 background strain, while the current study was conducted on the FVB.129 background strain (Bilousova et al., 2009). Yet, other studies have in fact found patterns of reduced anxiety in the *Fmr1* knockout similar to our study, in both the FVB (Liu & Smith, 2009) and the C57 background strain (Eadie et al., 2009). The potential reasons behind the discrepancy in results for MMP-9 are less apparent. MMP-9 is a protein involved in the degradation of the extracellular matrix and blood brain barrier leakage, with increased levels leading to immune cell extravasation (Wosik, Biernacki, Khouzam, & Prat, 2007). Contrary to the present study, other studies conducted in the same background strain have indicated increased hippocampal MMP-9

expression (Janusz et al., 2013; Sidhu, Dansie, Hickmott, Ethell, & Ethell, 2014). Additionally, based on the literature, we hypothesized that increased MMP-9 in the knockout would be reduced by treatment with omega-3 fatty acids, and instead found that treatment increased expression of MMP-9 (Shinto et al., 2009; Taguchi et al., 2014; Zhang et al., 2016). It is unclear why these discrepancies exist, though they could be due to the antibody used, as there was significant variation in values for MMP-9 expression. An alternate explanation could be that there may be a significant rebound in MMP-9 expression in response to early suppression by the omega-3 diet. In the prenatal paradigm, subjects had not received the diet for several weeks before tissue was collected. The previous studies conducted collected tissue immediately after the experimental manipulation. These methodological differences could account for the discrepancies demonstrated here and future studies should address this.

A fundamental tenet of our study was that loss of *Fmr1* would result in behavioral changes that mirror those previously seen in our lab as well as others. As hypothesized, we noted hyperactivity in the elevated plus maze, enhanced pre-pulse inhibition, a reduced startle threshold curve, and diminished fear learning. Moreover, no effect of loss of *Fmr1* was noted on either social behavior or stereotypy (data found in Appendix B). These results are consistent with previous experiments conducted in our lab (Nolan et al., 2017) and others (Frankland et al., 2004). However, there were some unexpected findings, perhaps due to the shifts in behavioral time points across the two paradigms. For example, our results for the elevated plus maze indicated that loss of *Fmr1* resulted in decreased anxiety only in the prenatal paradigm (around the age of PD60). However, when tested after PD90 (in the post-weaning paradigm), no effect of genotype was

detected. The finding from the prenatal paradigm is congruent with our previous findings, demonstrating decreased anxiety around the same time point (Nolan et al., 2017). Similarly, no impairments in cued recall were detected in the post-weaning paradigm for the delay fear conditioning task, however we did see this effect in the prenatal paradigm. The timing again lines up with the previous study in conducted in our lab, and suggests that these impairments may not hold the same pattern after 3 months of age (Nolan et al., 2017). While the reason for this is unclear, this lack of effect at a later timepoint could reflect a shift to a different expression of the fear response. Many factors, including sex and individual differences, have been shown to result in divergent expression of fear responses (Bush, Sotres-Bayon, & LeDoux, 2007; Gruene, Flick, Stefano, Shea, & Shansky, 2015). Overall, much of the work in the *Fmr1* knockout adult phenotype has focused on the early (~PD60) adult phenotype, and the results of the present study clearly indicate a need to include more time points to fully elucidate the role of *Fmr1* in fear learning.

While the current study provides proposed immune signaling as the main mechanism for the therapeutic potential of omega-3 fatty acids, it should be noted that other mechanisms, such as changes in ion channel functioning, may also be in operation. Omega-3 fatty acids like DHA can also participate in local messaging by binding to voltage-gated potassium channels (Poling, Vicini, Rogawski, & Salem, 1996). In fact, a recent study found that *in vitro* application of DHA in the cerebellum of *Fmr1* knockout mice of DHA reduced excessive inhibitory neurotransmission characteristic of this model (Yang et al., 2018). Subsequent *in vivo* experiments demonstrated that a one-week supplementation with 0.5% DHA-enriched chow attenuated behavioral changes in

acoustic startle responding, via binding to Kv1.2 channels (Yang et al., 2018). Links between DHA and the regulation of neuronal excitability via potassium channels have been examined in other disease models, with promising outcomes for hippocampal signaling (Yang et al., 2012). Future studies might consider how changes in potassium channel functioning in this model might be influenced by both post-weaning and prenatal dietary manipulation of omega-3 fatty acids in other brain areas like the hippocampus.

The current study also improves on the understanding of how consumption of the "Western diet" influences behavior. The control fat dietary manipulation included in the present study mirrors the typical "Western diet" (Monsanto et al., 2016). Previously, it had been surmised that the a high-fat diet was problematic for aspects of neurological functioning, resulting in significant deficits in learning and memory (Cordner & Tamashiro, 2015; Labouesse et al., 2013). Indeed, here we demonstrated that postnatal exposure to this diet exaggerated reduced anxiety characteristic of the *Fmr1* knockout. However, the results of the current study also complicate this thesis, finding that a post-weaning diet high in monounsaturated and saturated fats reverses deficits in startle responding. Moreover, when administered during the prenatal period, this improves deficits in ultrasonic vocalization production during the early neonatal period and can reduce anxiety and improve deficits in acquisition of a fear response in adulthood. Instead of solely focusing on the negative aspects a higher fat diet, this study seems to contribute to a larger body of evidence that implicates impairments in fatty acid metabolism for the etiology of neurodevelopmental disorders (Richardson & Ross, 2000). Going forward, the importance of a nuanced understanding of the role of fatty acid metabolism in neural functioning is clear.

Aside from the implications for FXS and ASD, the present study also introduces important experimental considerations for the field of behavioral neuroscience. It has long since been understood that many factors can influence the trajectory of a behavioral experiment [reviewed in (Van Meer & Raber, 2005)]. Environmental factors, such as the timing of the test in relations to the light cycle, noise in the animal facility, or sex of the experimenter, can have significant influences on the behavioral output, depending on the task. The current study adds to this literature, demonstrating that manipulating dietary fat can elicit large changes in behavior, on tasks such as the delay fear conditioning task and sensorimotor gating behavior, as well as significant changes in expression levels of inflammatory genes. There is significant variation in available "standard" laboratory chows and often this information is not included in the final published manuscript. However, the present study finds that prenatal exposure to a nonstandard diet can also elicit changes in ultrasonic vocalization production during the early postnatal period. Broadly, these findings support the need to consider dietary variables when characterizing the results of a behavioral experiment.

Altogether, the current study provides evidence of omega-3 fatty acids as a promising alternative to pharmaceuticals for patients with FXS. Beyond coping with the burden of the disease itself, the importance of finding affordable treatment options cannot be overstated. Nearly half of all families of children with FXS report a significant financial burden of the disease on their family, as out of pocket expenses can account for over 5% of family income (Ouyang, Grosse, Raspa, & Bailey, 2010). Moreover, medication or therapy can account for over 50% of these out-of-pocket expenses (Ouyang et al., 2010). This financial burden can disproportionately affect low-income families, as

low-income families with children with special health care needs are 11 times more likely than higher income families to have out-of-pocket medical expenditures exceeding 5% of income (Newacheck & Kim, 2005). Given the rising cost of pharmaceuticals (Lakdawalla, 2018), dietary interventions present as a more cost-effective, further improving the lives of individuals with Fragile X syndrome.

APPENDICES

APPENDIX A

Power Analyses

An a priori power analysis was conducted using G*Power 3.1.9.2 (Faul et al., 2007) for the 3 (Diet: Standard, Control Fat, Omega-3) x 2 (Genotype: WT, KO) mixed-model ANOVA to determine the appropriate number of subjects for the statistical analyses planned. The test family was set at F tests for an ANOVA: fixed effects, special, main effects and interactions. The input parameters were set as follows: $f = 0.40$, $\alpha = 0.05$ and power at 0.80, for 6 groups. For an 80% chance of finding a true effect, the output suggested a total sample size of 64, for a total of 11 for each of the 6 treatment combinations. Based on this, I aimed for a target sample size of 12 per group, to account for potential sample loss or death during experiments. The power for the intended design is 0.85.

APPENDIX B

Supplemental Data from this Project

Methods

Body Weight Analyses

During the post-weaning supplementation phase, subjects receiving the two experimental dietary conditions were monitored for changes in body weight from PD21 (weaning date) through behavioral testing, until sacrifice at the conclusion of all testing. For the prenatal paradigm, body weight was assessed weekly beginning at the first week of testing (PD60) until just prior to sacrifice. For the post-weaning paradigm, the final sample sizes were as follows: Standard WT = 11, Standard KO = 10, Control Fat WT = 16, Control Fat KO = 16, Omega-3 WT = 19, Omega-3 KO = 16. The final sample size for the prenatal paradigm was as follows: Standard WT = 23, Standard KO = 12, Control Fat WT = 16, Control Fat KO = 16, Omega-3 WT = 13, Omega-3 KO = 12.

Open Field

The open field test was conducted to evaluate the effects of the three diet conditions on activity levels. Prior to testing, animals were allowed to habituate to the testing room for at least 30 minutes. The testing arena consisted of a clear acrylic box (40x40x30cm). Background noise levels in the room were then limited to 60 dB. The lighting inside the test chamber was approximately 100 lux and uniform through the chamber. Subjects were placed into the testing arena and allowed to explore freely for 30

minutes and the experimenter was not present during the testing window. During the task, activity level variables (i.e. grooming, rearing, clockwise and counterclockwise revolutions) were measured and compiled by a computer operated optical animal activity system (Fusion by AccuScan Instruments, Inc.; USA). This system also measured other exploratory behaviors such as grooming, rearing, clockwise, and counterclockwise rotations, as well as stereotypic behavior, which accounts for repeated breaking of the same set of beams, (i.e. during grooming behavior). Following testing, subjects were returned to an alternate cage until all mice in the home cage were tested, before being returned to the home cage. Between subjects, the arena was thoroughly cleaned using a 30% isopropyl alcohol solution and dried thoroughly. For the post-weaning paradigm, the final sample sizes were as follows: Standard WT = 11, Standard KO = 10, Control Fat WT = 16, Control Fat KO = 16, Omega-3 WT = 19, Omega-3 KO = 16. The final sample size for the prenatal paradigm was as follows: Standard WT = 21, Standard KO = 12, Control Fat WT = 16, Control Fat KO = 16, Omega-3 WT = 14, Omega-3 KO = 14.

Elevated Plus Maze

In addition to how the data was extracted from the elevated plus maze task as described in Chapter 3, these videos were also scored later offline for head dip behavior and rearing activities by an experimenter blind to the experimental condition of the subject. Following testing, the test mice were returned to an alternate cage until all mice in the home cage were tested, before being returned to the home cage. Sample sizes for this assessment were specified in Chapter 3.

Nose Poke Assay

Nose poke behavior in a hole board arena was used as a measure of repetitive behavior. Subjects were given a 30-minute habituation period prior to testing. The testing apparatus consisted of a board with 16 equidistant holes that were 1" in diameter and approximately 0.75" in depth inserted into a clear plastic arena (40 x 40 x 30 cm). Behavior was considered a nose-poke when the subject inserts the nose as far in as the eye. During the 10-minute testing window, the number and location of these pokes was recorded by a researcher blind to the experimental condition of the subject. Following the conclusion of testing, the mice were returned to an alternate cage with other tested mice. The arena was cleaned thoroughly with 30% isopropyl alcohol between subjects. For the post-weaning paradigm, the final sample sizes were as follows: Standard WT = 10, Standard KO = 10, Control Fat WT = 16, Control Fat KO = 16, Omega-3 WT = 18, Omega-3 KO = 16. The final sample size for the prenatal paradigm was as follows: Standard WT = 24, Standard KO = 11, Control Fat WT = 16, Control Fat KO = 15, Omega-3 WT = 14, Omega-3 KO = 14.

Social Partition

To evaluate differences in sociability, animals were tested in the social partition paradigm. The animal was first housed overnight (approximately 24 hours) in a cage divided into two chambers by a clear partition with 0.6 cm diameter holes placed randomly, and a sex/weight/age-matched conspecific was placed in the other side. The next day the testing paradigm consisted of three testing phases. In each testing phase, the duration and frequency of visits to the partition were measured for 5 minutes. The first phase (familiar) measured the interaction at the partition with the mouse it has been

previously housed with for 24 hours. In the second phase (unfamiliar), the conspecific was replaced with a novel conspecific. The last phase (familiar 2), the previous conspecific was placed behind the partition and then the behavior of the experimental subject was measured on the same constructs as the other trials. Duration and frequency was recorded by a live observer using the Ethom computer software to measure the time and frequency of the social interaction behavior (Taiwanica, 2000). For the post-weaning paradigm, the final sample sizes were as follows: Standard WT = 11, Standard KO = 10, Control Fat WT = 16, Control Fat KO = 16, Omega-3 WT = 19, Omega-3 KO = 16. The final sample size for the prenatal paradigm was as follows: Standard WT = 24, Standard KO = 12, Control Fat WT = 16, Control Fat KO = 15, Omega-3 WT = 14, Omega-3 KO = 14.

Results

Body Weight Analyses

Post-weaning paradigm. During the post-weaning period (3 weeks of age or PD21) to 12 weeks of age (prior to behavioral testing), body weight measurements during this period were analyzed using a two-factor repeated measures ANOVA (Genotype [wildtype, *Fmr1* KO] x Diet [control fat, omega-3]) for all available data points (Figure B.1A). Results indicated that *Fmr1* KOs gained weight similarly to their wildtype counterparts, $F_{genotype}(1, 57) = 0.05$, $p = 0.83$, $F_{genotype \times time}(9, 513) = 0.27$, $p = 0.98$. However, dietary supplementation with omega-3 fatty acids did significantly increase body weight over time, $F_{diet}(1, 57) = 9.8$, $p = 0.003$, $F_{diet \times time}(9, 513) = 2.89$, $p = 0.002$. Diet did not interact with genotype, $F_{diet \times genotype}(2, 57) = 0.11$, $p = 0.74$, $F_{genotype \times diet \times}$

$_{time}(9, 513) = 0.74$, $p = 0.68$. At the final time point, final body weight was assessed prior to sacrifice for all three dietary manipulation groups for all available data points. Results indicated that post-weaning supplementation results in significantly increased body weight in animals treated with omega-3 fatty acids, compared to only the control fat condition, $F_{diet}(2, 81) = 3.20$, $p = 0.05$ (Figure B.1B). Loss of *Fmr1* did not impact body weight at this time, $F_{genotype}(1, 81) = 1.49$, $p = 0.23$, $F_{diet \times genotype}(2, 81) = 0.71$, $p = 0.50$.

Prenatal paradigm. For the prenatal paradigm, body weight was assessed beginning at the first day of testing (PD60) (Figure B.1C). Results indicated no lasting effect of diet on growth prior to that time, $F_{diet}(2, 91) = 0.13$, $p = 0.88$, and diet did not significantly interact with genotype, $F_{diet \times genotype}(2, 91) = 0.41$, $p = 0.67$. Loss of *Fmr1* had no effect on body weight measured at this time, $F_{genotype}(1, 91) = 0.01$, $p = 0.91$. This same finding persisted at the final time point prior to sacrifice as well (Figure B.1D): $F_{genotype}(1, 91) = 0.04$, $p = 0.85$; $F_{diet}(2, 91) = 1.27$, $p = 0.29$; $F_{diet \times genotype}(2, 91) = 0.70$, $p = 0.50$.

Open Field

Post-weaning paradigm. For the post-weaning paradigm, in the open field test, *Fmr1* KO mice demonstrated hyperactivity for movement time, $F_{genotype}(1, 87) = 4.63$, $p = 0.03$ (Figure B.2A). However, they did not show hyperactivity on any other variable measured: total distance, $F_{genotype}(1, 87) = 0.04$, $p = 0.84$ (Figure B.2B), number of rearings, $F_{genotype}(1, 87) = 3.34$, $p = 0.07$ (Figure B.2C), or stereotypy time, $F_{genotype}(1, 87) = 3.66$, $p = 0.06$ (Figure B.2D). These parameters were also not influenced by diet: total distance, $F_{diet}(2, 87) = 1.50$, $p = 0.23$, movement time, $F_{diet}(2, 87) = 0.82$, $p = 0.45$,

number of rearings, $F_{diet}(2, 87) = 0.22$, $p = 0.80$, or stereotypy time, $F_{diet}(2, 87) = 0.09$, $p = 0.92$. Genotype and diet did not interact on any of the variables either: total distance, $F_{diet \times genotype}(2, 87) = 0.61$, $p = 0.54$, movement time, $F_{diet \times genotype}(2, 87) = 1.34$, $p = 0.27$, number of rearings, $F_{diet \times genotype}(2, 87) = 1.08$, $p = 0.34$, or stereotypy time, $F_{diet \times genotype}(2, 87) = 2.08$, $p = 0.13$.

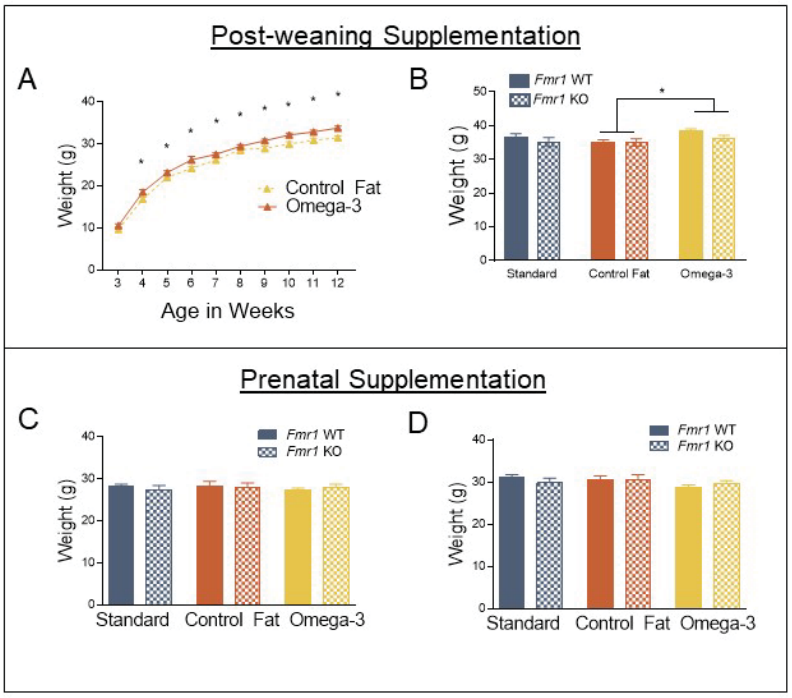

Figure B.1. Post-weaning supplementation with omega-3 fatty acids increases body weight, while prenatal supplementation has no impact on body weight. A. Weight gain was significantly higher in the omega-3 fatty acid condition. B. Final body weight prior to sacrifice was higher in subjects receiving the omega-3 fatty acid diet. C. No lasting effect of prenatal supplementation was detected prior to behavioral testing on PD60. D. No changes in body weight were detected prior to sacrifice. Data are expressed as mean ± SEM. * = $P < 0.05$. A designation of "b" indicates that this group differed from the "a" comparison group at the level of $p < 0.05$.

Prenatal paradigm. For the prenatal paradigm, in the open field test, loss of *Fmr1* did not significantly impact the time spent moving during the task, $F_{genotype}(1, 87) = 3.66$,

p = 0.06 (Figure B.2E). Dietary supplementation did not impact movement time, $F_{diet}(2, 87) = 0.08$, p = 0.93, nor was there an interaction of genotype and diet, $F_{genotype \times diet}(2, 87) = 0.25$, p = 0.78. However, some measures suggested that *Fmr1* KO mice were significantly hyperactive, showing increased total distance across the 30 minute testing period, $F_{genotype}(1, 87) = 27.35$, p = 0.001 (Figure B.2F). Dietary supplementation did not impact distance moved, $F_{diet}(2, 87) = 0.31$, p = 0.74, nor was there an interaction ,$F_{genotype \times diet}(2, 87) = 0.46$, p = 0.63. Number of rearings was not impacted by loss of *Fmr1*, $F_{genotype}(1, 87) = 1.63$, p = 0.21 (Figure B.2G). Dietary supplementation did not impact number of rearings, $F_{diet}(2, 87) = 2.07$, p = 0.13, nor was there a significant interaction, $F_{genotype \times diet}(2, 87) = 0.97$, p = 0.39. Time spent engaged in stereotypic behaviors was also not impacted by loss of *Fmr1*, $F_{genotype}(1, 87) = 0.20$, p = 0.89 (Figure B.2H). Dietary supplementation did not impact stereotypy time, $F_{diet}(2, 87) = 0.08$, p = 0.93, nor was there a significant interaction, $F_{genotype \times diet}(2, 87) = 0.39$, p = 0.68.

Elevated Plus Maze

Post-weaning paradigm. For the post-weaning paradigm, results for aspects of exploratory behavior indicated that similar to the effects on general locomotion, exposure to post-weaning omega-3 fatty acids, and to a lesser extent the control fat diet, significantly reduced exploration of the elevated plus maze. Specifically, results indicated a main effect of diet for time spent looking over the edge of the maze, $F_{diet}(2, 76) = 8.45$, $p < 0.001$, as well as the duration of rearing behavior in the closed arm of the maze, $F_{diet}(2, 76) = 7.01$, p = 0.002. Post-hoc analyses with LSD indicated that both the control

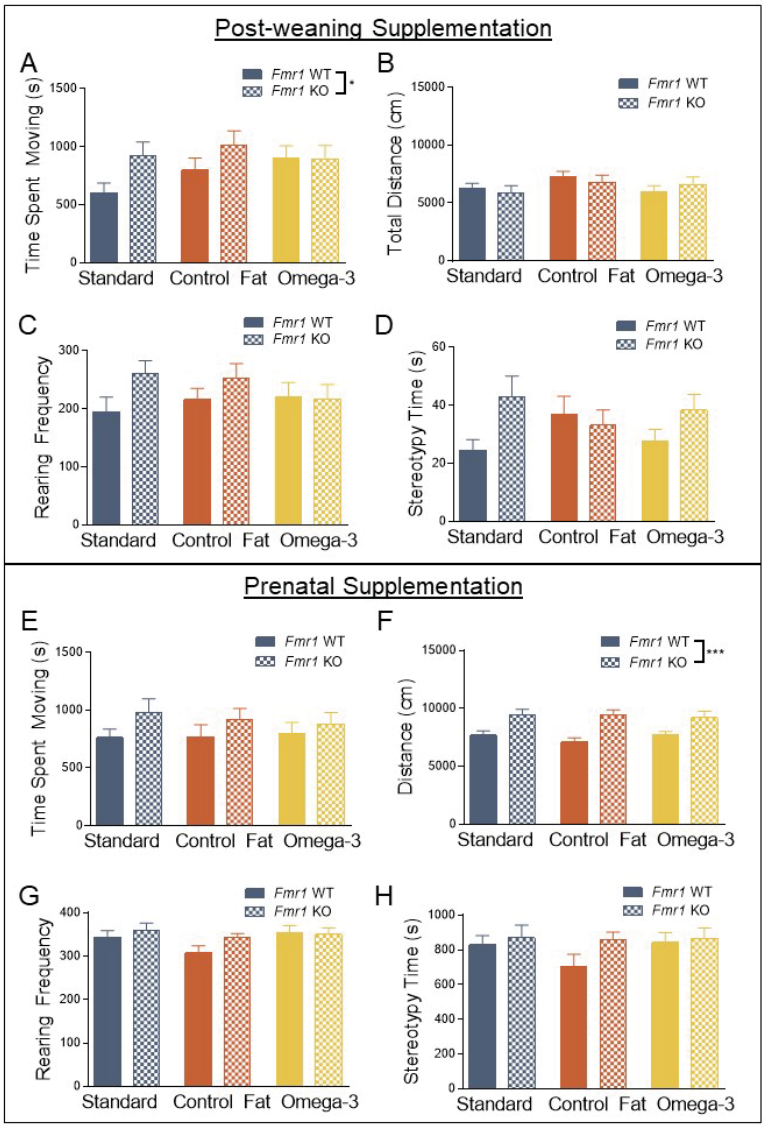

Figure B.2. In the open field, neither prenatal nor post-weaning dietary manipulation had an impact on hyperactivity in the *Fmr1* knockout. A. *Fmr1* knockouts demonstrate hyperactivity when measuring movement time in the open field. B. Total distance moved showed no significant differences. C. Number of rearings showed no significant differences. D. Stereotypy time showed no significant differences. E. No effect was detected for movement time in the prenatal paradigm. F. *Fmr1* knockouts demonstrate hyperactivity when measuring distance moved in the open field. G. No differences were detected for rearing frequency. H. No differences were detected for stereotypy time. Data are expressed as mean ± SEM. * = $P < 0.05$, *** = $P < 0.001$.
were significantly reduced compared to the standard diet.

fat (“b”) and omega-3 fatty acids (“b”) significantly reduced duration of head dips compared to the standard diet (“a”), at the level of $p < 0.01$ (Figure B.3A). Post-hoc analyses with LSD indicated a similar pattern for closed rearing duration for both control fat (“b”) and omega-3 dietary conditions (“b”), at the level of $p < 0.05$ (Figure B.3B). No effect of genotype was detected for either head dip duration, $F_{genotype}(1, 76) = 0.28$, $p = 0.60$, or duration of rearings, $F_{genotype}(1, 76) = 0.39$, $p = 0.54$. Moreover, no significant interaction was detected for head dip duration, $F_{genotype \times diet}(1, 76) = 0.23$, $p = 0.80$, as well as closed rear duration, $F_{genotype \times diet}(1, 76) = 1.75$, $p = 0.18$.

Prenatal paradigm. For the prenatal paradigm, results for exploratory behavior in the elevated plus maze indicated that *Fmr1* KOs spent more time exploring over the sides of the open arm, $F_{genotype}(1, 84) = 4.07$, $p = 0.05$ (Figure B.3C), and less time rearing in the closed arm, $F_{genotype}(1, 84) = 11.59$, $p = 0.001$ (Figure B.3D). These effects were significantly attenuated by exposure to the dietary manipulations, for both head dip duration, $F_{diet}(2, 84) = 4.44$, $p = 0.02$, as well as closed rearing duration, $F_{diet}(2, 84) = 4.94$, $p = 0.01$. Post-hoc analyses with LSD indicated that this effect was only true for the omega-3 diet compared to the standard diet, $p = 0.01$. However, for the closed rear duration, both control fat ($p = 0.001$) and omega-3 ($p = 0.002$) This impact of the diet was not additive for both head dip duration, $F_{genotype \times diet}(1, 84) = 0.60$, $p = 0.55$, as well as closed rear duration, $F_{genotype \times diet}(1, 84) = 0.26$, $p = 0.77$, suggesting it did not exacerbate the phenotype. Post-hoc analyses confirmed this assertation, demonstrating that all groups differed from the Standard WT condition, but did not differ among themselves, at the level of $p < 0.05$.

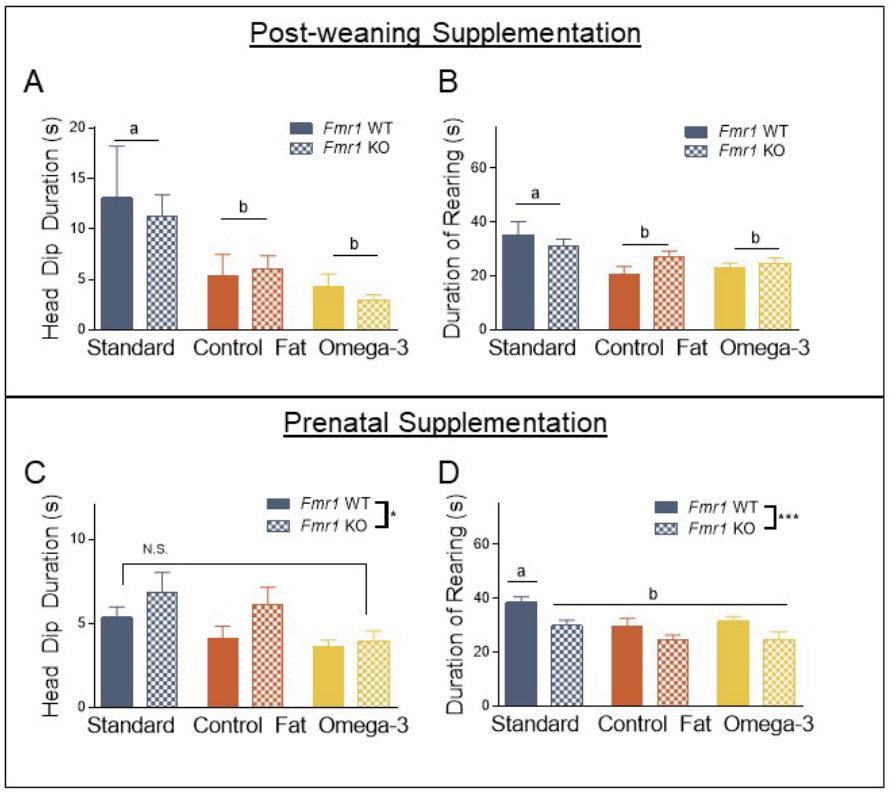

Figure B.3. Both post-weaning and prenatal exposures to omega-3 fatty acids and the control fat diet significantly reduce exploratory behavior in the elevated plus maze. A. Post-weaning exposure to both experimental diets reduced the duration spent looking over the elevated plus maze. B. This same exposure also reduced the number of rearings in the closed arm of the maze. C. *Fmr1* knockouts spent more time looking over the side of the maze, and this was attenuated by exposure to the omega-3 fatty acid diet. D. Loss of *Fmr1* and exposure to either of the experimental diets prenatally reduced rearing behavior, however these effects were not additive. Data are expressed as mean ± SEM. * = $P < 0.05$, *** = $P < 0.001$. A designation of "b" indicates that this group differed from the "a" comparison group at the level of $p < 0.05$.

Nose-Poke Assay

Post-weaning paradigm. For the post-weaning paradigm, results for the latency to hole poke indicated that deletion of *Fmr1* did not reduce the mean latency, $F_{genotype}(1, 80) = 1.50$, $p = 0.22$ (Figure B.4A). Diet did not impact this variable, $F_{diet}(2, 80) = 0.12$, $p = 0.89$. The combination of genotype and diet also did not impact this variable, $F_{genotype\ x}$

$_{diet}(2, 80) = 0.04$, $p = 0.97$. For total holes poked (Figure B.4B), deletion of *Fmr1* did not impact this variable, $F_{genotype}(1, 80) = 1.10$, $p = 0.30$. Diet also did not significantly affect the number of holes poked overall, $F_{diet}(2, 80) = 1.86$, $p = 0.16$. Moreover, the combination of genotype and diet did not impact the number of holes poked overall, $F_{genotype \times diet}(2, 80) = 0.34$, $p = 0.72$.

Prenatal paradigm. For prenatal paradigm, results for the latency to hole poke indicated that deletion of *Fmr1* had no impact on latency to engage in hole-poking behavior, $F_{genotype}(1, 89) = 1.03$, $p = 0.31$. However, diet significantly impacted this latency, $F_{diet}(2, 89) = 4.97$, $p = 0.01$. Further post-hoc multiple comparisons testing indicated that both control fat and omega-3 fatty acids reduced the latency to hole poke, at the level of $p < 0.05$ (Figure B.4C), and magnitude of this effect was similar between the two experimental groups. The unique combination of diet and genotype did not significantly influence the latency to engage in hole-poking behavior, $F_{genotype \times diet}(2, 89) = 0.38$, $p = 0.68$. Loss of *Fmr1* also did not influence the frequency of hole-poking behavior overall, $F_{genotype}(1, 89) = 2.05$, $p = 0.16$. Similar to latency, diet significantly impacted the number of hole-pokes, $F_{diet}(2, 89) = 7.71$, $p = 0.001$. Subsequent post-hoc multiple comparisons with LSD indicated that both omega-3 and control fat diets significantly increased hole-poking behavior (Figure B.4D), and the magnitude of this difference was similar for both groups, at the level of $p < 0.05$. To constitute an ASD-like increase in repetitive behaviors, and not more general increases in directed exploration, we next examined whether animals indicated a preference for any particular hole or type of hole according to a previous analysis paradigm (Moy et al., 2008). Visual inspection of

the data indicated that no group demonstrated “high” (> 12.5%) preference for any type of hole or specific hole (Figure B.4E).

Social Partition

Post-weaning paradigm. For the post-weaning paradigm, both duration, $F_{genotype}(1, 82) = 1.02$, $p = 0.32$ (Figure B.5A), and frequency, $F_{genotype}(1, 82) = 0.98$, $p = 0.32$ (Figure B.5B), were unaffected by loss of *Fmr1* across all trials. When examining across the three different types of trials, loss of *Fmr1* did not interact with trial for both duration of visits, $F_{genotype\ x\ trial}(2, 164) = 1.65$, $p = 0.20$, and the number of visits to the partition, $F_{genotype\ x\ trial}(2, 164) = 0.32$, $p = 0.73$. Diet did, however, significantly impact the duration, $F_{diet}(2, 82) = 26.95$, $p = 0.0001$ (Figure B.5A), and number of visits to the partition, $F_{diet}(2, 82) = 18.67$, $p = 0.0001$ (Figure B.5B), across all trials. Post-hoc results for multiple comparisons indicated that both omega-3 (“b”) and control fat diets (“b”) significantly increased both the duration and frequency of visits to the partition compared to the standard diet (“a”), at the level of $p < 0.05$. This effect was consistent across all three trials, as diet did not significantly interact with trial for both duration, $F_{diet\ x\ trial}(4, 164) = 2.12$ $p = 0.08$, as well as frequency of visits to the partition, $F_{diet\ x\ trial}(4, 164) = 0.58$, $p = 0.68$. Finally, the unique combination of genotype and diet did not impact either duration, $F_{genotype\ x\ diet}(2, 82) = 0.90$, $p = 0.41$, or frequency of visits, $F_{genotype\ x\ diet}(2, 82) = 2.31$, $p = 0.11$, across all trials. This combination also failed to interact significantly with trial, for both duration, $F_{genotype\ x\ diet\ x\ time}(4, 164) = 0.40$, $p = 0.81$, as well as frequency, $F_{genotype\ x\ diet\ x\ time}(4, 164) = 0.54$, $p = 0.71$.

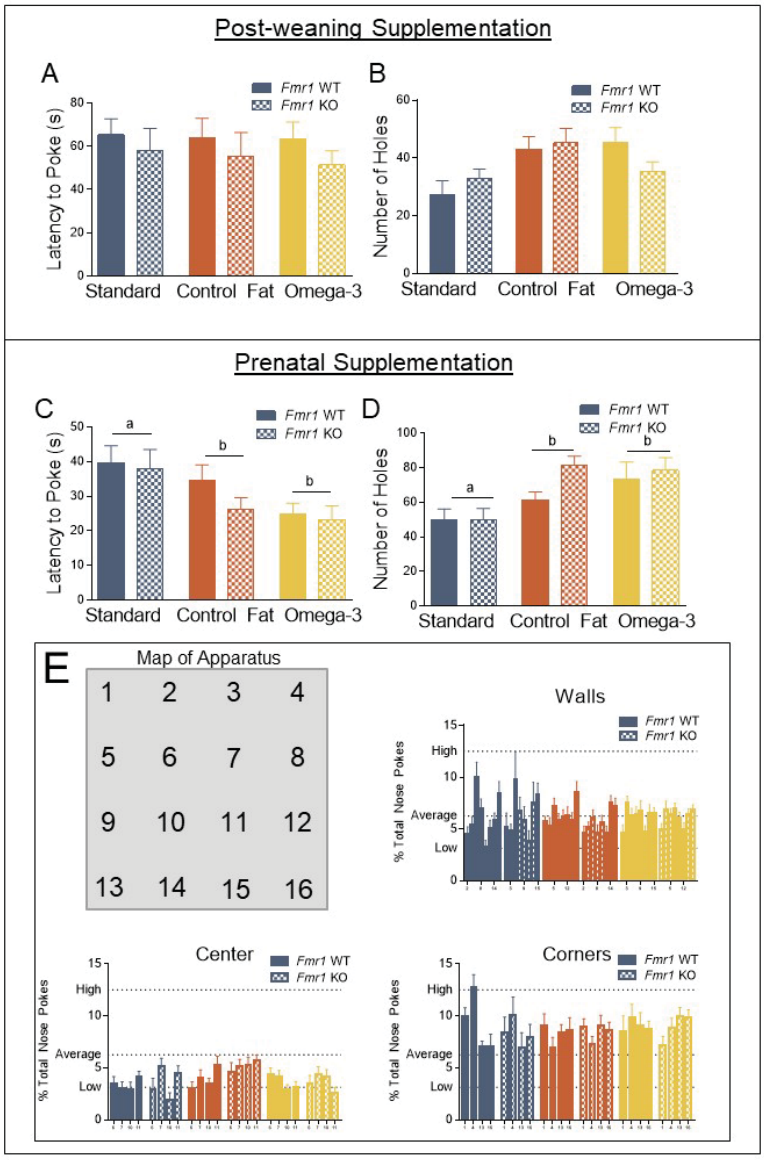

Figure B.4. Post-weaning supplementation with high fat diets does not affect nose poking behavior, while prenatal supplementation increases directed exploration but not ASD-like repetitive behavior. A. In the post-weaning paradigm, no effects were detected for latency to the first hole-poke. B. Similarly, no effects were detected for overall number of holes poked. C. Prenatal exposure to both experimental diets reduced the latency to hole-poke. D. Similarly, prenatal exposure to the experimental diets increased the number of overall hole-pokes. E. However, when assessing for potential indicators of repetitive hole-poking, visual inspection of the data indicates no preference for a type of hole or single location. Data are expressed as mean ± SEM.

Prenatal paradigm. For the prenatal paradigm, both duration, $F_{genotype}(1, 89) = 0.001$, $p = 0.98$ (Figure B.5C), and frequency, $F_{genotype}(1, 89) = 0.76$, $p = 0.39$ (Figure B.5D), were unaffected by loss of *Fmr1* across all trials. When examining across the three different types of trials, loss of *Fmr1* did not interact with trial for both duration of visits, $F_{genotype\ x\ trial}(2, 178) = 1.04$, $p = 0.36$, and the number of visits to the partition, $F_{genotype\ x\ trial}(2, 178) = 0.09$, $p = 0.92$. Diet also did not significantly impact the duration, $F_{diet}(2, 89) = 1.44$, $p = 0.24$, or number of visits to the partition, $F_{diet}(2, 89) = 0.75$, $p = 0.48$, across all trials. Similar to genotype, diet did not significantly interact with trial for both duration, $F_{diet\ x\ trial}(4, 178) = 0.95$, $p = 0.44$, as well as frequency of visits to the partition, $F_{diet\ x\ trial}(4, 178) = 0.53$, $p = 0.71$. Finally, the unique combination of genotype and diet did not impact either duration, $F_{genotype\ x\ diet}(2, 89) = 0.38$, $p = 0.69$, or frequency of visits, $F_{genotype\ x\ diet}(2, 89) = 0.02$, $p = 0.98$, across all trials. This combination also failed to interact significantly with trial, for both duration, $F_{genotype\ x\ diet\ x\ trial}(4, 178) = 0.65$, $p = 0.63$, as well as frequency, $F_{genotype\ x\ diet\ x\ trial}(4, 178) = 0.16$, $p = 0.96$.

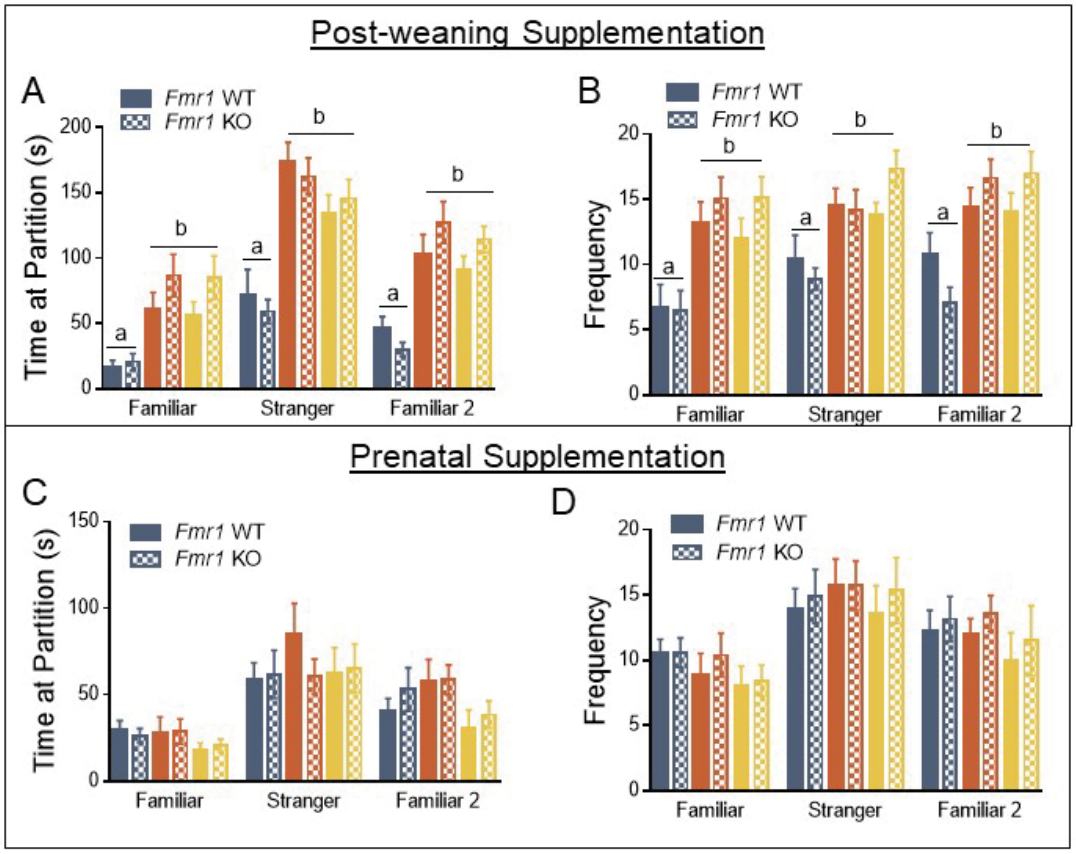

Figure B.5. Exposure to a high fat diet during the post-weaning paradigm significantly increases frequency and duration of visits to the partition across all three trials, while the prenatal exposure has no effect on sociability. A. Post-weaning exposure to both omega-3 and control fat diets significantly increased duration of visits to the partition. B. Similarly, post-weaning exposure to both experimental diets increased the frequency of visits to the partition. C. However, prenatal exposure had no impact on duration of visits to the partition. D. The frequency of visits was also unaffected by both genotype and dietary manipulations. Data are expressed as mean ± SEM. A designation of "b" indicates that this group differed from the "a" comparison group at the level of $p < 0.05$.

APPENDIX C

Pilot Study for MATLAB Analysis

Background

Many research groups utilize the Avisoft software and hardware to record and analyze the production of ultrasonic vocalizations in rodents, and data from the Avisoft program is typically cleaned and scored by a blind experimenter according to previous methods (Hodges et al., 2017; Reynolds, Nolan, Huebschman, Hodges, & Lugo, 2017; Scattoni, Gandhy, Ricceri, & Crawley, 2008). While the software is quite powerful, it also requires many hours of cleaning and fine-tuning for each file to ensure that the measurements appropriately reflect calls and not some sort of background noise. This time-intensive process is thus exposed to experimenter error and bias by the scorer. The Jarvis lab group has published and made freely available MATLAB code that captures most of these parameters automatically (Chabout et al., 2017). However, this tool has only been used for vocalizations from adult animals. To assess the potential efficacy of this method for our use, we conducted a pilot study to directly compare the measurements made by the two methodologies available, hereto after referred to as "Hand-Scoring Analysis" and "MATLAB Analysis". The results of the sensitivity analyses supported strong agreement between the two systems and thus, the MATLAB analysis was used in the above study to extract data from all files collected.

Methods

Animals

Male *Fmr1*$^{+/+}$ and female *Fmr1*$^{+/-}$ FVB.129P2-Pde6b+Tyrc-ch Fmr1tm1Cgr/J (Jackson Labs Stock No: 004624) mice originally from Jackson Lab were used as breeders to produce the following groups: male WT and male KO pups, female WT, and female HET pups. The final sample sizes were as follows: $n_{\text{male WT}} = 15$, $n_{\text{male KO}} = 14$, $n_{\text{female WT}} = 13$, $n_{\text{female HET}} = 12$. All pups were housed in individual cages with parents and littermates. The light cycle was kept at 12 hr. light, and the colony room was kept at an ambient temperature of 22º C. Animals had *ad libitum* access to food and water. All procedures performed were in accordance with Baylor University Institutional Animal Care and Use Committee, as well as the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All testing was conducted during the light cycle, specifically between 8 am and 5 pm. On postnatal day 7 (PD7), toes were clipped for identification and genotyping analysis. Pups were then returned to the home cage until testing on PD8.

Isolation-Induced Ultrasonic Vocalizations

Ultrasonic vocalizations are a form of prelingual communication produced in response to maternal separation from PD6 to PD14 (Branchi, Santucci, & Alleva, 2001). On PD8, pups were brought down from the colony room in their home cage and allowed to habituate to the testing room for 30 minutes prior to the testing period. At the time of testing, pups were separated from their parents into a clean housing pan with fresh bedding, warmed to ambient nesting temperature (approximately 35º C) using a heating pad. Vocalizations were recorded using a condenser microphone (CM16/CMPA, Avisoft

Bioacoustics, Germany). This microphone was connected to a recording interface (UltraSoundGate 116Hb, Avisoft Bioacoustics), that records USVs on a continuous spectrum from 0 – 125 kHz. Vocalizations were recorded for 2 minutes for each pup. Each pup was chosen randomly, recorded, weighed and then returned to the warmed cage with their littermate cage. A live experimenter remained in the room to monitor the gain and acquisition of the microphone. Pups were not away from the dam for longer than 30 minutes. After the last pup was tested, they were returned to the home cage with the parents.

Hand-Scoring Analysis

Following the conclusion of all testing, files were collected and Avisoft SASLab Pro software (Avisoft Bioacoustics, Germany) was used to convert all USV files (.wav) into spectrograms using a Fast Fourier transformation procedure. The parameters were set as follows for all lab studies to maintain consistency between experiments: FFT length = 1024, time window overlap = 75% (100% Frame, Hamming window), time resolution = 1ms. Sampling frequency was set at 22050. Additionally, call-types were manually identified by an experimenter blinded to treatment identity using previously described classification scheme (Scattoni et al., 2008). In addition to collecting information on call-type production, the following quantitative parameters were also measured: duration, peak frequency, peak amplitude and fundamental frequency across all calls using this analysis.

MATLAB Analysis

All WAV files were also cleaned and processed using MATLAB analysis, freely available from: (http://jarvislab.net/research/mouse-vocal-communication/). Sonograms were processed using the graphical user interface described previously (Chabout et al., 2017). In the Sonogram Parameters section, we set Min Frequency to 15,000 Hz, Max Frequency to 125,000 Hz, the sampling frequency to 256 kHz, and the Threshold to 0.3. In the Whistle Options section, we set the Purity Threshold to 0.075, the Min Duration of the syllable to 3 ms, the Min Frequency sweep to 20,000 Hz, and the Filter Duration to 3 ms. For consistency, we also set the Min Note Duration to 3 ms, and the Min Note Count to 1. Following sonogram processing, densite inter-syllable interval (ISI) was determined using the song-analysis guided analysis Excel file available at the same website. Previous works from the Jarvis lab set it at 0.25, and for the purposes of this study, we set it at 0.25 as well (Chabout et al., 2017).

Results

To determine the sensitivity of hand-scoring in Avisoft compared to automated processing in MATLAB, a Spearman's correlation was performed. This data was suggested based on the non-normal distribution of the data. This correlation was performed on data across three different parameters measured by both systems: number of USVs, fundamental frequency (kHz), and average duration of calls. Results indicated a significant association between the number of USVs detected in a file using hand-scoring methods and the number detected using MATLAB processing, $r_s(52) = 0.85$, $p < 0.0001$ (Figure C.1A). With regard to fundamental frequency, results indicated another significant association across the two systems, $r_s(52) = 0.91$, $p < 0.0001$ (Figure C.1B).

The average duration of calls detected in the two systems was also significantly associated, $r_s(52) = 0.42$, $p = 0.002$ (Figure C.1C). Overall, these results indicate that both systems are sensitive enough to be used for analysis of USVs.

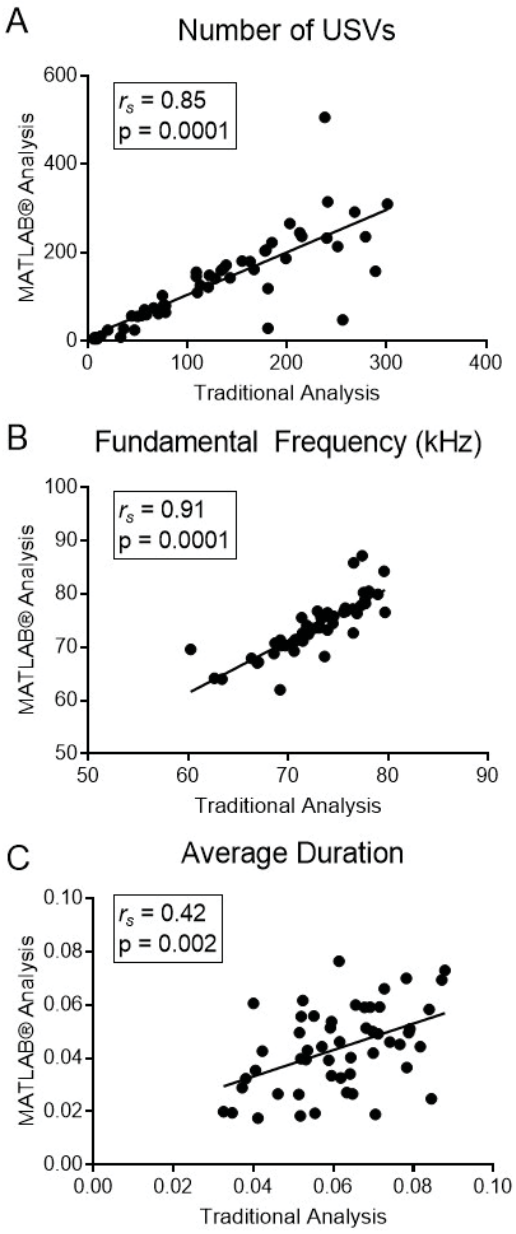

Figure C.1. Comparison of the Sensitivity of the Two Analyses. A. Results indicated a strong positive association between the number of USVs counted by the two methods. B. A strong correlation was detected between the average fundamental frequency assessed between the two systems. C. The average duration of calls detected between the two systems was significantly correlated.

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