Adiponectin Receptor Modulation as a Therapeutic Approach to Fragile X Syndrome

by

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We acknowledge and respect the lək̓ʷəŋən peoples on whose traditional territory the University stands and the Songhees, Esquimalt, and W̱SÁNEĆ peoples whose historical relationships with the land continue to this day.

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ABSTRACT

Fragile X Syndrome (FXS) is a leading cause of monogenic autism disorder (ASD) and intellectual disability (ID) caused by CGG trinucleotide repeat expansions in the 5' untranslated region of the *FMR1* gene. Methylation-dependent silencing of the *FMR1* gene and abolition of product protein FMRP translates to altered cognitive function in people with FXS. There is no cure and limited therapies for FXS. Abolition of FMRP causes defective protein translation control and cellular energy metabolism which translates to altered AMPA/NMDA ratios and diminished long-term potentiation (LTP) in the hippocampal dentate gyrus (DG) of *FMR1* knockout (KO) mice. This provides precedent for cell metabolic modulation as a therapeutic target in FXS.

Our work targets the adipocyte derived protein hormone adiponectin, an endogenous modulator of cellular autophagy, as a therapeutic compound for FXS. We evaluate the effect of a chronic 15-day AdipoRon treatment at a dose of 20mg/Kg on hippocampal DG LTP, DG synaptosomal and whole tissue lysis protein, and contextual and social anxiety-like behavior in adult male *FMR1* KO mice. We found that (1) AdipoRon has a unique, significant, and genotype-specific effect on anxiety-like behavior and (2) that differences in hippocampal LTP and proteomic signaling may exist but require further investigation. The direct effect of explicit exogenous adiponectin receptor modulation in FXS has not been investigated before. This novel inquiry into the use of AdipoRon as a therapeutic compound for FXS is a starting point, a valuable pilot study for future work, and a novel theoretical conceptualization of the neuroendocrine axis in FXS. This work could help to advance knowledge in the FXS field and contribute to an improved quality of life for patients with FXS and their families.

TABLE OF CONTENTS

LIST OF FIGURES

GLOSSARY Of ABBREVIATIONS

3CS	Three chamber sociability
ACSF	Artificial cerebrospinal fluid
Adip	AdipoRon (treatment group)
Akt	Protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4isoxazolepropionic acid
AMPK	5' AMP-activated protein kinase
ASD	Autism spectrum disorder
BP	Base pairs
BSA	Bovine serum albumin
CaMKII	Calcium/calmodulin kinase 2
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
CC	Cage control
CMC	Carboxymethylcellulose
CREB	cAMP response element-binding protein
Cre-lox	Cre recombinase-lox (editing system)
DG	Dentate gyrus
DMSO	Dimethyl sulfoxide
EEG	Electroencephalography
eIF4E	Eukaryotic translation initiation factor 4E
EPM	Elevated plus maze
ERK	Extracellular signal-regulated kinase
fEPSP	Field excitatory postsynaptic potential
FMR1	Fragile X messenger ribonucleoprotein 1 (gene)
fMRI	Functional magnetic resonance imaging
FMRP	Fragile X messenger ribonucleoprotein (protein)
FXPOI	Fragile X primary ovarian insufficiency
FXS	Fragile X Syndrome
FXTAS	Fragile X tremor ataxia syndrome

GABA	γ-Aminobutyric acid
GluA1/GluR1	Glutamate [AMPA] receptor 1
GluN2B	Glutamate [NMDA] receptor subunit epsilon-2
GPCR	G-protein coupled receptor
GSK3	Glycogen synthase kinase 3
GSK3β	Glycogen synthase kinase-3 beta
HFS	High frequency stimulation
ID	Intellectual disability
IGF1	Insulin-like growth factor 1
iGluR	Ionotropic glutamate receptor
IL6	Interleukin 6
iPSC	Induced pluripotent stem cell
KO	Knockout
LTD	Long term depression
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
mKREB	Modified Krebs–Henseleit solution
MPP	Medial perforant path
mTOR	(Mammalian/mechanistic) target of rapamycin
NEK	NIMA-related kinase 1
NMDA	N-methyl-D-aspartic acid
OF	Open field
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PM	Premutation
Pnd	Post-natal day
PP	Paired pulse
PP2A	Protein phosphatase 2A
PPI	Paired pulse inhibition
PSD	Postsynaptic density

PSD95	Postsynaptic density protein 95
S6K	Ribosomal protein S6 kinase
SNP	Synaptosome
SYN1	Synapsin 1
TBST	Tris-buffered saline-triton solution
Veh	Vehicle (treatment group)
WH	Whole homogenate (whole lysate)
WT	Wild type

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Certainly, a number of acknowledgements are in order. Dr. Christie, I hope this thesis feels in part like the fruit of your own labour because it certainly is. Thank you for your patience, your mentorship, and your support over the last few years. Your supervision has helped me grow as a researcher, as a scholar, and as a person – the outcomes of good mentorship. Thank you for giving me the trust and the space to learn and fail and try again. Thank you also for always having your office door open when I inevitably needed your help with something and, ultimately, for believing in me.

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To my parents, thank you for your love and support from a distance while I pursue my dreams. Thank you for making sure I'm eating, wearing sunscreen, and filling my taxes on time. Thank you for picking up the phone and always knowing what to say when I'm upset.

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DEDICATION

This thesis is dedicated to my late granny Sofia.

I raise my glass in your memory Babushka.

I love you. Thank you.

CHAPTER 1. INTRODUCTION

1.1 Epidemiology, Genetics, and Clinical Presentations of Fragile X Syndrome

1.1.1 Human Inheritance and Genotype

Fragile X syndrome (FXS) (The Dutch-Belgian Fragile X Consortium et al., 1994) is the most common monogenic cause of autism spectrum disorder (ASD), a leading cause of intellectual disability (ID), and the most common form of ID among men (Garber et al., 2008; Stone et al., 2021). The prevalence of FXS is estimated to be approximately 1:6000 men and 1:5000 women worldwide (J. Hunter et al., 2014). FXS results from a CGG repeat expansion mutation in the 5' untranslated region (UTR) of exon 1 in the *FMR1* gene at Xq27.3 (Garber et al., 2008; Stone et al., 2021). This is considered a full mutation and causes methylation of the *FMR1* promoter and abolition of the *FMR1* protein product Fragile X Messenger Ribonucleoprotein (FMRP) (Nobile et al., 2021). In FXS, CGG repeats in the promoter region of the *FMR1* gene are passed down from a parent with an expansion repeat to offspring; most commonly discussed as X-linked transmission from mother to son (Annear et al., 2022; Ciaccio et al., 2017). Premutation carriers are individuals with 60-200 repeats which does not confer characteristic features of FXS syndrome as with full mutations but does predispose these individuals to other *FMR1*-associated conditions including primary ovarian insufficiency (FXPOI) and fragile X-associated tremor/ataxia syndrome (FXTAS) (J. E. Hunter et al., 2019; Leehey, 2009). It is estimated that 1 in every 300 women is a permutation carrier (Annear et al., 2022; Ciaccio et al., 2017). When considering allelic instability, and the

mechanism by which the permutation allele becomes a full mutation in offspring, (Nolin et al., 1996) as summarized again by (Nolin et al., 2003), describes that while a permutation inherited from male carriers may display a range of outcomes including expanding or remain unchanged, a premutation inherited from female carriers is likely to expand. Additionally, allelic instability is a direct correlate to premutation length; more repeats in the allele corresponds with greater mutation instability (Hayward & Usdin, 2021; Zhao et al., 2019). Through X allele inactivation in early embryonic development, female inheritors of the faulty allele exhibit cellular mosaicism; whereby only a proportion of cells carry the mutation on the active allele (Pretto et al., 2014). Thus, females who inherit a premutation or even a full mutation allele on one X chromosome are less likely to exhibit any or all of the presentations of FXS and cognitive alterations associated with autism spectrum disorders (ASDs) (Nolin et al., Pretto et al., 2014;1996; Nolin et al., 2003).

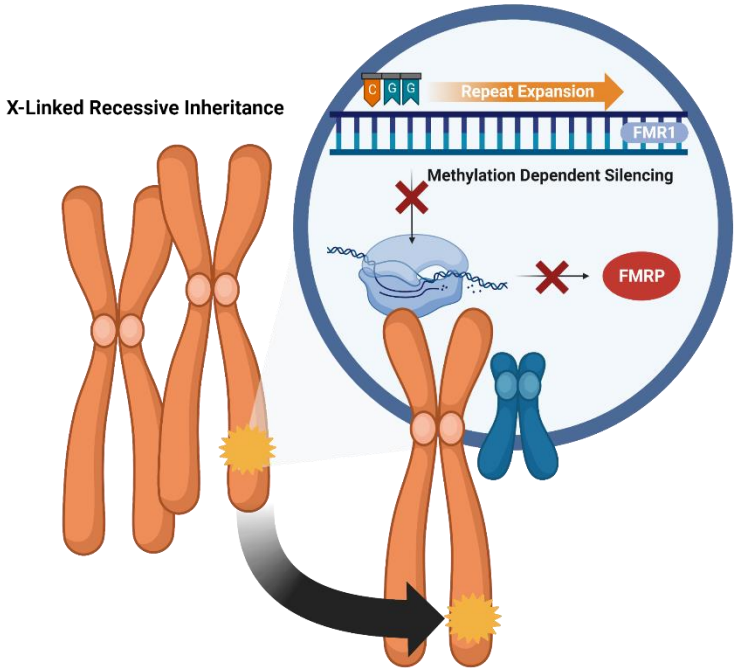


Figure 1. Brief mechanism of FXS inheritance. In this case, faulty allele passed from permutation (PM) XX parent to XY offspring. Full mutation (FM) causes methylation dependent silencing of *FMR1* gene and no product protein, FMRP, is produced.

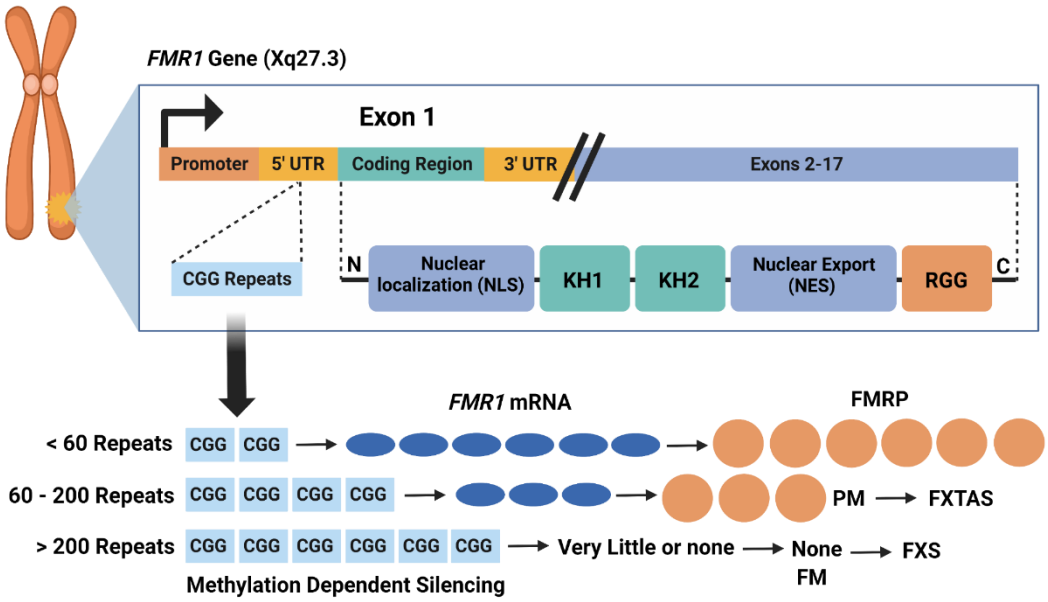


Figure 2. Recapitulation of *FMR1* gene mutations responsible for FXTAS and FXS. PM alleles exist in a range of approximately 60-200 CGG repeats and commonly present in FXTAS and otherwise asymptomatic carriers. FXS is consequential of <200 CGG repeats (FM) and subsequent gene silencing. FMRP contains nuclear localization and export domains (NLS & NES) as well as K homology domains (KH1 & KH2) which convey RNA binding function to FMRP.

1.1.2 Cognitive and Peripheral Features in Humans

Individuals diagnosed with FXS exhibit a wide range of phenotypic abnormalities which include ID, macroorchidism, and characteristic facial features (Stone et al., 2021). They may also experience cognitive and psychiatric symptoms, including learning disabilities, hyperactivity, and language deficits (Lozano et al., 2014). Many affected individuals also display social anxiety, gaze aversion which are characteristic of ASDs (Cregenzán-Royo et al., 2022; Klusek et al., 2020). Furthermore, children with FXS often exhibit sensory alterations, ranging from hypersensitivity to sensory stimuli to seizures (Hagerman & Stafstrom, 2009; Rais et al., 2018). Memory deficits and anxiety-related social behavior are key features of the FXS phenotype (Huddleston et al., 2014; Lozano et al., 2022). The deficiency in FMRP affects the anatomy and function of memory and

sensory processing regions in the brain, such as the hippocampus, prefrontal cortex, and amygdala (Cornish et al., 2001; Olmos-Serrano & Corbin, 2011). Additionally, seizure activity is observed to occur in 10-20% of individuals with FXS (Berry-Kravis et al., 2010). This is also found among *FMR1* knockout (KO) mice, which lack FMRP (L. Chen & Toth, 2001; Musumeci et al., 2000) and exhibit a reduced threshold for audiogenic seizures, indicating heightened neuronal excitability in the auditory cortex (L. Chen & Toth, 2001; Musumeci et al., 2000). Similarly, individuals with FXS show increased responses in the auditory cortex as measured by event-related brain potential recorded in EEG studies as demonstrated by (Castrén et al., 2003). Indeed, functional magnetic resonance imaging (fMRI) confirm spatially specific reduced activity in the hippocampus and basal forebrain during visual memory tasks in individuals with FXS (Greicius et al., 2004). Additionally, levels of FMRP in lymphocytes have been found to be directly related to the activation of specific brain regions during working memory tasks (Menon et al., 2000).

1.1.3 Mouse Models of FXS

FXS has been studied in patient volunteers, postmortem human tissue, human cell lines, and more recently, human cultured organoids (Dias et al., 2023; Kang et al., 2021; Kéri & Benedek, 2011). Despite this, and with the knowledge acquired through *in silico* models, the field still heavily relies on animal, and largely murine, models of FXS to investigate everything from drug pharmacodynamics to social aspects of FXS-associated ASDs. When it comes to modeling FXS in mice, there are four key considerations. First, the model must accurately represent the proteomic alterations which are present in humans. Second, the model must elicit functional characteristics contingent with those observed in humans. Third, the model must replicate, as much as possible, salient behavioral features of the FXS phenotype observed in and experienced by humans. Fourth,

the model must lend itself and respond to modulation by external effectors (i.e., pharmacology, lifestyle, genetic tools). Although there are a variety of model approaches, some show greater experimental success than others, and of these, two mouse models of FXS prevail; (1) a KO model and (2) an expansion repeat mutation model. Among the KO mouse models, there are constitutive and conditional variations that confer stable or inducible gene perturbation. The constitutive *FMR1* KO mouse model, first introduced by (The Dutch-Belgian Fragile X Consortium et al., 1994), is a popular approach for inducing FXS-associated alterations and observing gross *FMR1*-deletion-associated deficits. In parallel, conditional KO mouse models largely operate on the cre-loxP system under tissue-specific or doxycycline promoter-dependent conditions and center around recombinase activity-dependent excision of exons in the *FMR1* gene (Lovelace, Rais, et al., 2020; Zhong et al., 2018). However, no major evidence to date supports that there are significant differences in phenotype across existing KO models (Willemsen & Kooy, 2023). Moreover, a criticism of KO models is that they are not truly representative models of the human FXS phenotype (Curnow & Wang, 2022). Because they do not address gene repeat expansion, repeat-dependent methylation, and inactivation of the *FMR1* gene (Willemsen & Kooy, 2023). A knock in model of *FMR1* CGG repeat expansion was first developed by (Lavedan et al., 1998) but failed to achieve mutation-associated *FMR1* promoter methylation. Indeed, even among highly expanded alleles in KI models, researchers have recapitulated generational CGG repeat instability but not abolition of FMRP (Bontekoe et al., 2001; Colvin et al., 2022; Peier & Nelson, 2002). Thus, KI models are not suitable to model FMRP abolition and are better suited for modeling FXTAS and other *FMR1* premutation-associated pathologies (Lavedan et al., 1998).

1.1.4 Cognition and Behavior in FXS Mice

FXS presents a modeling challenge in recapitulating human phenotypes. Namely, it is uniquely challenging to model ASD features in mice because their presentations in humans vary in severity, across genetic history, and across social context. Taking a symptomatology rooted in the context of its perception among humans and attempting to recreate it in mice, understandably confers reproducibility issues, difficulty in observing a consistent phenotype, and a high likelihood of confounding factors altering behavioral outcomes. Indeed, literature indicates that multiple behavioral responses are dependent on mouse genetic background, including autistic-like traits which vary in type and severity across genetic backgrounds (Spencer et al., 2011). These challenges do not bar meaningful data but must be considered when attempting to establish baseline deficit or examining pharmaceutical efficacy. Overall, the most common constitutive *FMR1* KO mouse models exist on either C57BL/6 or FVB/N backgrounds; both strains do not reveal identical behavioral phenotypes (Errijgers & Kooy, 2004).

1.1.4.1 Anxiety and Social Interaction

Anxiety is a confirmed presentation among individuals with FXS, however it remains debated whether this characteristic can robustly be considered part of the murine phenotype given conflicting evidence in animal behavior experiments (Dahlhaus, 2018; Wadell et al., 2013). *FMR1* KO mice have been shown to spend more time in the center of the open field test relative to wild type (WT) which would suggest decreased anxiety (Dahlhaus, 2018; Liu & Smith, 2009). Likewise, exploration of anxiety behavior in the elevated zero maze, confirms that *FMR1* KO mice spent a greater percentage of time in the open aspect of the apparatus relative to WT (Liu & Smith, 2009). (Liu & Smith, 2009) further denote that in a three-chamber sociability test, *FMR1* KO mice

exhibited reduced initiation of social approach as well as reduced response to social novelty relative to WT mice. Work from our group demonstrates that *FMR1* KO mice display a deficit in social recognition memory but shows intact sociability when compared to WT mice; a presentation that was also found to be resistant to minocycline effects – a drug expected to reverse social deficits (S. Y. Yau et al., 2016). Conversely, social recognition in the same study was found to respond to modulation with minocycline (S. Y. Yau et al., 2016). These findings demonstrate that the social anxiety phenotype in mouse models of FXS are uniquely nuanced and that sociability and social novelty/social memory are distinct and sometimes contradictory to each other.

These findings are corroborated by the fact that in the mirrored chamber test, KO mice exhibit increased avoidance of the central mirrored chamber which suggests increased anxiety (Spencer et al., 2005). These findings suggest that *FMR1* KO mice do not necessarily present with increased environmental anxiety behavior but when faced with social interaction (or a mirror), and more specifically with social novelty, they may face greater deficits. This is further complicated however by the automated tube test by (de Esch et al., 2015) which demonstrates that in the event of social stress –like a forced social interaction– KO mice were more likely to win in a physical fight and are thus more likely to exhibit social dominance compared to WT mice (de Esch et al., 2015). While increased social dominance may appear to indicate decreased social anxiety, when coupled with decreased social approach and decreased response to social novelty, it could be argued that KO mice present with overall impaired social salience with a predisposition for antisocial aggression.

When considered from this perspective, animal models of social deficit corroborate findings of diminished sociability among people with a pronounced FXS phenotype. Given that mice are a

prey species, it is indeed abnormal for them to display decreased environmental anxiety; this can be considered a unique feature of the KO mouse phenotype. However, it remains unclear whether this behavioral finding can be considered a robust translational metric of the FXS model given that human anxiety, beyond social anxiety, is a more complex phenomenon than the subversion of prey behavior in mice. It follows that in therapeutic studies involving mouse anxiety behavior tests, interpretations of social and environmental anxiety must be contextually decoupled.

1.1.4.2 Hyperactivity and Repetitive behavior

Work from our group by (Eadie et al., 2012) confirms that there is no singular concerted basal phenotype in a mouse model of FXS and cautions that careful investigation of both behavior and biological characteristics is required to observe minor differences. (Eadie et al., 2012) propose that by knowing where to look, it is possible to observe subtle changes between FXS models and WT mice. They further describe that basic behaviors in FXS mice can be quantified using a modified SHIRPA test battery, a proposed protocol for comprehensive phenotype assessment developed by (Rogers et al., 1997). The SHIRPA battery includes physiological metrics such as body weight, respiration, waste output, heart rate, and skin color as well as behavioral metrics including stereotaxis, startle response, tail elevation, and righting reflex among many others (Rogers et al., 1997;Eadie et al., 2012). Overall, *FMR1* KO mice do not present with significant phenotype differences from wild-type mice, and so careful investigations are needed to examine more subtle differences (Eadie et al., 2012).

Hyperactivity is a chief characteristic of FXS in humans and is generally thought to robustly translate to the KO mouse model (Dahlhaus, 2018; Chromik et al., 2019). This is indeed observed

among our own work by (Ghilan et al., 2018) which found that *FMR1* KO mice display hyperlocomotion and altered thigmotaxis. Despite this conserved behavioral presentation, the degree of hyperactivity in these mouse models varies considerably across studies (Cogram et al., 2019; Kooy et al., 1996). Overall, KO mice do indeed demonstrate increased locomotion in the open field and light-dark test confirming a sustained hyperactive phenotype (Ding et al., 2014; Ghilan et al., 2018). Repetitive behavior stereotypies in humans, like hand flapping, have been shown to be consistent with severe FXS presentations (Garber et al., 2008) while *FMR1* KO mice exhibit these features through repetitive behaviors including increased self-grooming and increased burying of marbles in the marble burying test (Thomas et al., 2009). Like memory tasks however, repetitive behaviors in KO appear to be largely genotype background-dependent; largely in marble burying tests (Huebschman et al., 2022).

1.1.4.3 Learning and Memory

Initial studies using the original constitutive *FMR1* KO mouse model showed deficits in spatial learning and memory as evaluated using the Morris water maze (D'Hooge et al., 1997; The Dutch-Belgian Fragile X Consortium et al., 1994). The current FXS research landscape recognizes these discrepancies and notes that they may arise from the use of *FMR1* KO mice bred onto different genetic backgrounds. Indeed, our group's work also finds an unperturbed phenotypic result in Morris and plus water maze among *FMR1* KO mice on a C57BL/6 background (Ghilan et al., 2018). This confirms early previous work by (Dobkin et al., 2000; Paradee et al., 1999) which demonstrated differences in Morris water maze behavioral outcomes among mice bred differentially on C57BL/6 and 129P2 genetic backgrounds.

Additionally, *FMR1* KO mice on the C57BL/6 background show a deficit in contextual and passive avoidance tests which may be taken to enrich understanding of the paradoxical nature of attenuated anxiety behavior (Ding et al., 2014). Given that *FMR1* KO mice display decreased context-based fear-cues, it follows that they may display less anxiety behavior. Additionally, we demonstrate that learning and memory, as measured through electrophysiology in *FMR1* KO mice, is uniquely perturbed by stress when compared to WT mice (Ghilan et al., 2015). This suggests that not only do *FMR1* KO mice display unique, and sometimes paradoxical, anxiety behavior from WT mice, they also do so in a genotype-stress interaction-induced way (Ghilan et al., 2015). Thus, while learning and memory alterations are consistent presentations in humans with FXS, mouse models only partially replicate these behavioral alterations, largely, in a context and background genotype-dependent manner.

1.1.4.4 Seizures

In *FMR1* KO mice, seizures can be induced via audiogenic stimuli, however C57BL/6J mice are rare to display this particular phenotype compared to the FVB/N which are more prone to the audiogenic seizures and more closely represent the increased epileptic susceptibility of children with FXS (Kasugai et al., 2007; Van der Aa & Kooy, 2020). Likewise, a difference in the pre-pulse inhibition of the acoustic startle response is demonstrated in *FMR1* KO mice which is contingent with the decreased PPI documented among patients with FXS (Frankland et al., 2004).

1.2 Molecules and Function: Synaptic deficits in FXS and Therapeutics

FMRP is a cytoplasmic protein expressed throughout the body but to the highest extent in cells of the testes and the brain. FMRP is a ubiquitous molecular chaperone, regulator of mRNA translocation, and a critical player in second messenger signaling (Fernández et al., 2013; B. Xu et al., 2018). It consists of four canonical mRNA binding motifs which contain one arginine-glycine (RGG) domain and three K homology (KH) domains; both structural characteristics of proteins involved in transcriptional and translational regulation as well as mRNA storage and stability (E. Chen & Joseph, 2015; Richter & Zhao, 2021). FMRP is thought to bind approximately 4% of all mRNA in the mammalian brain and is posited to play a major role in externalization of mRNAs from the cell nucleus and localization to the synapse (Brown et al., n.d.; M. Kim et al., 2009). It serves a crucial role as a regulator of translation and protein folding; thus, its absence contributes considerably to functional abnormalities associated with FXS (Athar & Joseph, 2020). FMRP stalls the translation of a variety of many proteins, but notably, those pertinent to the generation and maintenance of long-term bidirectional plasticity.

1.2.1 Synaptic Plasticity

Dendritic spines are the primary post-synaptic site of contact between neurons; they are pertinent effectors in neurotransmission, and by consequence, synaptic plasticity (Runge et al., 2020). The number, density, and morphology of dendritic spines are important markers of network function, disease states in neuropsychiatric conditions, and overall brain health. Indeed, perturbation in the aforementioned spine characteristics in any given neural circuit, are contingent with well described

neuropathologies including schizophrenia and Alzheimer's (Dorostkar et al., 2015; Glausier & Lewis, 2013).

In both humans and among animal models, FXS is well characterized by a high density of immature (filopodia-like) dendritic spines in the primary somatosensory cortex, the amygdala, the cerebellum, in granule hippocampal cells, as well as in layers 2, 3, and 5 of the cortex and in hippocampal pyramidal cells (C. X. He & Portera-Cailliau, 2013; Hinton et al., 1991; Irwin et al., 2000; Oddi et al., 2015). While altered cortical dendritic morphology has been reliably established across studies, including by our group (S.-Y. Yau et al., 2018; S. Y. Yau et al., 2018), hippocampal alterations in dendritic spine morphology remain conflicting across groups and publications hedging primarily on developmental time point at which measurements occur and secondarily on mouse genetic background and hippocampal subregions (C. X. He & Portera-Cailliau, 2013). Historically, the dendritic spine morphology of FXS has been pondered as the source of functional synaptic deficits underlying FXS (Portera-Cailliau, 2012). While this is an understandable perspective given that dendritic spines serve central functions in synaptic transmission, advances in proteomics and mechanistic exploration indicate that the spine phenotype in FXS more certainly is a parallel consequence of FMRP abolition rather than the major cause behind synaptic alterations (Portera-Cailliau, 2012; S.-Y. Yau et al., 2018; S. Y. Yau et al., 2018).

The conventional understanding of memory encompasses the neural process by which acquired information is received, stored, and retrieved. Memory is a functional manifestation of synaptic plasticity – an activity-dependent strengthening or weakening of the synapse to suit contextual need. Long term potentiation (LTP) is the process of activity-dependent strengthening of a synaptic circuit which leads to a sustained increase in signal transmission. While LTP is very well discussed

in countless reviews, textbooks, and papers (Abraham, 2003; Pittenger & Kandel, 2003), and while different types of LTP exist, the general mechanism of LTP induction is as follows: Upon stimulation, glutamate released from the pre-synaptic neuron activates ionotropic glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on the post-synaptic neuron which allow an influx of Na^+ into the cell to cause depolarization. This causes metabotropic N-methyl-D-aspartate (NMDA) receptors to be relieved of their Mg^{2+} block and they flux Na^+ and Ca^{2+} through the NMDA receptor. Ca^{2+} associates with calmodulin (CaM) and together Ca^{2+}/CaM bind to the CaMKII kinase to trigger phosphorylation of CaMKII and initiate constitutive CaMKII activity. Constitutively active CaMKII subsequently interacts with NMDA receptors and phosphorylates the (GluA1) subunit of AMPA receptors which causes AMPA receptor externalization to the cell membrane. At the same time, constitutive CaMKII activity drives cyclic adenosine monophosphate (cAMP) dependent second messenger cascades which modulate transcriptional control of genes relevant to sustaining the localized maintenance of structural, functional, and energetic needs of the newly potentiated synapse. Herein, in the generation of LTP, dendritic spines are formed and remodeled (Toni et al., 2001). A broader discussion of spines is beyond the scope of this thesis dissertation but remains a big source of inquiry in the FXS research field. Namely, what came first, synaptic dysfunction or dendritic spine alterations, given that they are mutually perpetuating? See (C. X. He & Portera-Cailliau, 2013) for a comprehensive review on the topic. Nevertheless, dendritic spine alterations are a documented, well observed, somewhat controversial, and complex phenomenon in FXS; they lend a salient route for exploration when considering the alterations in synaptic plasticity to be discussed below.

1.2.1.1 Glutamatergic Transmission

Glutamate is the chief excitatory neurotransmitter of the brain who's inotropic (iGluRs) and metabotropic (mGluRs) receptor subtypes, are intrinsically implicated in the generation of bidirectional synaptic plasticity. mGluRs are G-protein coupled receptors (GPCRs) which are comprise GluR1, GluR2, and GluR3 and further make up eight known receptors. They are all characteristic in their slow glutamate response kinetics (Niswender & Conn, 2010; Purves et al., 2001).

Synaptic plasticity comprises the molecular underpinning of learning and memory and is, by consequence, a central tenet underlying the involvement of FMRP interaction with GluRs and their role in the functional deficits associated with FXS (Antar et al., 2004; Todd et al., 2003). Influential work exploring plasticity in FXS showed increased GluA1 and GluR5 in hippocampal neurons of *FMR1* KO mice – setting in motion the basis for the mGluR hypothesis of FXS synaptic dysfunction (Huber et al., 2002; Auerbach & Bear, 2010; Dölen & Bear, 2008). The mGluR hypothesis of FXS proposes that mGluR1 expression is regulated by FMRP, and that loss of FMRP results in increased expression of mGluR1 in *FMR1* KO neurons and a consequent enhancement of mGluR-dependent LTD (Auerbach & Bear, 2010; Dölen & Bear, 2008). The abnormal increase in mGluR-dependent LTD coupled with FMRP loss and cognitive deficit has persisted in the field of FXS research and has been explored over decades in many studies (Choi et al., 2011; Dölen et al., 2007; Osterweil et al., 2010). While the mGluR hypothesis of FXS has perpetuated throughout research to this day, the replicability of these findings have been called into question and mounting evidence exposes key gaps which remain unaddressed by the mGluR hypothesis and ultimately destabilize its perspective on the underlying pathophysiology of FXS on the basis of reproducibility across labs and translational failure into human iPSC models (Achuta et al., 2017).

Likewise, the efficacy of mGluR1 antagonists in clinical trials have not shown promise in improving FXS presentations in humans (Berry-Kravis et al., 2016).

Ionotropic Glutamate receptors (iGluRs) comprise AMPA, NMDA, and kainate receptors (Newcomer et al., 2000). There is mounting evidence for the involvement of altered iGluR function in FXS pathophysiology, including diminished hippocampal LTP, as demonstrated in early works by (Hu et al., 2008; H. Y. Lee et al., 2011; Shang et al., 2009) and more recent work by (Bostrom et al., 2013; S.-Y. Yau et al., 2018; S. Y. Yau et al., 2018). (Bostrom et al., 2013) demonstrate NMDA involvement in the functional deficits of FXS by showing diminished GluA1 containing AMPARs in the hippocampal DG, coupled with a regional reduction in GluN1, Glun2A, and GluN2B NMDAR subunits and diminished GluA1 phosphorylation among *FMR1* KO mice. Likewise, (S.-Y. Yau et al., 2018) show basal deficit among mature granule cells in the outer hippocampal granule cell layer which displayed significantly smaller post synaptic NMDA-dependent currents, a higher AMPA to NMDA ratio, and decrease dendritic complexity in *FMR1* KO mice compared to WT mice. Finally, (S. Y. Yau et al., 2018) corroborated this NMDA deficit by showing that enhancement of NMDAR-dependent LTP in the DG of *FMR1* KO mice also rescued performance in novel object recognition. Together, these studies implicate NMDA alterations as a central player in FXS and demonstrate that modulating NMDA signaling can restore some molecular and functional characteristics of FXS.

1.2.1.2 GABAergic Transmission

Gamma-(γ)-aminobutyric acid (GABA) is the chief inhibitory neurotransmitter of the brain and comprises two families of receptors ionotropic (GABAA) and metabotropic (GABAB). GABAA

receptors work to inhibit neuronal activity by responding to fast Cl^- dependent cell hyperpolarization while GABAB receptors serve the same function but respond to G protein coupled K^+ channel activation (Terunuma, 2018; Wu & Sun, 2015) .

In FXS, both GABA receptors (both A and B type) are perturbed during embryonic development and in adulthood (Paluszkiewicz et al., 2011). Indeed, FMRP abolition is found to present with a decrease in in GABA receptor subunit expression including δ, α1/3/4, β1/2, and γ1/2 across the amygdala, cerebellum, and the hippocampus (Braat et al., 2015; Deng et al., 2022; D'Hulst et al., 2006; D'Hulst & Kooy, 2007). The exact mechanism by which GABAergic dysfunction occurs in FXS remains contested and complicated by the limited evidence that observed alterations in *FMR1* KO mice exist in humans with FXS too (Cea-Del Rio et al., 2020; D'Hulst et al., 2015; Gibson et al., 2008). Since GABAA receptors are intimately involved in excitatory/inhibitory homeostasis and neurodevelopment –namely the developmental GABA polarization switch– GABAergic alterations in the FXS brain contribute to altered synaptic function including reduced inhibitory post-synaptic currents in *FMR1* KO mice (Sabanov et al., 2017). In parallel, it is proposed that GABAB subunit expression alterations may contribute to a delayed developmental GABA shift which would certainly impact chloride gradient regulation throughout development and into adulthood. A delayed developmental GABA shift has indeed been shown in *FMR1* KO mice manifesting in delayed maturation of Cl^- currents and a longer period of neuronal depolarization (Q. He et al., 2014). This finding may speak to known characteristics including delayed synaptic maturation and likely contributes to the etiology underlying the dendritic spine phenotype associated with FXS. While molecular and functional GABAergic alterations are demonstrated in

the studies discussed above, much remains unknown about FMRP-GABA interactions and considerable further investigation is required.

1.2.2 Molecules, Signal Transduction, and Therapeutic Targets

Through both direct association and secondary signal transduction, FMRP interacts with multiple described target mRNAs which are known to play significant and broad roles in generation, maintenance, and fine tuning of bidirectional plasticity. These relationships are documented, as we previously discussed, at the level of basal translation gating but also through association with highly specialized mRNA substrates implicated with maintenance of synaptic cytoarchitecture and function. Given that FMRP perturbation causes proteomic alterations locally and as well as through second messenger ripples, our foray into novel neuropharmaceuticals aims to probe relevant biomarkers at varying degrees of distance from effector receptors along secondary signal transduction cascades focusing both on cellular metabolic proteins as well as those with concrete roles in generation and maintenance of activity dependent plasticity. Below, we discuss salient biomarkers for therapeutic investigation and discuss previous attempts –where such exist– and research trends in the neuropharmaceutical field as they relate to FXS. The regulation of FMRP itself is driven by post-translational modifications (Darnell et al., 2011; Narayanan et al., 2007; Sidorov et al., 2013). FMRP phosphorylation and dephosphorylation is modulated in response to synaptic activity, demonstrating that synaptic stimulation interacts with local translation in a traceable manner along key intermediate signaling proteins (Darnell et al., 2011; Narayanan et al., 2007; Sidorov et al., 2013). The primary molecular alteration associated with FXS in this framework is the gross upregulation of activity dependent translation that goes unchecked in the absence of FMRP.

1.2.2.1 mTOR

Excitatory synaptic activity sets off second messenger cascades which modulate cell metabolic pathways relevant to activity-dependent *de novo* protein synthesis. The mechanistic target of rapamycin (aka mammalian target of rapamycin) (mTOR) signaling pathway is a chief regulator of basal cell function including energy metabolism, proliferation, and second messenger transduction (Garza-Lombó et al., 2018). Phosphorylation of mTOR drives S6K-dependent phosphorylation, and thus activation of FMRP (Hoeffer & Klann, 2010). As such, mTOR drives the activity of its own counteracting repressor protein, and in doing so presents an important mechanism by which neurons maintain the tight spatial and temporal control of activity-dependent translation necessary to elicit and fine tune synaptic plasticity events at both pre and post synaptic sites (Dossou & Basu, 2019; Henry et al., 2012).

In both human and animal models of FXS, elevated mTOR signaling has been identified. Although rapamycin, an mTOR inhibitor, may initially seem like a potential solution to counteract this runaway signaling, its use has proven ineffective in restoring behavioral phenotypes in some mouse models (Saré et al., 2017). This highlights the delicate nature of mTOR signaling, as both overstimulation and complete inhibition are detrimental (Saré et al., 2017). Despite these challenges, exploring mTOR-targeting molecules remains worthwhile, as progress has been made in this field. Examples of this are with IGF 1 analog trofinetide which normalized ERK and Akt signaling, improved dendritic spine morphology, and ameliorated deficits in mouse learning, memory, hyperactivity, and social behavior (Deacon et al., 2015). Similar findings were recapitulated in human clinical trials (Berry-Kravis et al., 2020). Likewise, S6K inhibitors improved social behavior and dendritic spine characteristics in mice (Bhattacharya et al., 2016).

Likewise, work in our group and others' has demonstrated that modulation of mTOR with the tetracycline antibiotic minocycline restores neuronal structure, NMDAR function, and social recognition in *FMR1* KO mice (Lovelace, Ethell, et al., 2020; S. Y. Yau et al., 2016; S. Y. Yau et al., 2018). These findings underscore the involvement of mTOR signaling in FXS pathophysiology, particularly in relation to neurogenesis, neuronal branching, and molecular mechanisms underlying synaptic plasticity.

1.2.2.2 MAPK

The mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling cascade is crucial for cellular processes in neural cells, including neurogenic progenitor proliferation and differentiation as well as in the generation of learning and memory (Zhang & Liu, 2002).

Both the mTOR and the MAPK/ERK pathways are central drivers of the neuron's response to excitatory activity and chiefly involved in basal metabolic events which underlie the initiation of transcriptional and translational events which generate and maintain synaptic plasticity (Albert-Gascó et al., 2020; Papa et al., 2019). Similarly, the mTOR and the MAPK/ERK pathways are gated by FMRP in its regulatory capacity and are thus major, if not primary, sources of the gross and constitutive upregulation of transcriptional events observed in FXS – as well as major drivers of characteristic functional deficits (Casingal et al 2020; Thomas et al., 2023; Wang et al 2012). Given the impact of MAPK dysregulation, there is a unfulfilled niche in therapeutic molecules targeting this signal transduction cascade for FXS therapies; especially in the realm of direct or indirect MAPK inhibition. The antidiabetic, Metformin has emerged as a prominent pharmaceutical agent in FXS research. Its diverse off-target effects on the metabolic-

neuropsychiatric axis have made it a subject of investigation in various neuropsychiatric conditions and in FXS (Gantois et al., 2017, 2019; N. Li et al., 2022).

Metformin inhibits respiratory chain complex 1 in the mitochondrial electron transport chain, which causes activation of AMP-activated protein kinase (AMPK) and diminished cellar energy metabolism (Vial et al., 2019; Y. Wang et al., 2019). Metformin's interaction with the FXS brain remains not fully understood, but it has been observed to modulate metabolic and autophagic cellular function as evidenced by it's ability to downregulate MAPK, mTOR, and eIF4E signaling (C. C. Lu et al., 2019). In animal models of FXS, metformin, similar to minocycline (S. Y. Yau et al., 2016; S. Y. Yau et al., 2018), has shown success in restoring structural and functional defects, and rescuing repetitive behavior, social behavior deficits, and dendritic spine morphology (Gantois et al., 2017). Clinical trials are currently underway investigating metformin, and while tolerability has been high, the overall improvements in aberrant neural function and behavior in humans remain to be characterized. Thus, as an important signaling intermediate between external stimuli and cellular response along the AMPK-mTOR signaling cascade, MAPK/ERK serves not only as a litmus test of sorts to observe cellular state, but also in an of itself a potential therapeutic target.

1.2.2.3 PSD95

The synaptic scaffolding protein post-synaptic density protein 95 (PSD95) is highly abundant in the postsynaptic density (PSD) and plays an important role in the establishment and maintenance of bidirectional synaptic plasticity through the regulation of receptor and secondary signaling protein localization (Iasevoli et al., 2013). The post-synaptic aspect of excitatory synapses at dendritic spines, contains a high localization and concentration of receptors for neurotransmitters and affiliated cellular machinery for second messenger signaling which rely on PSD-95 and

affiliated proteins for correct distribution and organization (Barrera-Ocampo & Chater, 2013; Pchitskaya & Bezprozvanny, 2020). Indeed, (Bagni & Zukin, 2019) describe how impaired homeostatic regulation of PSD95 may contribute to defective spine and synapse maturation and remodeling in *FMR1* KO mice. Previous work by (Ifrim et al. 2015) suggest that ubiquitin proteasome-mediated degradation of PSD95 is decreased in *FMR1* KO mice and that basal translation of PSD95 is altered in *FMR1* KO neurons (Ifrim et al., 2015). Since PSD95 mRNA is dendritically localized, these studies suggest that PSD95 mRNA translational regulation and stabilization is impaired at synapses in FXS (Ifrim et al., 2015; Zalfa et al., 2007).

1.2.2.4 Glun2B

FMRP exerts regulatory control over the NMDAR-associated synaptic signaling interactome (Darnell & Klann, 2013). Moreover, FMRP interacts with NMDAR subunit mRNA suggesting implication for NMDARs in the pathophysiology of FXS, as discussed by (Edbauer et al., 2010). Notably, alterations in subunit levels have been observed across distinct brain regions of *FMR1* KO mice, notably an increase in GluN1 and GluN2B within the hippocampus, as highlighted by (Schütt et al., 2009a; Schütt et al., 2009b) and recapitulated by our group (Bostrom et al., 2013).

Existing evidence supports the bidirectional stimulation between NMDARs and mGluRs which centers the mGluR hypothesis of FXS in a dysregulated NMDA signaling (Alagarsamy et al., 1999; Collett & Collingridge, 2002). This interplay between mGluRs and NMDARs forms a feedback loop which is thought to modulate NMDAR-dependent and mGluR-dependent forms of synaptic plasticity, potentially contributing to the pathological responses induced by NMDA receptor activation (Gladding et al., 2009; Nicoll et al., 1998; Toft et al., 2016).

In relation to this, it has been shown that blocking GluN2B-containing NMDARs in the hippocampus of *FMR1* KO mice negated the enhanced mGluR-dependent LTD, which points to the hyperactivity of the GluN2B-signaling pathway in *FMR1* KO mice and its involvement in the generation of functional deficits associated with FXS (Bostrom et al., 2013; Toft et al., 2016). The upregulation of GluN2A and GluN2B proteins in the hippocampus of *FMR1* KO mice, as independently confirmed by (Schütt et al., 2009a) and (Toft, Lundbye, and Banke, 2016), further supports this observation. (Toft, Lundbye, and Banke, 2016) propose a model wherein dysregulation of NMDARs hampers the proper regulation of mGluR-LTD, consequently leading to neuronal hyperexcitability (Schütt et al., 2009a; Toft et al., 2016). Furthermore, their findings highlight the role of dysregulated NMDAR signaling as the underlying cause of enhanced mGluR-LTD in *FMR1* KO mice and underscore the potential therapeutic target in FXS research of manipulating interactions along the NMDAR- mGluR axis.

1.2.2.5 Syn1

While the majority of studied molecular abnormalities in FXS center on post-synaptic cytoarchitecture as driving elements behind functional deficits, there is evidence that pre-synaptic alterations also modulate the rate and extent of neurotransmitter release, vesicle composition and thus ultimately neurotransmission in FXS (Bonnycastle et al., 2021). Therefore, and in the context of deficits in plasticity, it's relevant to consider the consequence of FMRP abolition on the properties of neuronal activity and function including transmitter release probability and vesicle composition. Synapsins are a family of proteins which play a role in synaptic vesicle localization and thus control the number of fusion-bound vesicles at the active zone (Bloom et al., 2003; Ceccaldi et al., 1995). At presynaptic terminals, synapsins associate with the surface of synaptic vesicles and are documented to make up a sizeable proportion of total synaptic vesicle protein and

regulate synaptic vesicle fusion (Khvotchev & Sun, 2009;Coleman & Bykhovskaia, 2009; Hendricks & Shi, 2014). In a mosaic mouse model of *FMR1* deficiency, (Hanson and Madisson, 2007) conducted electrophysiological recordings on CA3 pyramidal neurons to find a decrease in the proportion of active synaptic connections by nearly two-fold between *FMR1* expressing neurons and *FMR1* KO neurons. Notably in this study, the average amplitude of excitatory post-synaptic currents did not exhibit any significant change in correlation with FMRP expression. These results, as described by its authors, suggest that the primary consequence of *FMR1* knockout is a reduction in synaptogenesis but not necessarily post-synaptic responses (Hanson & Madison, 2007).

1.2.2.6 GluA1

Among AMPARs, their four subunits GluA1/A2/A3/A4 form functionally distinct tetramers and make up for the majority of fast excitatory signaling throughout the brain (Gan et al., 2015; Diering et al., 2016). AMPAR subunits undergo a plethora of posttranslational modifications that profoundly influence protein trafficking and receptor abundance (W. Lu & Roche, 2012). As pointed out initially by (Diering & Huganir, 2018; Henley & Wilkinson, 2013) and summarized by (Qu et al., 2021) post-translational modifications can have an impact on channel properties, but functional changes in AMPARs implicated in bidirectional plasticity are a consequence of differences in their synaptic abundance. Indeed, (Wang et al., 2015) describe abnormal internalization of surface GluA1 in the prefrontal cortex of *FMR1*KO; this is contingent with the mechanisms underlying increased hippocampal LTD described by (Bear et al., 2004; Huber et al., 2002). This is corroborated in the work of (Bostrom et al., 2013) previously discussed in section 1.2.1.1.

1.2.2.7 AMPK

Adenosine mono-phosphate-activated protein kinase (AMPK) is a heterotrimeric serine-threonine protein kinase which contains an α, β, and γ subunit (Herzig & Shaw, 2018). The α-subunit confers the protein kinase activity, the β-subunit allows AMPK to participate in glycogen metabolism, and the γ-subunit allows AMPK to act as an energy sensor and respond to ATP abundance in the cell (Hudson et al., 2003). AMPK acts as a crucial downstream mediator of mitochondrial respiration status and initiates cellular adaptations in response to alterations in nutrient availability and environmental cues (Gao, Cantó, Houtkooper, 2014). Given its central role in cellular metabolism and its influence on mTOR activity, the steady-state expression and phosphorylation of AMPK are pertinent to investigating molecular abnormalities associated with FXS.

As kinase inhibitors continue to be a prominent approach in FXS therapeutic development, AMPK represents a valuable protein target for investigation due to its involvement in early synaptic inputs and activity-dependent cellular functions. Notably, a study conducted by (Monyak et al., 2017) implicated dysregulated AMPK signaling in a Drosophila model of FXS. In this study, KO flies showed improved LTP in response to being given Metformin; presumably through modulation of AMPK and mTOR signaling. While this study is on flies and not mice or humans, it does document a traceable mechanism by which AMPK alterations are contingent with FMRP abolition. Thus, they demonstrate that AMPK serves as an essential intermediate for evaluating novel drug mechanisms in the context of cellular energetics, as it establishes a direct correlation between compound action and the underlying metabolic response of the cells. Likewise, studies by

1.2.2.8 GSK3β

Glycogen synthase kinase 3 (GSK3), which comprises two paralogs (GSK3α and GSK3β), is vital protein involved in glycogen metabolism through the association with glycogen synthase (Parker et al., 1983). Both isoforms are widely expressed in various brain regions and play essential roles in brain bioenergetics, synaptic plasticity, proliferation, and survival (Salcedo-Tello et al., 2011). Studies using murine models have shown that GSK3 inhibitors significantly improve behavioral deficits in a variety of pathologies, including FXS (Guo et al., 2012; King & Jope, 2013; Nakao et al., 2020). Specifically, lithium treatment has been found to remediate learning, memory, and social behaviors in this model (King & Jope, 2013). (King & Jope, 2013) demonstrated that while novel object exploration and spatial processing increased in *FMR1* KO mice following lithium treatment, these improvements regressed to impaired levels after treatment cessation.

Similar findings are documented by (Yuskaitis et al., 2010) where they document that lithium – by way of GSK3 inhibition – restored behavioral deficits in social and anxiety-like behavior as well as increased hippocampal abundance of brain derived neurotrophic factor. These findings suggest that modulation of GSK3 may exert their effect through a mechanism that supports pro-neurogenic effects in the hippocampus. Their work however, took place in *FMR1* KO mice on an FVB background. Given the previously discussed difference that strain background can have on behavioral outcomes in FXS models, it is uncertain whether these finding may be strain specific.

Lithium is described to inhibit both GSK3α and GSK3β of GSK (Freland & Beaulieu, 2012). Interestingly, (Camphill et al., 2020) investigated this relationship even further by demonstrating that inhibition of each paralog appears to produce a unique effect in *FMR1* KO mice (on a C57BL/6 genetic background). Namely, they show that selective inhibition of GSK3α specifically, and not

GSK3 β, rescues protein synthesis and audiogenic seizures (Camphill et al., 2020). These findings suggest the effects of lithium in restoring FXS pathophysiology in mouse models may be GSK3 subunit specific and that this may require special considerations in the application of lithium given it's potential for toxicity and side effects.

1.2.2.9 cAMP and CREB

Cyclic adenosine monophosphate (cAMP) plays a pivotal role in mediating synaptic transmission and regulating various aspects of neuronal function by controlling the metabolic state of target neurons and neurotransmitter synthesis, as well as neuronal growth and differentiation (Shahoha et al., 2022). The primary mechanism through which cAMP exerts its effect is through activating cAMP-dependent protein kinase (PKA) which then phosphorylates target kinases, transcriptional factors, and ion channels (Shahoha et al., 2022; Sassone-Corsi, 2012). Long term effects associated with cAMP activity include *de novo* protein synthesis and is mediated by the nuclear transcription factor cAMP response element-binding protein (CREB) (H. Wang et al., 2018).

Active CREB binds the promoter cAMP response element (CRE) region in target genes and drives transcription (Shaywitz & Greenberg, 1999; H. Wang et al., 2018; Dyson & Wright, 2016). NMDA activation-dependent modulation of the cAMP-PKA-CREB pathway is a well-established regulator of synaptic plasticity – namely LTP – and links neural firing to downstream memory generation. Thus, CREB acts as a crucial link between short-term plasticity-related molecular events and transcription-dependent LTP (Abel & Nguyen, 2008; Kandel, 2012; Mohanan et al., 2022).

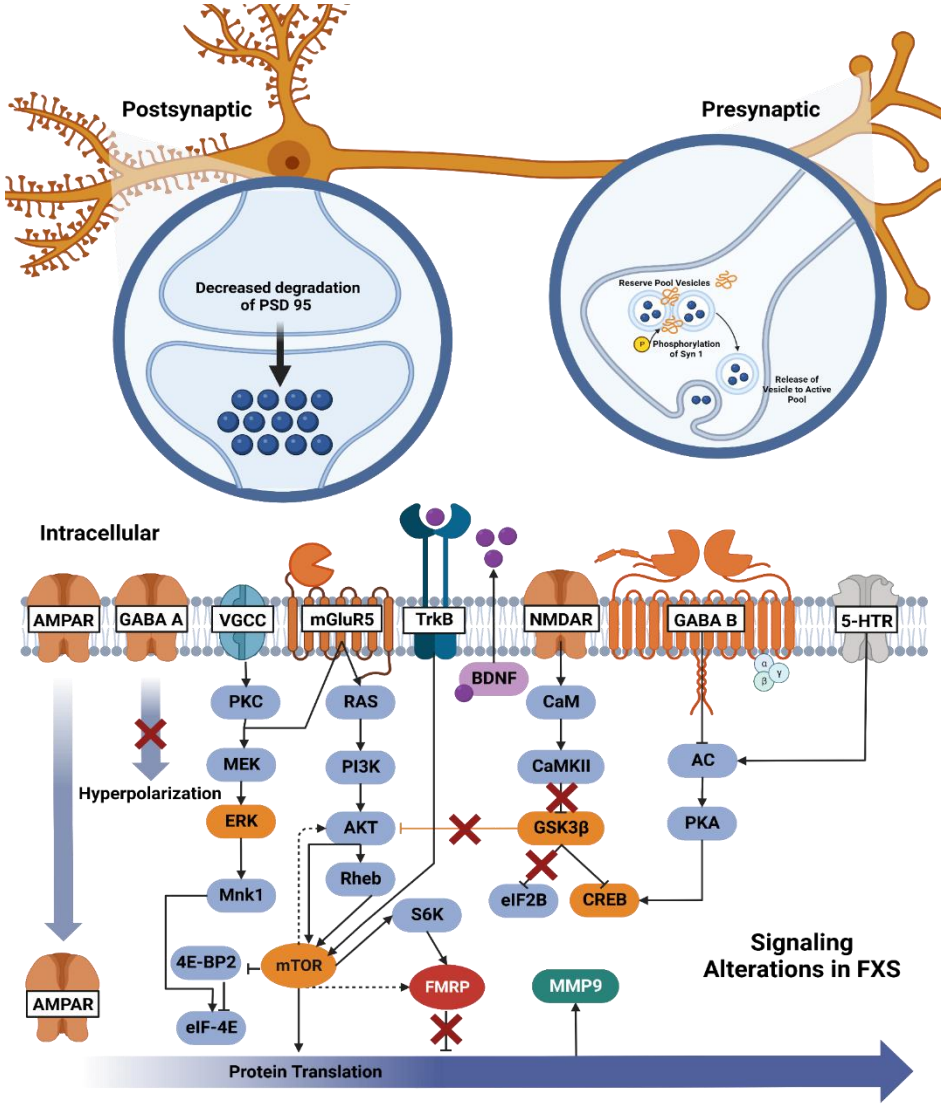


Figure 3. Summary of molecular alterations of FXS. Pre and post-synaptic deficits including altered PSD95 and SYN1 function convey altered chemical transmission and probability of neurotransmitter release. FMRP-abolition-associated changes in intracellular proteins which impart neuronal metabolic functions and contribute to synaptic plasticity including ERK1/2/MAKP, GSK3B, CREB, and mTOR are key contributors to FXS pathophysiology. Druggable targets including intermediate signaling proteins and receptors discussed here are indicated in orange.

1.3 The Neuroendocrine Axis: Cellular Autophagy, Hormone Signaling, and Cognition

The FXS research field has quickly evolved over the previous decades with exponential expansion in the number and variety of compounds being investigated as therapies. Despite this, antipsychotics remain the only approved therapeutics for FXS (Erickson et al., 2010; Protic et al., 2019). While antipsychotics have proven efficacy among some patients for some characteristics of FXS including aggression and mood instability, they fail to address basal molecular disruptions implicated in FXS etiology (Berry-Kravis et al., 2012; Dominick et al., 2018; Y. Kim et al., 2022). Pharmacological and genetic therapies represent the present landscape and next frontier of the field with a great deal of exploration occurring at the level of basic science as well as in clinical trials. We stand on the shoulders of giants in discussing a gap in therapeutic development that is: neuroendocrine modulation.

1.3.1 Autophagy

FMRP associates with a multitude of different mRNA targets including those at the most basal levels of cellular signaling. Abolition of FMRP has been shown to produce alterations in extremely reductive cellular processes including mitochondrial respiration, cellular energy metabolism, and lysosomal machinery (Shen et al., 2019; Weisz et al., 2018; Yan et al., 2018). Indeed, FMRP abolition has been demonstrated to be associated with dysregulated mitochondrial respiration as evidenced by increased internal proton leakage leading to cellular energy state decoupling and increased aerobic mitochondrial respiration (Mithal & Chandel, 2020; Shen et al., 2019; Weisz et al., 2018; Yan et al., 2018). This is well supported by the documented mTOR signaling alterations which are central to the FXS signaling landscape, and due to mTOR's modulatory role in cell

autophagy (Y. C. Kim & Guan, 2015; Zhu et al., 2019). It has predictably been demonstrated among patients with ASD and among transgenic mice which show constitutive mTOR upregulation, that cellular autophagy is dysregulated in an mTOR-dependent fashion (Tang et al., 2014). Similar findings are documented by (Yan et al., 2018) when they demonstrate that activation of autophagy in *FMR1* KO mice restores synaptic and cognitive deficits (Mithal & Chandel, 2020; Shen et al., 2019; Weisz et al., 2018; Yan et al., 2018).

By nature, cellular autophagy represents a chief cellular survival and repair mechanism. Under conditions of energy depletion or low oxygen, cellular survival depends on a metabolic shift distinguished by post-translational modification of AMPK, inhibition of mTOR, and ultimately a decrease in protein synthesis and proliferative processes (J. Kim et al., 2011; Mizushima, 2007). Autophagy prompts the cell to engage in energy conservation, mitochondrial biogenesis, and lysosome-mediated recycling of intracellular macromolecules (F. L. Roberts & Markby, 2021). These processes are very powerful regulators of cell signaling and have thus been hallmark therapeutic targets for adjacent pathologies including neurodegenerative disease, epilepsy, and metabolic disorders (Alam et al., 2021; Corti et al., 2020). Therefore, autophagy presents a particularly conspicuous target for therapeutics in FXS syndrome. Some studies directly targeting autophagy have shown promise in murine models including the use of the antidiabetic compound metformin and lentiviral-mediated mTOR inhibition (Gantois et al., 2017; Takeda et al., 2023; Yan et al., 2018). While clinical trials with metformin are underway, additional and alternative candidate drugs for this application are worthwhile for the continued elucidation of mechanisms and to expand the treatment toolbox of therapeutic compounds for FXS and ASDs more broadly.

A key mediator of autophagic activity is metabolic stress; the environmental signals received by the cell about nutrient and oxygen availability. Metabolic stressors including starvation, physical exertion, and ketogenic diets are potent inducers and regulators of autophagic activity in cells across the body (Alirezaei et al., 2010; Halling & Pilegaard, 2017; Liśkiewicz et al., 2021). All of these autophagic modulators share the common principle of metabolic modulation in regards to their known effects on glucose metabolism, lipid mobilization, and inflammasome biomarker profiles (Lahiri et al., 2019; Singh, 2010). Over the decade, our group has extensively investigated the effects of exercise on neurogenesis (Pinar et al., 2018; Triviño-Paredes et al., 2016) , the effects of fasting on synaptic plasticity in FXS (unpublished), and most recently, the application of these principles to therapeutics in FXS.

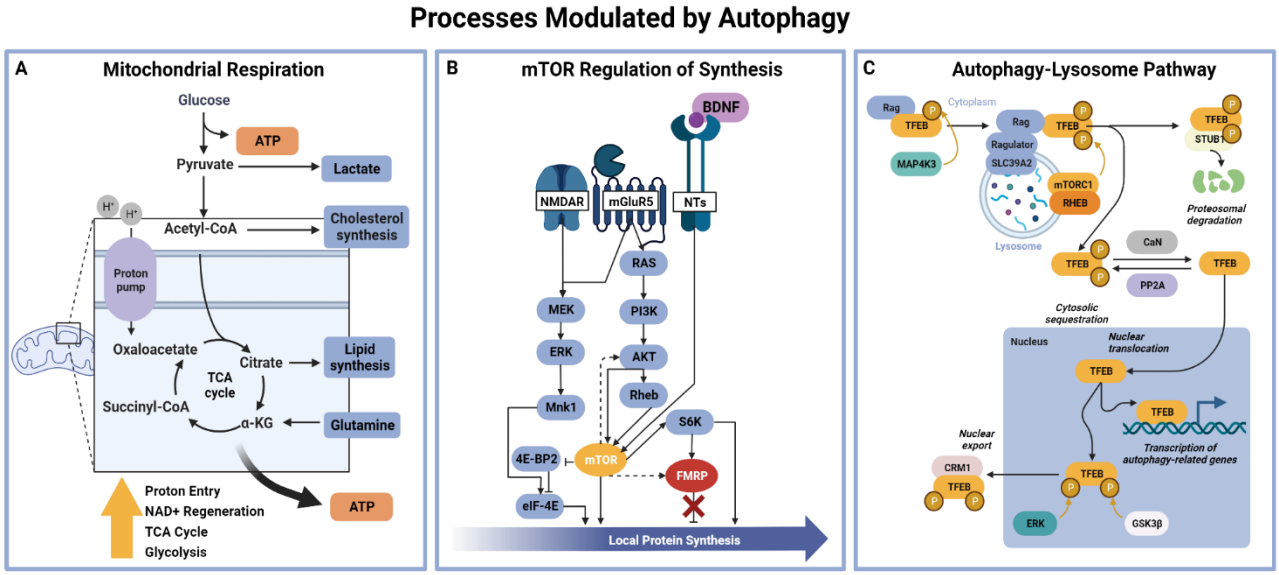


Figure 4. Fasting, exercise, ketosis and other metabolic stressors exert a pro-autophagic effect on cellular processes (A) mitochondrial respiration, (B) mTOR-dependent protein synthesis, and (C) lysosomal degradation and cycling.

1.3.2 The Neuroendocrine Axis

Metabolic stress induced by lifestyle including exercise and therapeutic diets hold meaningful value as treatment approaches however represent a major translational challenge for therapeutics because they are untargeted and may present too much of a barrier to patients to be easily adopted. Thus, we examine the numerous intermediate effectors of autophagy signaling for a localized and druggable therapeutic target. Our previous studies have led us to focus on neuroendocrine modulators of autophagy – that is, intermediate biomolecules which actuate the metabolic stress into cellular autophagy. One targeted mechanism of neuroendocrine-mediated autophagy modulation that has emerged of interest to us is adiponectin. Adiponectin is an adipokine protein lipid hormone released by adipocytes during states of metabolic stress (Becic et al., 2018). Together with other adipokines like leptin, interleukin-6 (IL6), and tissue necrosis factor (TNF), adiponectin plays an important regulatory role in cell survival, cellular glucose metabolism and insulin sensitivity regulation, and inflammation (Roy et al., 2023). After synthesis and excretion from adipocytes, adiponectin binds to cellular adiponectin receptors 1 and 2 (ADIPOR1/2) (Kadowaki & Yamauchi, 2011). Through AMPK-mediated signaling, adiponectin inhibits mTOR activity and induces pro-autophagic signal transduction cascades (Achari & Jain, 2017; Kadowaki & Yamauchi, 2011). Thus, adiponectin represents one targetable mechanism by which metabolic stress acts to induce cellular autophagy and presents an opportunity for pharmacological investigation of adiponectin receptor modulation as a druggable mechanism in FXS. The use of synthetic adiponectin receptor agonist, AdipoRon, shows promising results in murine models of autophagy-associated benefits in diabetes, obesity, and depression (T. H. Lee, Ahadullah, et al., 2021; Okada-Iwabu et al., 2013). Despite its therapeutic potential in FXS, no published record of AdipoRon (or any other adiponectin receptor agonist) exploration in FXS exists. In a proteomic

evaluation of human blood, there is evidence that patients with FXS present with decreased circulating serum adiponectin and increased serum leptin compared to age matched controls (Lisik et al., 2016). This suggests that FMRP may be somehow involved in adiponectin protein synthesis, adiponectin receptor function, or perhaps receptor distribution; and that adiponectin signaling may play a part in cellular autophagy alterations observed in FXS. Previous pilot work demonstrates that among field recordings in slices from *FMR1* KO mice, a twenty-minute incubation with AdipoRon ameliorates deficits in hippocampal LTP. This prompted us to pursue a broad experimental in vivo exploration of AdipoRon in an *FMR1* KO mouse model of FXS.

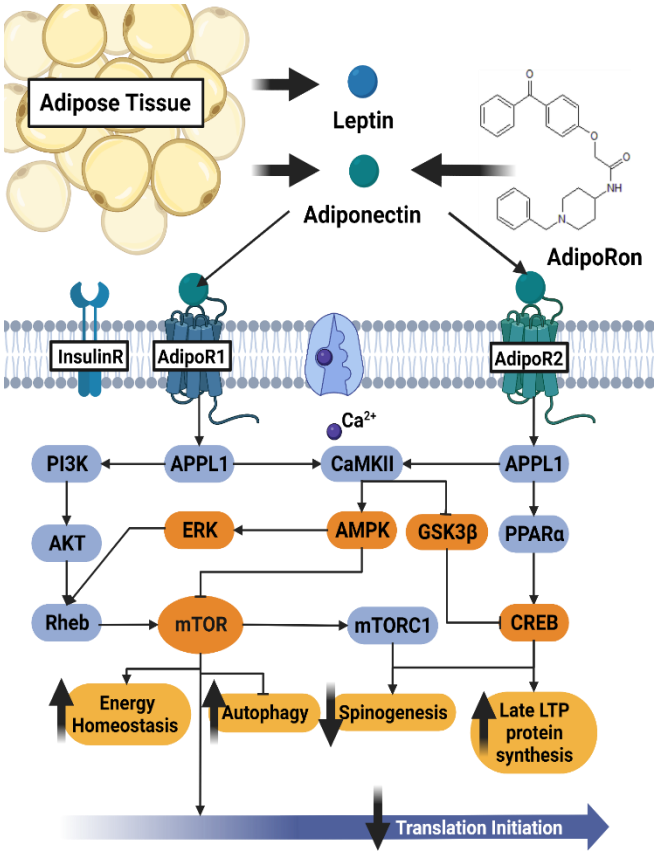


Figure 5. Proposed mechanism of action of AdipoRon. In agreement with known mechanism of endogenous adiponectin, Adiponectin receptor modulation engages molecular mechanism of autophagy which feed forward into generation and support of synaptic plasticity.

In this study, we explore three questions which reflect our three primary research aims.

1. Can exogenous adiponectin modulation through chronic 15-day administration of AdipoRon at 20 mg/Kg restore diminished LTP in the hippocampal DG of adult male *FMR1* KO mice?

2. Does exogenous adiponectin modulation through chronic 15-day administration of AdipoRon at 20 mg/Kg impart changes in plasticity-related proteins through modulatory effects on cellular metabolism and autophagic flux in the hippocampal DG of adult male *FMR1* KO mice?

3. Does exogenous adiponectin modulation through chronic 15-day administration of AdipoRon at 20 mg/Kg restore any documented alterations in behavior, and more specifically anxiety behavior, among adult male *FMR1* KO mice?

CHAPTER 2. METHODS

2.1 Animals, Genotyping, and AdipoRon Treatment

2.1.1 Animal Breeding and Maintenance

FMR1 hemizygous male KO mice were generated in house by crossing female *FMR1* homozygous mice with male WT mice (C57Bl/6J) from established colonies. All mice were housed with constant humidity at 22°C on a 12 hour light-dark cycle. Mice were housed in standard cages containing 3-5 mice from mixed litters –to minimize litter confounds— and had *ad libitum* access to mouse chow and water. All experiments were carried out in adult mice at post-natal day (pnd) 56-84 in accordance with international standards on animal welfare, guidelines set by the Canadian Council on Animal Care, and approved by the Animal Care Committee at the University of Victoria (Appendix 1).

2.1.2 Genotyping

Genotyping was conducted on ear snip tissue using protocols specified in purchased DNA isolation kit (Invitrogen, Ontario, Canada) and polymerase chain reaction (PCR) analysis as described previously (S. Y. Yau et al., 2016). The following cycling parameters were used: denaturation at 94°C for 5 minutes, followed by 35 cycles of 60 seconds at 94°C, 90 seconds at 65°C, and 150 seconds at 72°C. The primers used to probe the KO allele and WT allele of 450bp and 800bp respectively are below.

Allele	Primer Sequence
WT - Forward	5’-GTGGTTAGCTAAAGTGAGGATGAT-3’
WT - Reverse	5’- CAGGTTTGTTGGGATTAACAGATC-3’
KO - Forward	5’-ATCTAGTCAYGCTATGGATATCAGC-3’
KO - Reverse	5’-GTGGGCTCTATGGCTTCTGAGG-3’

2.1.3 AdipoRon Preparation and Administration

Powdered AdipoRon (#15941, Cayman) was reconstituted, at 75°C to a concentration of 2mg/ml in 5% DMSO, 0.5% carboxymethylcellulose (CMC) in sterile saline. Mice received intraperitoneal (IP) injections daily with 20 mg/Kg for 15 days (T. H. Lee, Ahadullah, et al., 2021). Special effort was paid to maintaining consistent injection time –during light cycle– over the treatment duration to ensure consistent peak and trough drug metabolism. Of note, injection sites were alternated each day to allow for recovery and minimize animal stress. Age matched vehicle and cage control mice were measured in parallel in every experiment. Vehicle mice were injected with 10µl/g: 5% DMSO, 0.5% carboxymethylcellulose (CMC) in sterile saline, while cage control mice received no injections prior to experimental assays.

2.2 Behavior

2.2.1 Open Field

To assess for hyperlocomotion and anxiety like behavior, mice underwent an open field (OF) test on day 13 of 15 of treatment. The test was performed during the light cycle approximately 1.5 to 2 hours after injection of AdipoRon or Vehicle (Ng et al., 2021). Mice were placed in a 60 cm diameter round field with a white floor and dark, tall, and opaque walls. Visual recording of the mouse began three seconds after the mouse was detected to be in the field. The researcher left the room, and the mouse was allowed to roam freely about the open field for 10 minutes. Analysis was conducted using EthoVision XT 11.5 software (Noldus, Netherlands). For analysis, the metrics measured were velocity, distance traveled, and proportion of time spent in the center zone (inner region of the circle of half the diameter of the field).

2.2.2 Elevated Plus Maze

The next day after OF, mice were evaluated for anxiety like behavior using the elevated plus maze on day 14 of 15 of treatment. The test was performed during the light cycle approximately 1.5 to 2 hours after injection of AdipoRon or Vehicle (Ng et al., 2021). The maze apparatus consisted of a plus shaped white opaque platform. Two opposing arms of the plus contained high, opaque walls, while the other opposing two arms of the apparatus were open to the room. Mice were placed in the chamber with their head facing the same open arm each trial. Mice were allowed free movement about the maze for 5 minutes. Analysis was conducted using EthoVision XT 11.5 software (Noldus, Netherlands). For analysis, the metrics measured were velocity, distance traveled, and time spent in open and closed arms of the maze.

2.2.3 Three Chamber Sociability

To assess for sociability and social anxiety behavior, mice were subjected to a three chamber sociability test. In house, a sociability chamber box was built at 60cm x 40cm x 22cm with three distinct separations: each measuring 20cm. The experimental animal was placed in the middle segment on the box and allowed to habituate for 5 minutes prior to the beginning of the trial. The sociability test then consisted of two phases. To test for social approach, the experimental mouse was granted access to the remaining two chambers. In one, the mouse was given access to exploration of an empty cage while in the other, the mouse was given access to exploration of a cage mate mouse in a perforated plexiglass cage. For 10 minutes, the mouse was allowed to explore freely in the absence of the researcher. The measured metrics were the amount of time spent in each segment of the chamber to assess whether mice show social preference for the familiar cage mate over an empty space. In the second trial of the sociability test, and to test for social novelty, the mouse was now given access to the same cage mate as before as well as a new –age matched—WT stranger mouse in the previously empty plexiglass container. Again, the mouse was allowed to freely explore for 10 minutes in the absence of the researcher. The measured metrics were the amount of time spent in each augment of the chamber to assess whether mice show social novelty preference for a new mouse over a familiar, and previously explored, cage mate.

2.3 Electrophysiology

2.3.1. Slice Preparation

Mice were deeply anesthetized with isoflurane and decapitated to yield fresh hippocampal electrophysiological sections. The brain was rapidly extracted and cooled to 4°C in oxygenated (95% O2 /5% CO2) artificial cerebrospinal fluid (ACSF) consisting of 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaHPO4, 25 mM NaHCO3, 2 mM CaCl2, 1.3 mM MgCl2, and 10 mM dextrose, pH 7.3. Transverse 300 μm hippocampal sections were cut in ice-cold ACSF with Vibratome 1500 (Ted Pella Inc., Redding, California, USA) as described in previous works from our lab (Bostrom et al., 2013; S. Y. Yau et al., 2018). Slices were placed in recovery solution of continuously oxygenated ACSF at 32°C for 1 hour minimum.

2.3.2. *In Vitro* Field Electrophysiology

An upright light microscope (Olympus BX5OWI) was used to place the concentric bipolar (FHC, Bowdoinham, ME) and borosilicate glass recording electrode (1 MΩ) filled with ACSF and placed about 200 μm from the stimulating electrode in the medial perforant pathway (MPP) of the dentate gyrus. Slices were maintained at a perfusion rate of 2ml/min for the duration of the recording with continuous suction of used ACSF. Field excitatory postsynaptic potentials were (fEPSPs) recorded using an Axon MultiClamp 700B amplifier on a PC running Clampex 10.2 software (Molecular Devices, CA, USA). fEPSPs were produced through delivery of a 120 μs 0.067 Hz pulse every 15 seconds to the medial perforant path (MPP) of the hippocampal dentate gyrus (DG) via stimulating electrode. Electrodes were determined through attainment of a minimum fEPSP of amplitude 0.7

mV. A stable baseline was recorded for at least 15 minutes using a magnitude which produced a response of 50% of the maximum amplitude obtained for synaptic potentiation.

A paired pulse (PP) ratio and input-output (I/O) were applied to the slice upon baseline to observe changes in synaptic transmission across experimental conditions. For PP, the mean slope of the second pulse was divided by the mean slope of the first pulse. I/O represents ten pulses of incrementally increasing pulse width starting at 0.3 μs up to 300 μs. For LTP induction –and to mitigate tonic inhibition– in the hippocampal DG, slices were continuously washed with 5 μM GABAA antagonist bicuculline methionine (Sigma-Aldrich, Ontario, Canada) for 20 minutes. LTP was induced by applying a high frequency stimulus (HFS) consisting of a tetanizing stimulus of 4 trains of 50 pulses at 100 Hz, 30 seconds apart. Post-conditioning, slices were returned back to the 0.12 ms at 0.067 Hz and onto regular ACSF and slice response was recorded for 60 minutes. Slices were deemed fit for analysis if the amount of potentiation at 60 minutes after HFS exceeded 0%. For analysis, the slope of the fEPSP was measured in pClamp 11 software (Molecular Devices, San Jose, California, USA) and binned to 1 minute intervals. Post-conditioning recordings were reported and plotted as a percent change from the average preconditioning slope (%LTP).

2.4 Protein Preparation and Western Blotting

2.4.1 Preparation of Synaptosomal (SNP) and Whole Tissue Homogenate (WH)

Following sacrifice, the DG was isolated from the hippocampus on ice and frozen in liquid nitrogen. The frozen samples were stored at -80˚C until further use (one hemisphere from each brain was designated for SNP fractions, while the other hemisphere was allocated for WH

analysis). The tissue was thawed on ice and homogenized using a Potter-Elvehjem homogenizer in chilled modified Krebs-Henseleit buffer (mKREBS) supplemented with a protease inhibitor cocktail (#1860932, Thermoscientific, Waltham, Massachusetts, USA). The mKREBS buffer contained: in mM: 118.5 NaCl, 4.70 KCl, 1.18 MgCl2·6H2O, 2.50 CaCl2·2H2O, 1.18 KH2PO4, 24.90 NaHCO3, 10.00 glucose, pH adjusted to ~7.40 using 1.0 N HCl. The homogenate was passed through sequential filtrations using 100 μm pore nylon filters (NY1H02500; EMD Millipore) and 5 μm nitrocellulose Durapore membrane filters (SBLP01300; Millipore). The filtrate was then centrifuged at 1000 × g for 15 minutes at 4°C. Following centrifugation, the resulting pellet was resuspended in the mKREBS buffer containing protease and phosphatase inhibitors for subsequent Western blot analysis. The total protein content in each sample was quantified using the DC Protein Assay (Biorad). For both WH and SNP samples, 10 μg of protein was subjected to electrophoretic separation using 10% TGX Stain-Free™ FastCast™ Acrylamide Solutions (BioRad, Hercules, California, USA) at 200 V for 60 minutes. Following the electrophoresis, the proteins were transferred onto 0.2 μm PVDF membranes (#1704272, BioRad, Hercules, California, USA) using the Trans-Blot Turbo Transfer System (BioRad, Hercules, California, USA) through a semi-dry method.

2.4.2 Western Blotting and Resolution

Membranes were blocked with 5% (w/v) BSA for 1 hour at room temperature. Membranes were incubated in primary antibodies overnight at 4°C at a 1:1000 concentration in 5% (w/v) BSA. Proteins measured in the hippocampus were mTOR (#2972S, CST), p-mTOR (#2971S, CST), CREB (#4820S, CST), p-CREB (#9198S, CST), GluA1 (#13185S, CST), p-GluA1 (#75574S, CST), p-Erk1/2 (#9101S, CST), and GluN2b (UC Davis). After primary incubation, blots were washed in tris-buffered saline with 1% [v/v] tween (TBST), and subsequently incubated for 1 hour

at room temperature with horseradish peroxidase-linked goat anti-mouse or goat anti-rabbit secondary at 1:5000 in blocking buffer. After sequential washes, membranes were resolved with Luminata Crescendo or Classico (#WBLUR0500 and #WBLUC0500), Millipore Sigma, Burlington, Massachusetts, USA) chemiluminescent detection. Technical duplicates or triplicates were analyzed for every protein where possible from each animal. Beta actin was used as a house-keeping standard for all proteins probed (#4967S, NEB). The SynGene imaging system was used to capture all images, and densitometry measurements using FIJI was employed for quantification of Western blot bands.

2.5 Statistical Analysis

2.5.1. Analysis of Behavior

Statistics were conducted on PRISM Graph Pad 6 and 9. Two-way ANOVAs were used to assess differences between conditions (WT and *FMR1* KO) as well as treatment groups (Vehicle or AdipoRon) across experiments and trials. Tukey's *post-hoc* tests were used for multiple comparisons between conditions and treatments where ANOVA showed significance. All data are expressed using mean ± standard error of mean (SEM) and significance is defined as $p<0.05$.

2.5.2 Analysis of Electrophysiology

Statistics were conducted on PRISM Graph Pad 6 and 9. Mean fEPSP traces and %LTP were calculated from slice replicates and two tailed student's t-test or Two-way ANOVA were used to make single or multiple comparisons respectively. Tukey's *post-hoc* tests were used for multiple comparisons between conditions and treatments where ANOVA showed significance. Any outliers beyond -2.5 or +2.5 standard deviations away from the mean were excluded from analysis. All

data are expressed using mean ± standard error of mean (SEM) and significance is defined as $p<0.05$.

2.5.3 Analysis of Western Blotting

Statistics were conducted on PRISM Graph Pad 6 and 9. For Western blotting, housekeeping-normalized replicates were pooled to derive the mean relative abundance which was then compared using the two tailed student's t-test to make comparisons between treatment conditions but within the same genotype. All data are expressed using mean ± standard error of mean (SEM) and significance is defined as $p<0.05$.

CHAPTER 3. RESULTS

3.1 Genotype and Treatment Condition Interact to Produce Unique Behavioral Phenotype of Anxiety-Like Behavior

3.1.1 Open Field

In analysis of time spent in the center of the OF (Figure 6. A1), no significant difference in time spent in center was observed between Veh and Adip treatment groups (n=10 mice per group, F (1, 36) = 0.1298, P=0.7208; two-way ANOVA) across WT and *FMR1* KO conditions. A genotype condition effect was observed between WT and *FMR1* KO mice where *FMR1* KO mice spend less time in the center of the open field (n=10 mice per group, F (1, 36) = 5.930, P=0.020, two-way ANOVA). There is a small but not significant increase in time spent in the center in the Adip treatment group relative to the Veh treatment group. No significant interaction is observed between treatment and genotype condition F (1, 36) = 01.784, p=0.190; two-way ANOVA). Interaction plot (Fig 6. A2) indicated non-parallelity of lines that intersect, indicating trend towards interaction between independent variables but not found to be statistically significant. When analyzing the same group of mice for distance traveled, no significant treatment effect between Veh and Adip groups, genotype condition effect between WT and *FMR1* KO mice, or independent variable interaction across conditions (n=10 mice per group, condition: F (1, 36)=2.065, p=0.1594; treatment: F (1, 36) = 2.632, p=0.1135; interaction: F (1, 36) = 0.2983, p=0.588; two-way ANOVA) (Fig 5, B1). Interaction plot (Fig 6. B2) depicts two downwards sloping nonparallel lines indicating trend towards both drug and treatment effects but not towards independent treatment/condition interaction. Heat maps (Fig 6. C) indicate position in the open field as a

function of time where color transitions from cool to warm as a function of time spent still in a singular place.

3.1.2 Elevated Plus Maze

Analysis of time spent in the closed arm of the EPM indicates no significant treatment effect across mice in the Veh and Adip groups (n=10 mice in each group, F (1, 36) = 1.722, p=0.1977; two-way ANOVA) (Figure 7. A1). There is however, a significant genotype condition main effect observed in time spent in the closed arm of the EPM between WT and *FMR1* KO mice (n=10 mice in each group, F (1, 36) = 13.340, p=0.008; two-way ANOVA). Likewise, a significant decrease is observed between WT Adip group mice and *FMR1* KO groups (*post-hoc*: Tukey's multiple comparison, p=0.010). Interaction plot of time spent in the closed arm of EPM. Lines display non-parallelity and do not intersect, indicating no main effect interactions and an emerging genotype condition main effect which ANOVA confirmed to be statistically significant (Fig 7. A2). Heat maps (Fig 7. A3) indicate position in the open field as a function of time where color transitions from cool to warm as a function of time spent still in a singular place.

3.1.3 Three Chamber Sociability

In analysis of time spent with a stranger in round one of the three chamber sociability test (Figure 8, A1) no significant treatment effect was observed among mice within the WT and *FMR1* KO conditions (n=8 mice in each group, F (1, 28) = 2.836, p=0.1033; two-way ANOVA). A genotype condition main effect was observed between WT and *FMR1* KO groups, where *FMR1* KO mice were found to spend more time with the stranger mouse than WT mice (n=8 mice per group, F (1, 28) = 12.07, p=0.001; two-way ANOVA). Likewise, pairwise comparison reveals that *FMR1* Adip group mice spent significantly more time with the stranger mouse than WT Adip group mice (*post-*

hoc: Tukey's multiple comparisons; p=0.0213). No significant interaction effect was observed between main effects as F (1, 28) = 0.8364, p=0.368; two way ANOVA) as supported by linear interaction of plot (Fig 8. A2) which depicts two non-parallel, non-intersecting lines. Follow up testing in round two of the three chamber sociability test (Fig 8. B1) indicates no main effects of treatment or genotype conditions (n=8 mice in each group; genotype condition: F (1, 28) = 0.1110, p=0.7415; treatment: F (1, 28) = 0.1632, p=0.6893; two-way ANOVA). However, a significant interaction effect was found to exist between treatment and genotype condition F (1, 20) = 7.505, p=0.0106; two-way ANOVA) and is confirmed by an intersection interaction plot (Fig 8. B2).

An examination of novel preference (Fig 8. C1) applied the following function to the previously described data: [(time spent with novel stranger)/(time spent with novel stranger + time spent with familiar stranger)] to generate a proportion measurement of preference of novel stranger over familiar stranger. Novel preference is found be subject to an interaction between genotype condition and treatment F (1, 28) = 9.432, p=0.0004, two-way ANOVA) as well as pairwise comparison effects between WT Veh and KO Veh groups (*post-hoc*: Tukey's multiple comparison; p=0.0061) as well as WT Veh ad WT Adip groups (*post-hoc*: Tukey's multiple comparison; p=0.0395). The interaction plot (Fig 8. C2) demonstrates two intersecting linear plots shown to have significance with ANOVA. Heat maps (Fig 8. A3, B3) indicate position in the three chamber box as a function of time across both trials where color transitions from cool to warm as a function of time spent still in a singular place.

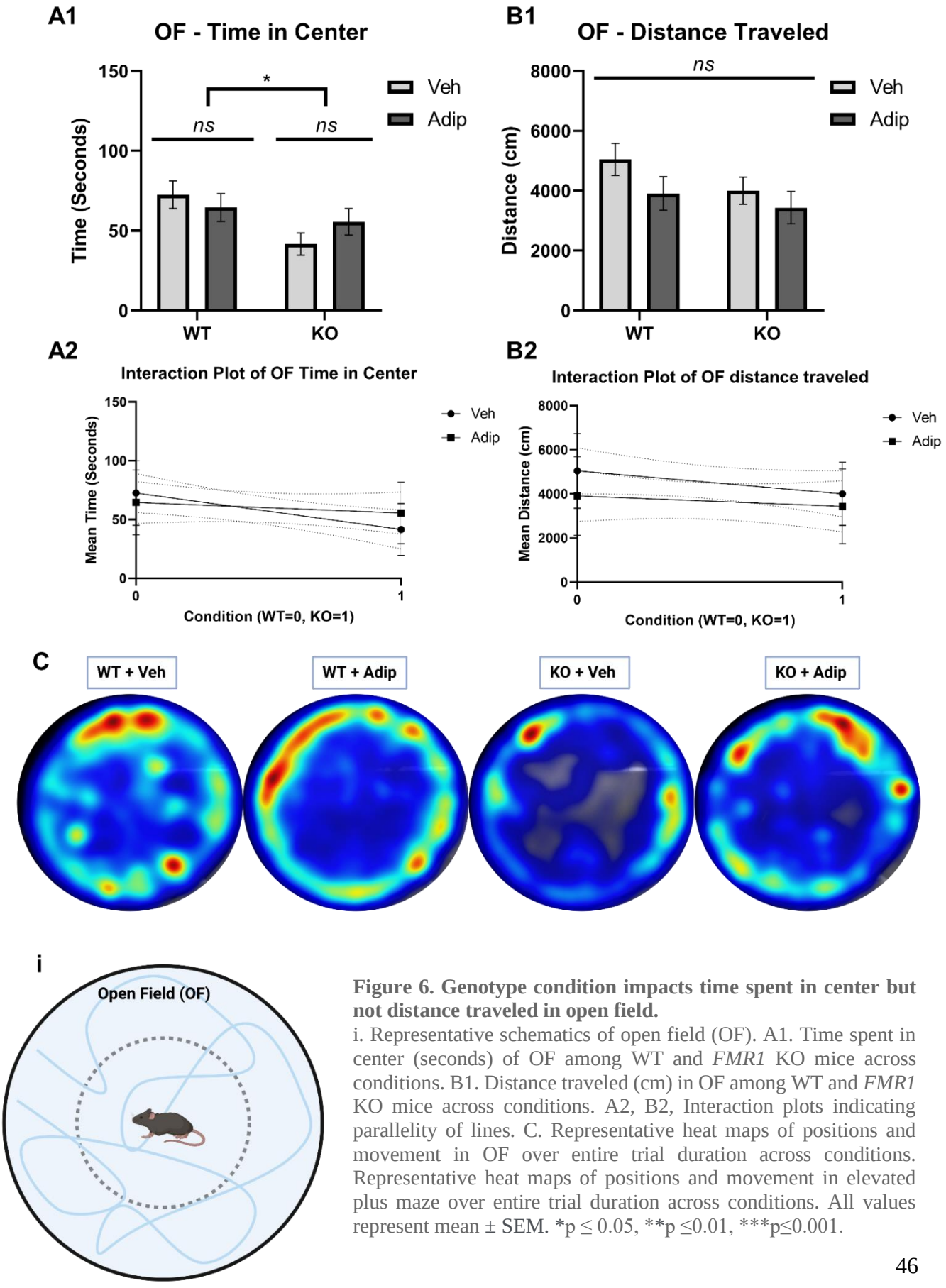


Figure 6. Genotype condition impacts time spent in center but not distance traveled in open field.

i. Representative schematics of open field (OF). A1. Time spent in center (seconds) of OF among WT and *FMR1* KO mice across conditions. B1. Distance traveled (cm) in OF among WT and *FMR1* KO mice across conditions. A2, B2, Interaction plots indicating parallelity of lines. C. Representative heat maps of positions and movement in OF over entire trial duration across conditions. Representative heat maps of positions and movement in elevated plus maze over entire trial duration across conditions. All values represent mean ± SEM. *p ≤ 0.05, **p ≤0.01, ***p≤0.001.

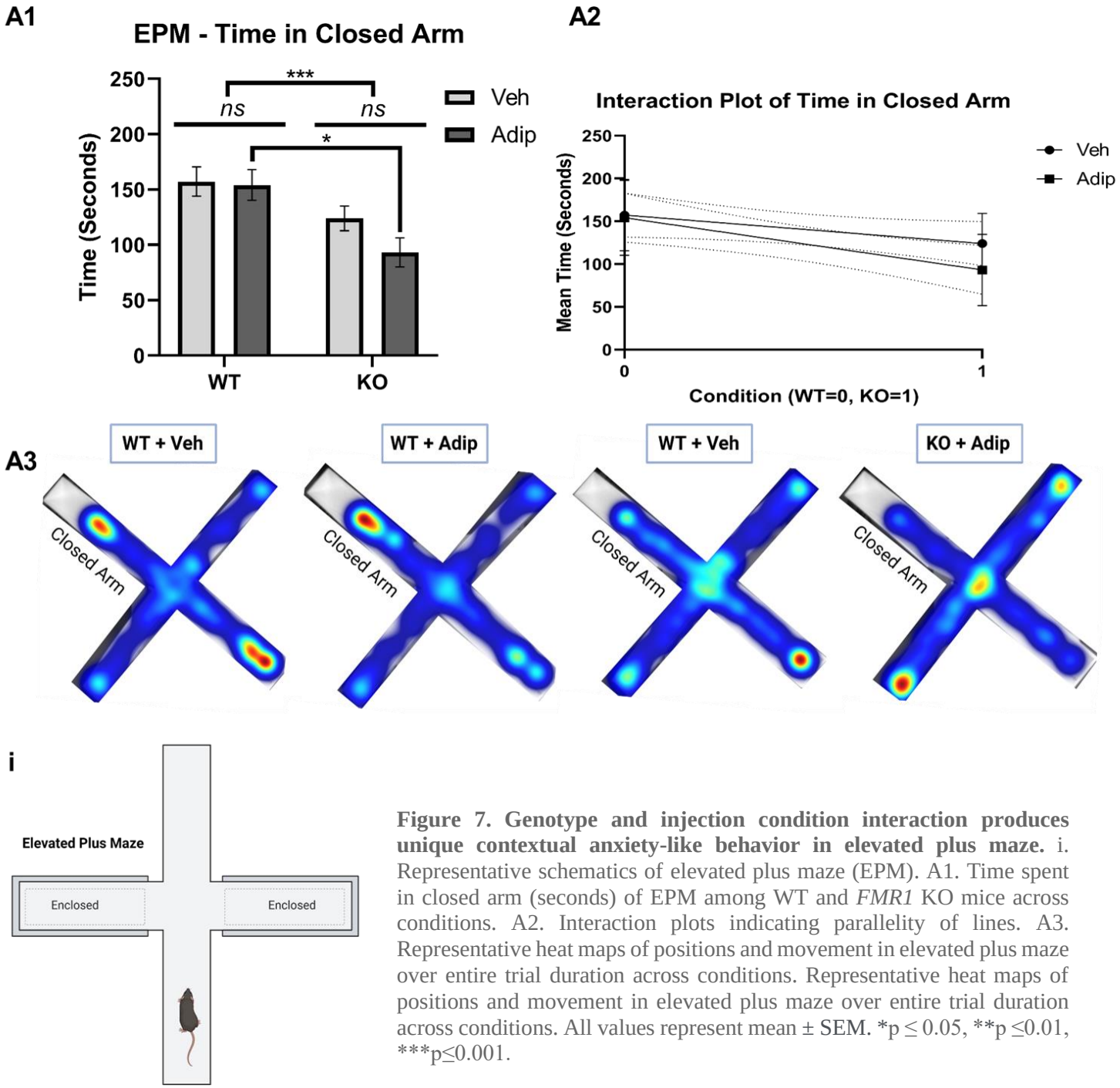
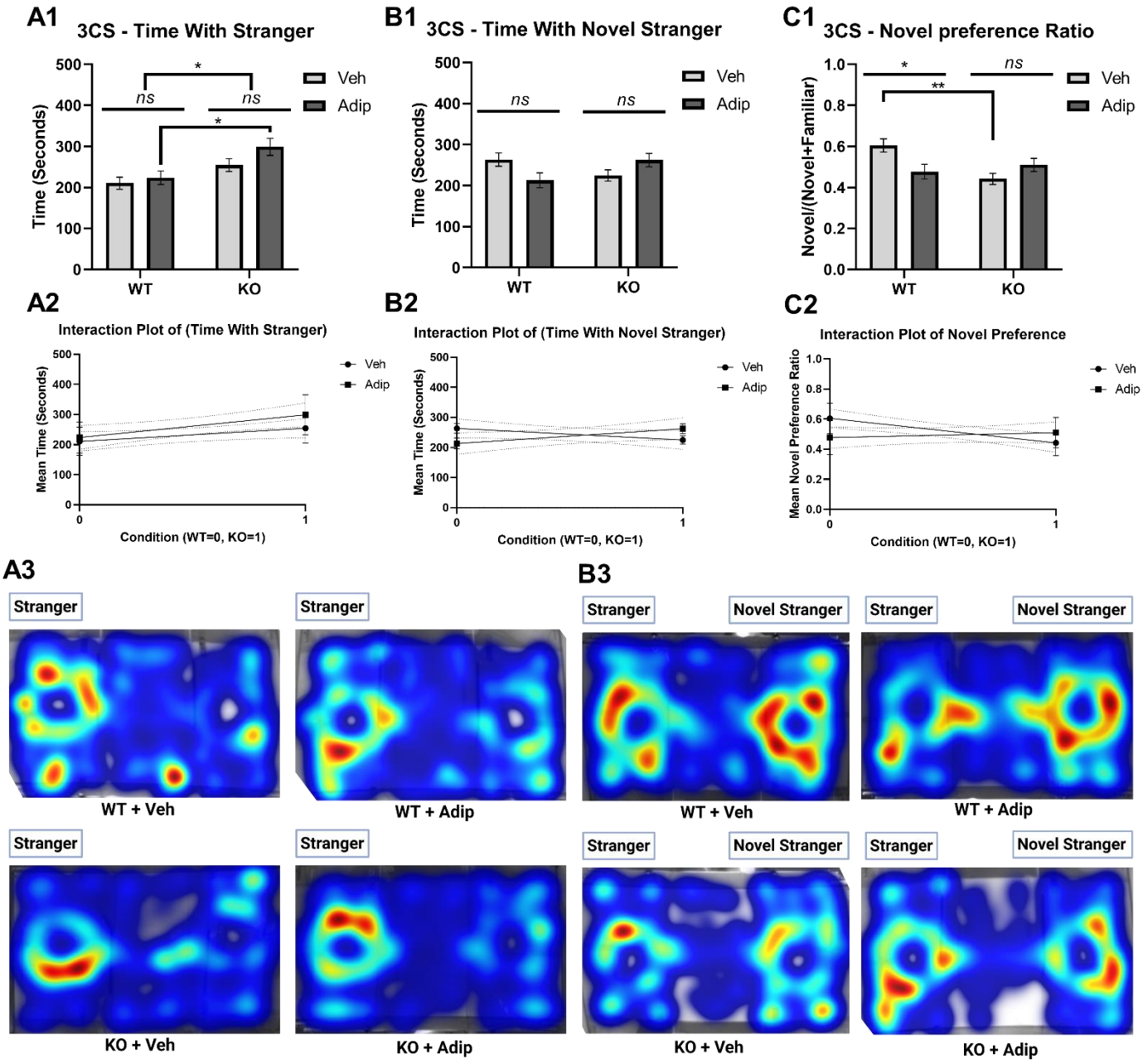


Figure 7. Genotype and injection condition interaction produces unique contextual anxiety-like behavior in elevated plus maze. i. Representative schematics of elevated plus maze (EPM). A1. Time spent in closed arm (seconds) of EPM among WT and *FMR1* KO mice across conditions. A2. Interaction plots indicating parallelity of lines. A3. Representative heat maps of positions and movement in elevated plus maze over entire trial duration across conditions. Representative heat maps of positions and movement in elevated plus maze over entire trial duration across conditions. All values represent mean ± SEM. *p ≤ 0.05, **p ≤0.01, ***p≤0.001.



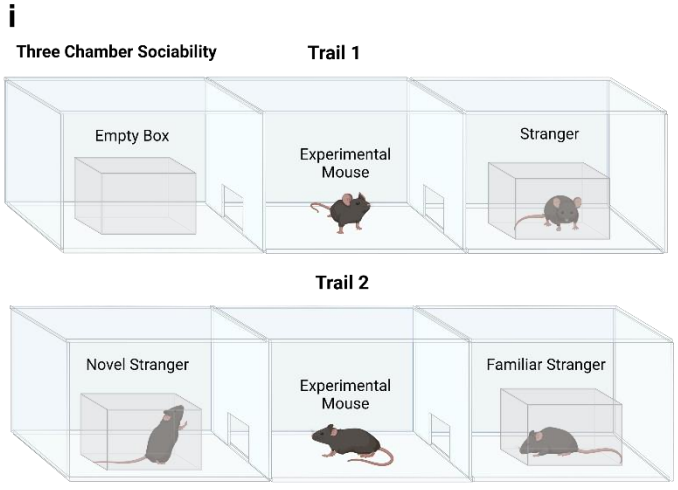


Figure 8. Genotype and injection condition interact: Unique social anxiety-like behavior in three chamber sociability test. i. Representative schematics of three chamber box and trail conditions. A1. Time spent (seconds) with stranger among WT and *FMR1* KO mice across conditions. B1. Time spent (seconds) with novel stranger cross conditions. A2. B2. C2. Interaction plots indicating parallelity of lines. A3, B3. Representative heat maps of positions and movement in three chamber box over entire trial duration across conditions. All values represent mean ± SEM. *p ≤ 0.05, **p ≤0.01, ***p≤0.001.

3.2 AdipoRon Does Not Restore LTP in the Hippocampal DG of Adult Male *FMR1* KO Mice

Slices from WT and *FMR1* KO mice across all treatment conditions (CC, Veh, Adip) were used for *in vitro* electrophysiology in the MPP of the hippocampal dentate gyrus. The analysis observes the comparison between Veh and Adip groups to reflect the primary question in aim 1 which seeks to evaluate whether the active injection ingredient, AdipoRon, is able to significantly alter –and more specifically increase– long term potentiation among KO mice when compared to vehicle injection (Figure 9. A, B, C). A significant basal state difference was observed between mean % LTP of WT CC mice and KO CC mice (n=10 and 11 slices for WT and KO respectively, p=0.0295 student's two tailed t-test) (Figure 9. A1, A2). In WT mice across Veh and Adip treatment groups, not statistically significant between the means of both groups (Figure 9. B1, B2) (n=7 and 11 slices for WT and KO respectively, p=0.4025; unpaired student's two tailed t-test) (Figure 9. B1, B2). In In *FMR1* KO mice across Veh and Adip treatment groups, not statistically significant between the means of both groups (Figure 9. C1, C2) (n=7 and 9 slices for WT and KO respectively, p=0.3032; unpaired student's two tailed t-test) (Figure 9. C1, C2). Comparison between all treatment groups across conditions indicates a statistically significant genotype effect between WT and *FMR1* KO mice (Genotype effect: F(1,50) = 0.2712 p=0.020, Two-way ANOVA). Comparison between CC WT and CC *FMR1* KO approaches significance (*post-hoc*: Tukey's multiple comparison, p=0.051) (Figure 10).

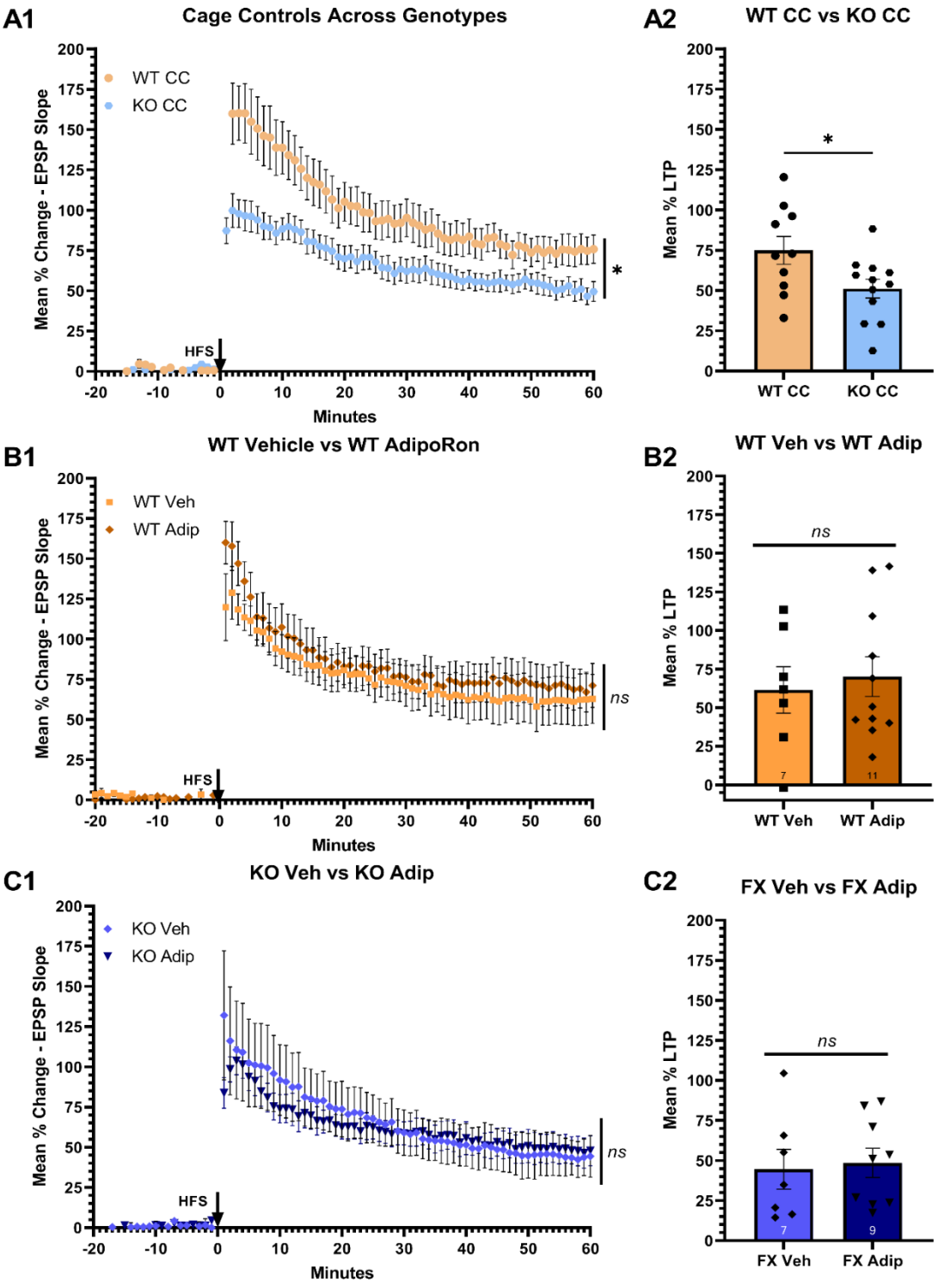


Figure 9. AdipoRon does not restore hippocampal LTP. A1. Mean % change in basal fEPSP slope in WT and *FMR1* KO cage control mice. A1. Mean % basal LTP in WT and *FMR1* KO cage control mice. B1. Mean % change in fEPSP slope in WT mice across Veh and Adip treatment groups. B1. Mean % LTP in WT mice across Veh and Adip treatment groups. C1. Mean % change in fEPSP slope in *FMR1* KO mice across Veh and Adip treatment groups. C2. Mean % LTP in *FMR1* KO mice across Veh and Adip treatment groups. In A1, B1, C1, data binned to reflect 1-minute intervals across 60 minutes. All values represent mean ± SEM. *p ≤ 0.05, **p ≤0.01, ***p≤0.001.

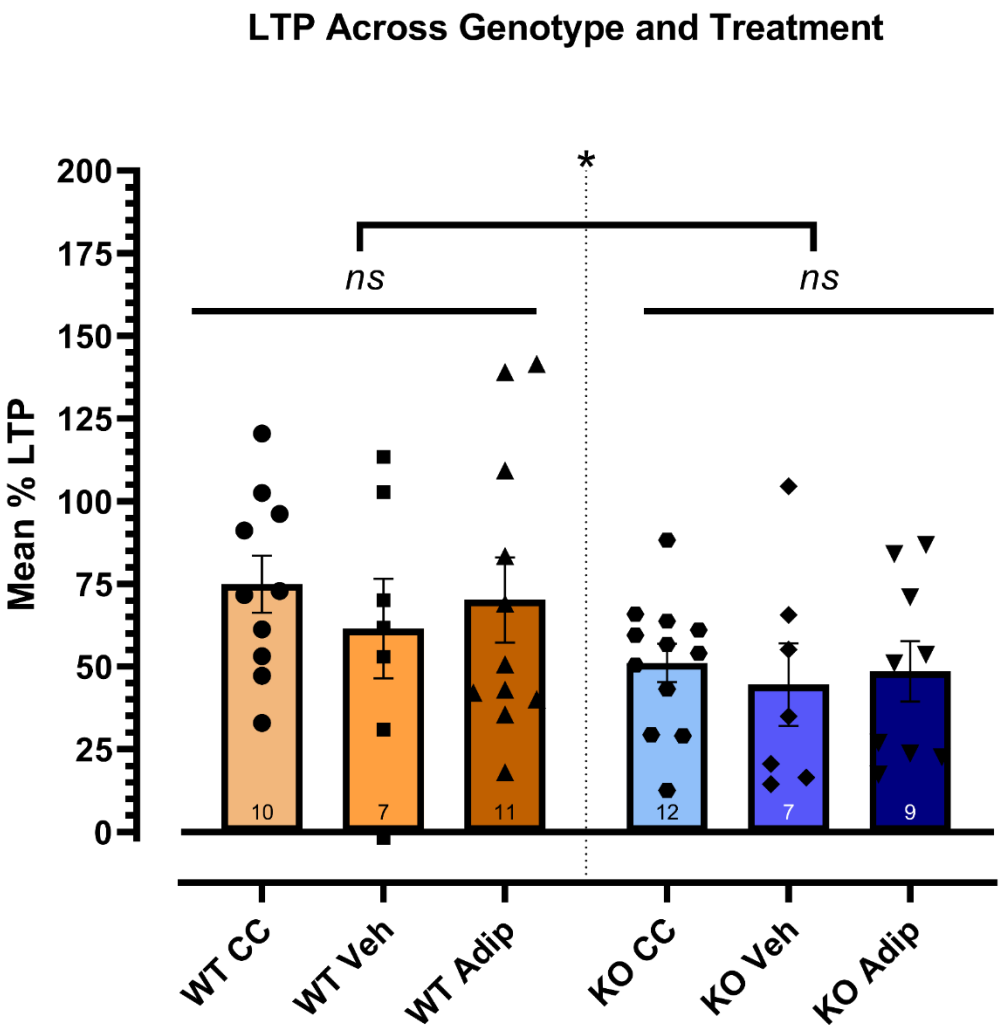


Figure 10. Strain effect observed between WT and *FMR1* KO mice. No significant difference observed between CC, Veh, and Adip treatment groups within the same strain but strain main effect exists. All values represent mean ± SEM. *p ≤ 0.05, **p ≤0.01, ***p≤0.001.

3.3 AdipoRon Does Not Significantly Change Autophagic and Plasticity-Related Proteins in the Hippocampal DG of Adult Male *FMR1* KO Mice

Fresh DG brain tissue from mice across conditions and treatment groups was used to probe whole tissue and synaptosomal fractions for biomarker proteins in FXS, cell autophagy, and synaptic plasticity.

3.3.1 Synaptosomes

No significant effect of AdipoRon is observed on relative abundance in the majority synaptic fraction proteins. No difference was found in SYN 1 across conditions and Veh/Adip treatment groups in synaptosomal DG fractions (WT: n=5, p=0.6355; unpaired student's two tailed t-test) (KO: n=5, p=0.7303 unpaired student's two tailed t-test) (Figure 12. A). While insignificant, SYN1 abundance appears to trend upwards among Adip mice relative to Veh mice in both WT and KO conditions. PSD95 abundance is marginally decreased by AdipoRon relative to Vehicle in WT mice but inversely increased slightly among AdipoRon treated KO animals. Despite these minor trends, no statistically significant difference was found across all treatment groups and conditions (WT: n=4, p=0.8155; unpaired student's two tailed t-test) (KO: n=5, p=0.6922 unpaired student's two tailed t-test) (Figure 12. B). Synaptosomal abundance of NMDA receptor subunit, GluN2B, is not significantly affected by AdipoRon treatment as observed among Veh and Adip mice in both WT and KO conditions (WT: n=4, p=0.3148; unpaired student's two tailed t-test) (KO: n=4, p=0.4820 unpaired student's two tailed t-test) (Figure 12. B). GluN2B abundance is slightly increased by AdipoRon relative to Vehicle in WT mice and inversely decreased slightly in AdipoRon treated KO mice (Figure 12. C). Total AMPA receptor subunit GluA1 protein and its

phosphorylated state (pGluA1) is found to be slightly diminished among KO mice in the Adip treatment group relative to the KO Veh treatment group (n=4, p=0.4597; unpaired student's two tailed t-test). The same was not observed in the WT conditions across treatments, which remained nearly unchanged (n=3, p=0.9595 unpaired student's two tailed t-test) (Fig 12. D1, D2). Despite observed trends, no statistically significant differences are deduced in relative basal GluA1 and pGluA1 abundance. Likewise, the phosphorylated proportion of total protein, denoted as a ratio of pGluA1/GluA1, is not found to be significantly affected by AdipoRon treatment across conditions (WT: n=3, p=0.9740; unpaired student's two tailed t-test) (KO: n=3, p=0.8132 unpaired student's two tailed t-test) (Fig 12. D3).

Both total and phosphorylated mTOR protein abundance exhibits inverse response to AdipoRon treatment. In WT animals, mTOR and pmTOR abundance is diminished among the Adip treatment group relative to the Veh group (pmTOR: n=2, p=0.1587; unpaired student's two tailed t-test) (mTOR: n=2, p=0.2687 unpaired student's two tailed t-test) (Fig 12. E1, E2). Conversely, among KO animals, the inverse is observed whereby relative mTOR and pmTOR abundance is increased in the Adip group in the KO condition is increased relative to the Veh group (pmTOR: n=2, p=0.5707 unpaired student's two tailed t-test) (mTOR: n=2, p=0.0970 unpaired student's two tailed t-test). The phosphorylated proportion of total mTOR is diminished in the Adip group relative to the Veh group in both WT (p=0.9101; unpaired student's two tailed t-test) and KO (p=0.8857; unpaired student's two tailed t-test) conditions (Fig 12. E3). The relative abundance of pAMPK protein remains nearly unchanged between the Adip and Veh groups across both WT (pAMPK: n=3, p=0.9520; unpaired student's two tailed t-test) and KO conditions (pAMPK:n= 3,p=0.9497; unpaired student's two tailed t-test) (Fig 12. F1). Total AMPK abundance, however,

is diminished in the Adip group relative to the Veh group in both the WT (AMPK: n=3, p=0.1970; unpaired student's two tailed t-test) and KO groups – with KO group showing significant effect (AMPK: n=3, p=0.0247; unpaired student's two tailed t-test) (Fig 12. F2). Likewise, the phosphorylated proportion of AMPK, pAMPK/AMPK, indicates increased phosphorylation in the Adip group in both the WT (p=0.0931; unpaired student's two tailed t-test) and KO conditions relative to the Veh group (p=0.0680; unpaired student's two tailed t-test) (Fig 12. F3) – neither of which were significant.

The Adip groups of relative pGSK3**β** abundance are increased relative to the Veh groups in both the WT (n=2, p=0.3927; unpaired student's two tailed t-test) and KO conditions (n=2, p=0.6353; unpaired student's two tailed t-test) (Fig 12. G1) but not to a statistically significant extent. The relative abundance of total GSK3**β** is nearly unchanged in the Adip group of the WT condition (n=2, p=0.9280; unpaired student's two tailed t-test) but is increased, to a non-significant extent, in the KO condition (n=2, p=0.7600; unpaired student's two tailed t-test) (Fig 12. G2). The phosphorylated proportion of total pGSK3**β**, pGSK3**β**/GSK3**β**, is increased in the Adip group relative to the Veh group in both WT (p=0.4259; unpaired student's two tailed t-test) and KO conditions (p=0.7068; unpaired student's two tailed t-test) (Fig 12. G3).

Relative abundance of pCREB is found to be increased among KO mice in the Adip treatment group relative to the KO Veh treatment group but not significantly so (n=4, p=0.6798; unpaired student's two tailed t-test) (Fig 12. H1). Inversely, the relative abundance of total CREB protein is slightly increased in the Adip group of the WT conditions relative to the Veh group (n=4, p=0.5495; unpaired student's two tailed t-test) while slightly decreased in the Adip group of the

KO condition relative to the Veh group (n=4, p=0.7086; unpaired student's two tailed t-test) (Fig 12. H2). The phosphorylated proportion of total pCREB, pCREB/CREB, is increased in the Adip group relative to the Veh group in both WT (p=0.5764; unpaired student's two tailed t-test) and KO conditions (p=0.3908; unpaired student's two tailed t-test) (Fig 12. H3).

The relative abundance of pERK is marginally decreased in the Adip group of both WT (n=4, p=0.7155; unpaired student's two tailed t-test) and KO conditions (n=4, p=0.6577; unpaired student's two tailed t-test) relative to the Veh group (Fig 12. I1). Conversely, the relative abundance of ERK is marginally increased in the Adip group of both WT (n=4, p=0.6792; unpaired student's two tailed t-test) and KO conditions (n=4, p=0.5977; unpaired student's two tailed t-test) relative to the Veh group (Fig 12. I2). The abundance of total ERK that is phosphorylated, pERK/ERK, is slightly diminished in the Adip group relative to the Veh group in both the WT (p=0.8800; unpaired student's two tailed t-test) and KO conditions (p=0.6199; unpaired student's two tailed t-test) (Fig 12. I3). No statistical significance is inferred among the above discussed observations. Among all relative protein abundances probed across synaptosomal fractions, only total AMPK showed a statistically significant difference between vehicle and AdipoRon treated KO animals.

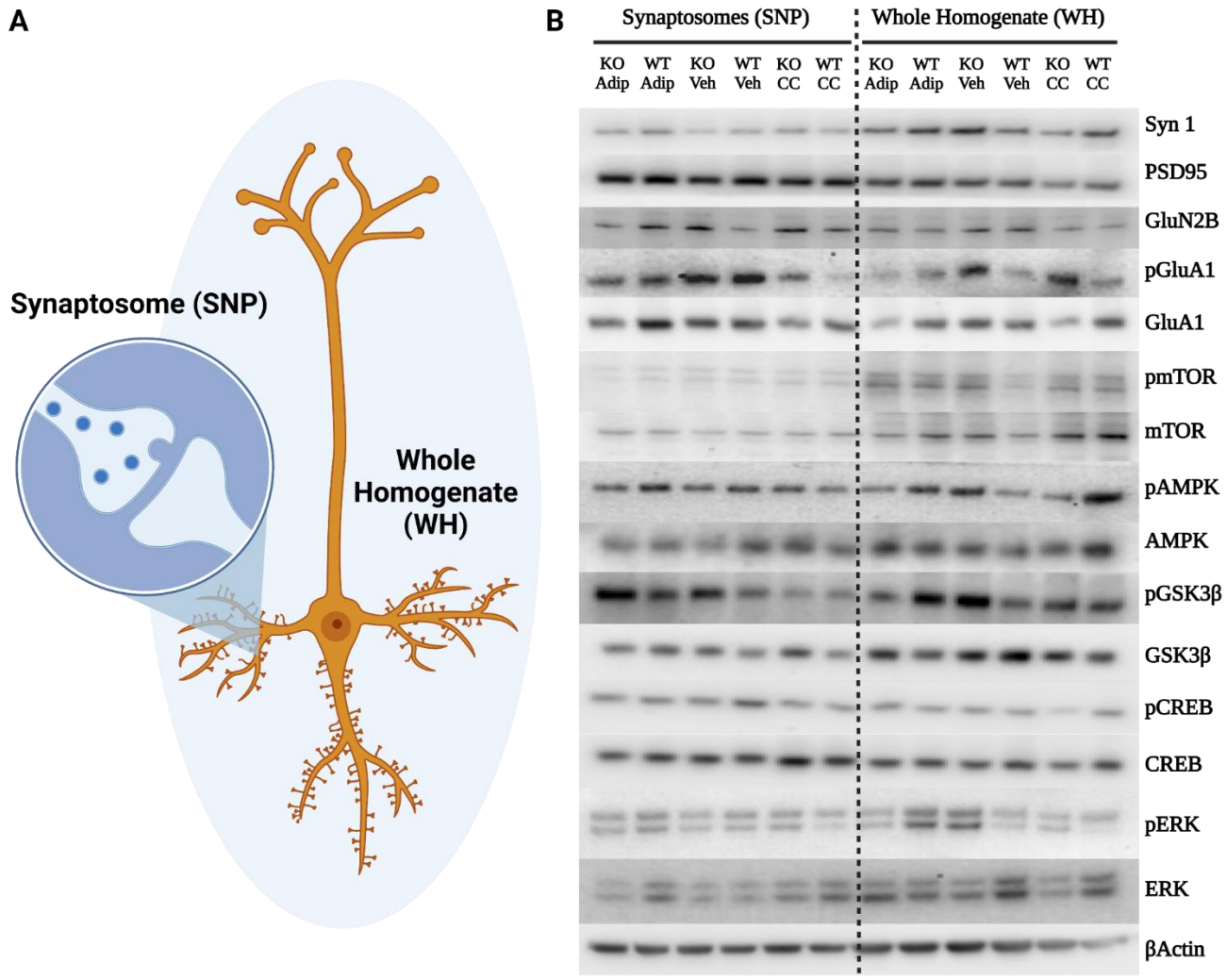
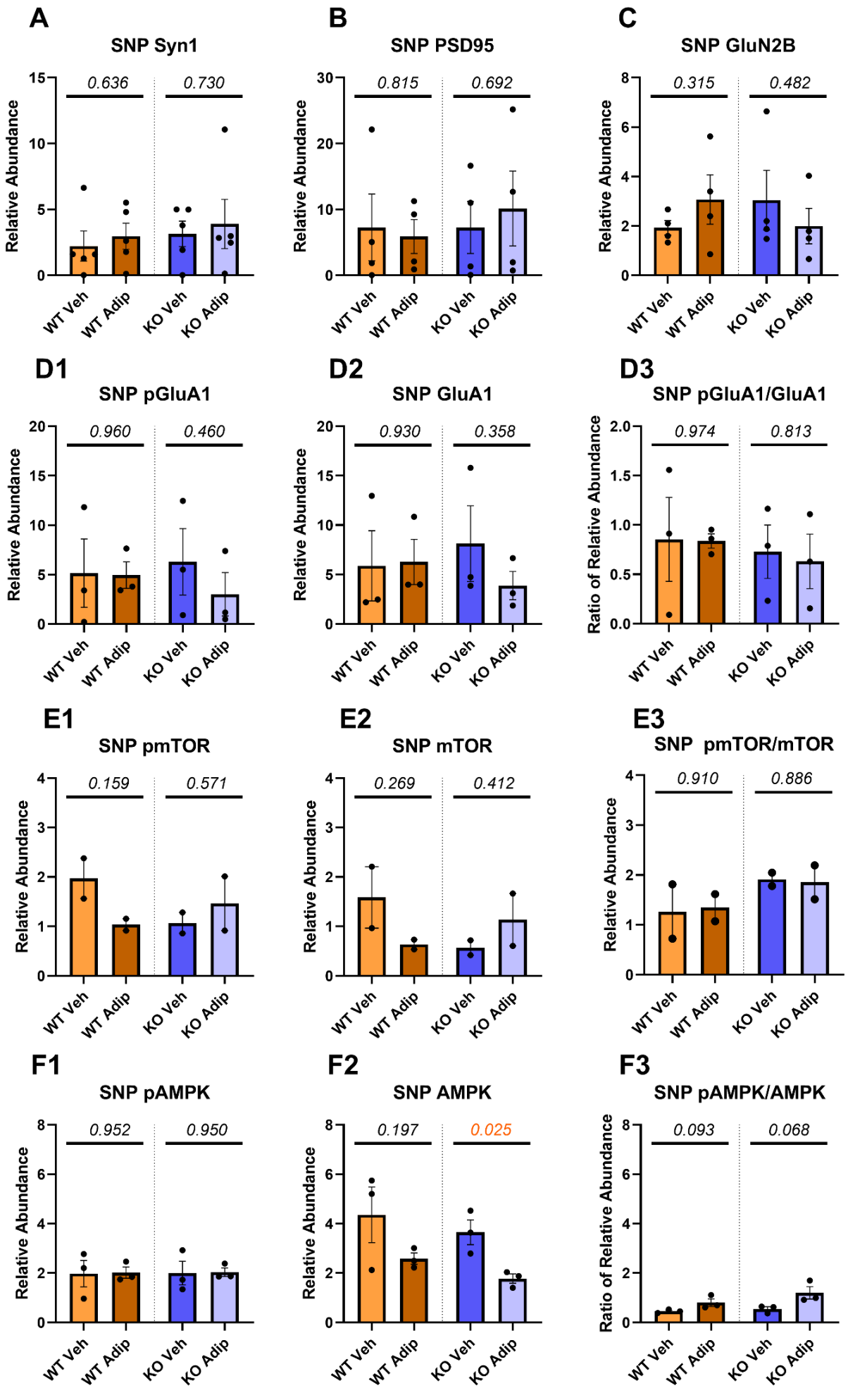


Figure 11. A. Schematic representation of synaptosomal and whole tissue fractions. B. Representative figure of proteins probed by Western blotting and their phosphorylated states. Proteins assayed include, SYN 1, PSD95, GluN2B, pGluA1, GluA1, pMTOR, mTOR, pAMPK, AMPK, pGSK3β, GSK3β, pCREB, CREB, pERK, ERK, and housekeeping protein βActin.


A
SNP Syn1
Relative Abundance
0.636
0.730
B
SNP PSD95
Relative Abundance
0.815
0.692
C
SNP GluN2B
Relative Abundance
0.315
0.482
D1
SNP pGluA1
Relative Abundance
0.960
0.460
D2
SNP GluA1
Relative Abundance
0.930
0.358
D3
SNP pGluA1/GluA1
Ratio of Relative Abundance
0.974
0.813
E1
SNP pmTOR
Relative Abundance
0.159
0.571
E2
SNP mTOR
Relative Abundance
0.269
0.412
E3
SNP pmTOR/mTOR
Relative Abundance
0.910
0.886
F1
SNP pAMPK
Relative Abundance
0.952
0.950
F2
SNP AMPK
Relative Abundance
0.197
0.025
F3
SNP pAMPK/AMPK
Ratio of Relative Abundance
0.093
0.068
WT Veh
WT Adip
KO Veh
KO Adip

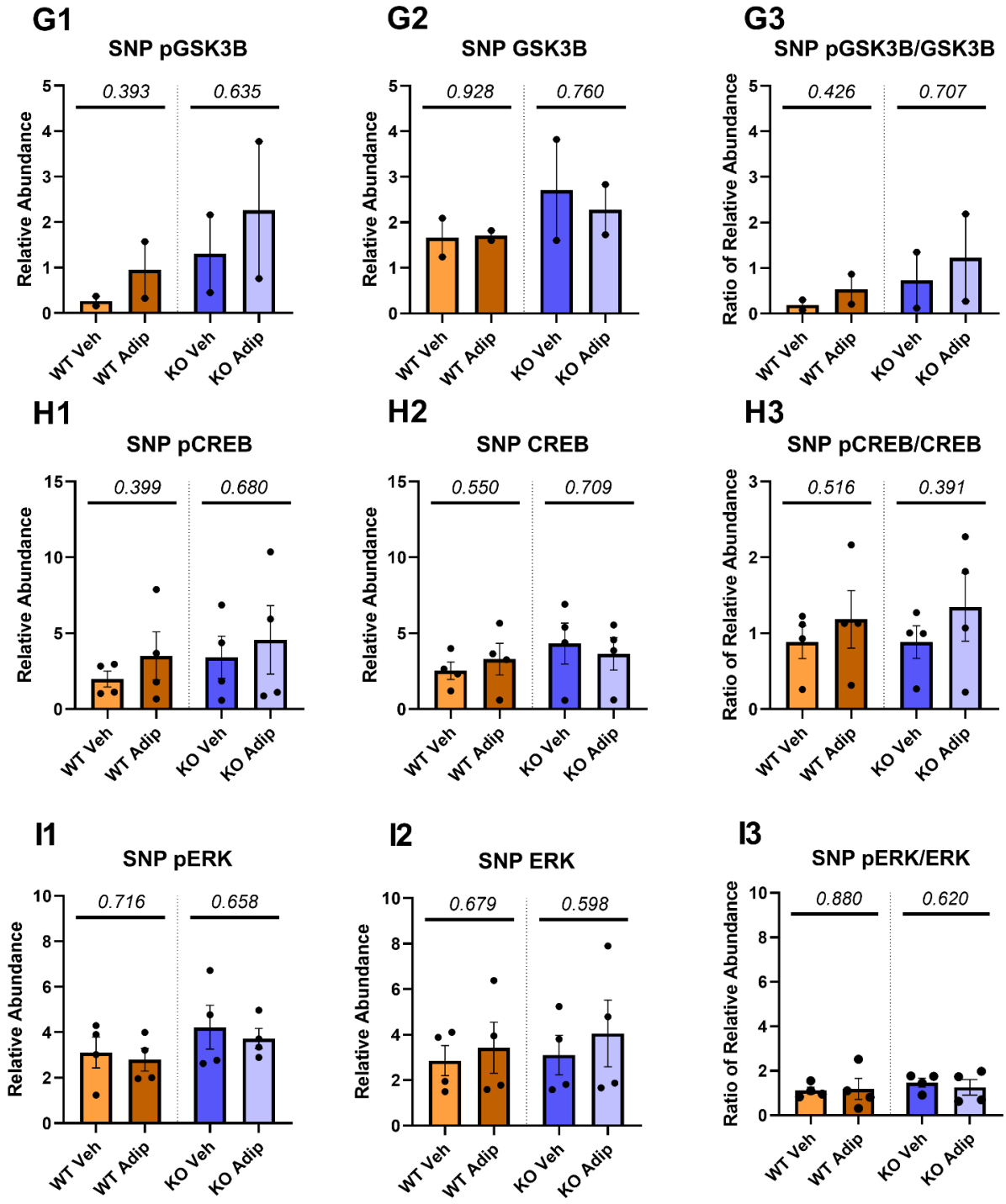


Figure 12. No Change in relative abundance of most proteins in synaptosome fractions. All measurements represented in WT and *FMR1* mice across treatment conditions (Vehicle, AdipoRon) A. Relative abundance of SYN1. B. Relative abundance of PSD95. C. Relative abundance of GluN2B. D1. Relative abundance of pGluA1. D2. Relative abundance of GluA1. D3. Proportion of phosphorylated total protein pGluA1/GluA1. E1. Relative abundance of pmTOR. E2. Relative abundance of mTOR. E3. Proportion of phosphorylated total protein pmTOR/mTOR. F1. Relative abundance of pAMPK. F2. Relative abundance of AMPK. F3. Proportion of phosphorylated total protein pAMPK/AMPK. G1. Relative abundance of pGSK3β. G2. Relative abundance of GSK3β. G3. Proportion of phosphorylated total protein pGSK3β/GSK3β. H1. Relative abundance of pCREB. H2. Relative abundance of CREB. H3. Proportion of phosphorylated total protein pCREB/CREB. I1. Relative abundance of pERK. I2. Relative abundance of ERK. I3. Proportion of phosphorylated total protein pERK/ERK. Among all graphs, every point represents one animal; bars represent mean ± SEM. P value for each t-test represented with decimal above each graphed.

3.3.2 Whole Homogenate

The relative abundance of SYN 1 remains consistent across treatment group and condition with only a marginal decrease in the Adip group relative to the Veh group in the WT condition (n=5, p=0.9068; unpaired student's two tailed t-test) (Figure 13. A) and a marginal increase in the Adip group relative to the Veh group in the KO condition (n=5, p=0.8290 unpaired student's two tailed t-test) (Figure 13. A). PSD95 abundance is slightly diminished in the Adip group relative to the Veh group in both the WT (n=5, p=0.7848 unpaired student's two tailed t-test) and KO conditions (n=5, p=0.8847 unpaired student's two tailed t-test) but not to a significant extent (Figure 13. B). The relative abundance of GluN2B is marginally increased in the Adip group relative to the Veh group in WT mice and inversely decreased slightly in KO mice (n=5, p=0.6881 unpaired student's two tailed t-test) (Figure 13. C).

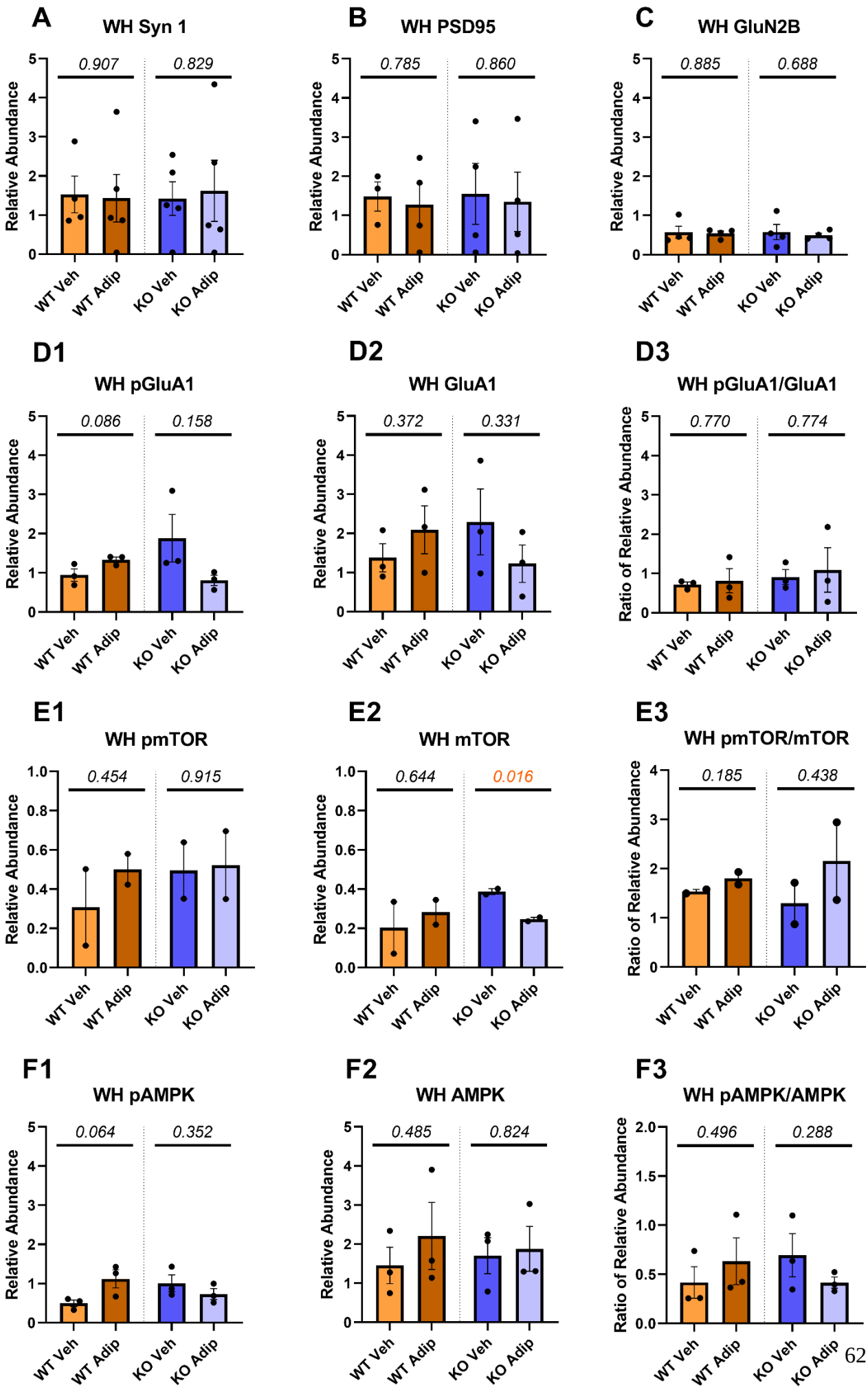
Among pGluA1, protein is found to be slightly increased in WT mice in the Adip treatment group relative to the WT Veh treatment group (n=3, p=0.0862 unpaired student's two tailed t-test) (Fig 13. D1). The inverse was observed in the KO condition among the Adip group compared to the Veh group (n=3, p=0.1582 unpaired student's two tailed t-test) (Fig 13. D1). Total GluA1 protein was observed to be slightly increased in the WT Adip treatment group and oppositely decreased in the KO Adip treatment group relative to WT (n=3, p=0.3722 and p=0.3314 unpaired student's two tailed t-test) (Fig 13. D2). The phosphorylated proportion of total GluA1, pGluA1/GluA1, is slightly increased in the Adip group relative to the Veh treatment group in both the WT (p=0.7697 unpaired student's two tailed t-test) and the KO groups (n=3, p=0.7738 unpaired student's two tailed t-test) (Fig 13. D3). The relative abundance of pmTOR is increased among the Adip group relative to the Veh group (n=2, p=0.4538 unpaired student's two tailed t-test) in the WT condition

but remains nearly unchanged in the KO condition (n=2, p=0.9146 unpaired student's two tailed t-test) (Fig 13. E1). In total mTOR protein, the Adip group has increased relative abundance compared to the Veh group in the WT condition (n=2, p=0.6441 unpaired student's two tailed t-test) but the inverse is observed to a statistically significant extent in the KO condition (n=2, p=0.0157 unpaired student's two tailed t-test) (Fig 13. E2). The phosphorylated proportion of total mTOR, pmTOR/mTOR, is increased in the Adip group relative to the Veh group in both WT (p=0.1853 unpaired student's two tailed t-test) and KO (p=0.4378 unpaired student's two tailed t-test) conditions (Fig 13. E3).

The relative abundance of pAMPK is found to be slightly increased in WT mice in the Adip treatment group relative to the WT Veh treatment group (n=3, p=0.0640 unpaired student's two tailed t-test). The inverse was observed in the KO condition among the Adip group compared to the Veh group (n=3, p=0.3818 unpaired student's two tailed t-test) (Fig 13. F1). The relative abundance of total AMPK Adip groups is increased relative to the Veh groups in both the WT (n=3, p=0.4845 unpaired student's two tailed t-test) and KO conditions (n=3, p=0.8244 unpaired student's two tailed t-test) (Fig 13. F2). The phosphorylated proportion of AMPK, pAMPK/AMPK, indicates increased phosphorylation in the Adip group relative to the Vhe group in the WT condition (p=0.4956 unpaired student's two tailed t-test) and decreased phosphorylation in the Adip group relative to the Vhe group in the KO condition (p=0.2882 unpaired student's two tailed t-test) (Fig 13. F3). None of these differences display statistical significance. The Adip groups of relative pGSK3**β** abundance are increased relative to the Veh groups in the the WT (n=2, p=0.54331 unpaired student's two tailed t-test) condition and decreased in the KO condition (n=2. p=0.1020 unpaired student's two tailed t-test) (Fig 13. G1). The relative abundance of total GSK3**β**

is diminished in the Adip group relative to the Veh group of both the WT (n=2, p=0.7768 unpaired student's two tailed t-test) and KO conditions (n=2, p=0.9156 unpaired student's two tailed t-test) (Fig 13. G2). The phosphorylated proportion of total pGSK3**β**, pGSK3**β**/GSK3**β**, is increased in the Adip group relative to the Veh group in the WT condition (p=0.6679 unpaired student's two tailed t-test) and decreased in the KO condition (p=0.5465 unpaired student's two tailed t-test) (Fig 13. G3). Relative abundance of pCREB and CREB are found to be nearly unchanged across treatment groups and conditions (Fig 13. H1, H2). The phosphorylated proportion of total pCREB, pCREB/CREB, is slightly decreased in the Adip group relative to the Veh group in both WT (n=4, p=0.4895 unpaired student's two tailed t-test) and KO conditions (n=4, p=0.8184 unpaired student's two tailed t-test) (Fig 13. H3).

The relative abundance of pERK is marginally increased in the Adip group of the WT (n=4, p=0.6051 unpaired student's two tailed t-test) condition and marginally decreased in the KO conditions (n=4, p=0.6013 unpaired student's two tailed t-test) relative to the Veh group (Fig 13. I1) The relative abundance of ERK is nearly unchanged in the Adip group of the WT condition (n=4, p=0.8180 unpaired student's two tailed t-test) and increased in the KO condition (n=4, p=0.2828 unpaired student's two tailed t-test) relative to the Veh group (Fig 13. I2) The abundance of total ERK that is phosphorylated, pERK/ERK, is nearly unchanged across treatment groups in the WT condition (p=0.8345 unpaired student's two tailed t-test) and diminished in the Adip group relative to the Veh group in the KO condition (p=0.2558 unpaired student's two tailed t-test) (Fig 13. I3). No statistical significance is inferred among the above discussed observations. Among all relative protein abundances probed across whole homogenate, only total mTOR showed a statistically significant difference between vehicle and AdipoRon treated KO animals.


A
WH Syn 1
0.907
0.829
B
WH PSD95
0.785
0.860
C
WH GluN2B
0.885
0.688
D1
WH pGluA1
0.086
0.158
D2
WH GluA1
0.372
0.331
D3
WH pGluA1/GluA1
0.770
0.774
E1
WH pmTOR
0.454
0.915
E2
WH mTOR
0.644
0.016
E3
WH pmTOR/mTOR
0.185
0.438
F1
WH pAMPK
0.064
0.352
F2
WH AMPK
0.485
0.824
F3
WH pAMPK/AMPK
0.496
0.288
Relative Abundance
Ratio of Relative Abundance
WT Veh
WT Adip
KO Veh
KO Adip

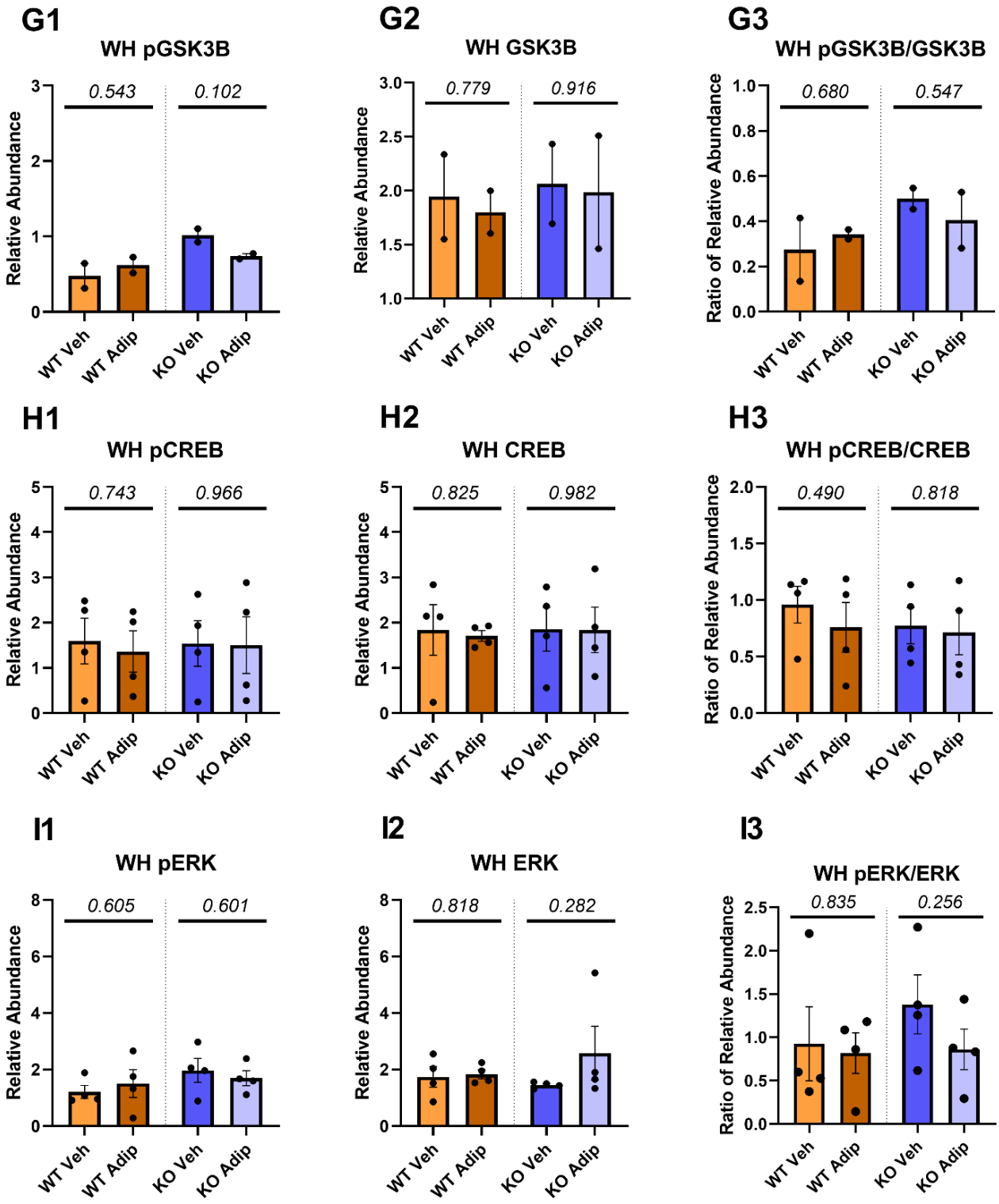


Figure 13. No change in relative abundance of proteins in whole homogenate lysate. All measurements represented in WT and *FMR1* mice across treatment conditions (Vehicle, AdipoRon) A. Relative abundance of SYN1. B. Relative abundance of PSD95. C. Relative abundance of GluN2B. D1. Relative abundance of pGluA1. D2. Relative abundance of GluA1. D3. Proportion of phosphorylated total protein pGluA1/GluA1. E1. Relative abundance of pmTOR. E2. Relative abundance of mTOR. E3. Proportion of phosphorylated total protein pmTOR/mTOR. F1. Relative abundance of pAMPK. F2. Relative abundance of AMPK. F3. Proportion of phosphorylated total protein pAMPK/AMPK. G1. Relative abundance of pGSK3β. G2. Relative abundance of GSK3β. G3. Proportion of phosphorylated total protein pGSK3β/GSK3β. H1. Relative abundance of pCREB. H2. Relative abundance of CREB. H3. Proportion of phosphorylated total protein pCREB/CREB. I1. Relative abundance of pERK. I2. Relative abundance of ERK. I3. Proportion of phosphorylated total protein pERK/ERK. Among all graphs, every point represents one animal; bars represent mean ± SEM. P value for each t-test represented with decimal above each graphed.

CHAPTER 4. DISCUSSION

To our knowledge, this study is the first of its kind in attempting to explore a therapeutic function of exogenous *in vivo* synthetic adiponectin receptor modulation through administration of AdipoRon in an *FMR1* KO mouse model of FXS. Thus, each experiment serves as a pilot study where the primary aim was to elucidate threads of reasoning along this endeavor to guide the next line of questioning. Thus, we discuss the major findings of this study in context of their relevance to the biology within which they operate as well as contextualize them within discussion of future experiments and clinical translation.

4.1 Major Findings

4.1.1 Genotype and Injection Conditions Interact to Produce a Unique Behavioral Phenotype: Key Insight into Phenotype Variability, Therapeutic Considerations, and Translational Applications

Our behavioral data suitably reflects what is discussed at length in previous sections of this thesis and a well described phenomenon in the field of FXS research; it is challenging to model FXS behavioral phenotypes in mice. In our work, we have selected some of the most salient and well documented behaviors to assess our compound of interest, AdipoRon. Besides audiogenic seizures, which have the advantage of having a binary presentation, tests for anxiety-like behaviors like OF, EPM, 3CS are described to highlight a reliable and reproducible anxiety behavior in *FMR1* KO mice.

The OF test gives insight into the anxiety behavior, the locomotion, and exploratory behavior of an animal. In rodents, it is proposed that anxiety should translate to diminished exploration of the center of the field and a propensity to hug the walls of the apparatus. While the robustness of this particular finding as a characteristic phenotype presentation in FXS mouse model is contested, our own data demonstrate decreased exploration in the center of the field in *FMR1* KO mice. Our OF findings demonstrate a genotype effect where the genotype of the mouse, WT or *FMR1* KO, produces the majority of differences in how long a mouse spends exploring the center of the open field. Likewise, we observe a slight increase in the amount of time an *FMR1* KO mouse spends in the center of the field in the AdipoRon treated group, albeit not to a significant extent. Thus, it could be argued based on our findings, that *FMR1* KO mice exhibit increased anxiety behavior and that AdipoRon treatment trends towards significance in remediating it.

This finding is starkly and paradoxically contrasted by our genotype main effect EPM findings which demonstrate that *FMR1* KO mice spend less time in the closed arm of the EPM compared to WT suggesting less anxiety-like behavior and an increased propensity to explore a novel open space. To further complicate this finding, AdipoRon appears to significantly potentiate this effect and produces an even lesser propensity to remain in the closed arms of the EPM. This contradicts our OF findings not only in the basal anxiety-like behavior of mice, but also in the effect that our experimental drug produces. Finally, we examine a social anxiety paradigm through application of the three chamber sociability assay to our therapeutic exploration. Here, we observe that *FMR1* KO mice exhibit increased exploration of the stranger mouse and that AdipRon treated mice of both genotypes spend more time exploring the stranger mouse relative to vehicle mice.

Moreover, we observe competing effects with a strong main effect interaction in the second round of the three-chamber sociability test where AdipoRon appears to diminish exploration of a novel stranger among WT mice and increase it among *FMR1* KO mice. Finally, we document that while *FMR1* KO mice show a trend towards increased sociability when compared to WT mice, they show significantly reduced social novelty preference relative to WT.

Notably, we observe a trend towards, or else a significant effect, in main effect interactions across multiple of our trials. That is to say that where trends or other significant effects can be observed, AdipoRon appears to act on the *FMR1* KO mouse uniquely from WT and sometimes in the opposite way. While our proteomic findings failed to concretely demonstrate significant relationships, their trends do appear to corroborate these behavioral findings. Likewise, electrophysiological findings demonstrate minor trends to suggest that AdipoRon could play some modulatory role on synaptic plasticity if observed under the right conditions. Given these observations, we explicitly look to the possibility for stress confounds, unique dose responses, regional and temporal variability in function, proteomic function, and experimental design as sources of failure, and worthwhile opportunities for further exploration.

4.1.2 Chronic IP AdipoRon Administration at 20mg/Kg Does Not Restore Observed Deficit in Hippocampal LTP in *FMR1* KO Mice

Experiments in this study investigating induction of hippocampal LTP in the DG of adult mice across WT and *FMR1* KO conditions among CC mice indicate a significant reduction in % LTP among *FMR1* KO mice compared to WT mice. This corroborates previous studies which demonstrate significantly diminished LTP in the DG of *FMR1* KO mice, including those from our own group which we discussed above. We recapitulate that a DG LTP deficit does exist, that a

genotype effect is observed across WT and *FMR1* KO mice, and that no significant difference is observed between treatment groups among mice of the same genotype. We observe a non-significant trend towards diminished LTP among both WT and *FMR1* KO mice in the vehicle group and a non-significant trend towards increased LTP among the AdipoRon treated mice of both genotypes.

A likely reason for this finding is a small effect size and thus insufficient slice number to observe a minor effect that AdipoRon could be having given the directionality of observed trends.
Indeed, previous work from our lab documents electrophysiology slice numbers ranging from 15 to 30 in a given condition (S.-Y. Yau et al., 2018). In contrast, our current study –as an exploratory pilot study of a novel drug– was too conservative in this respect and examines at most 12 slices in a given group. This finding is further compounded by the described decrease in probability of achieving LTP in mature granule cells compared to immature granule cells which is likely to translate to a higher proportion of slices which fail to potentiate (Aziz et al., 2019; Burke & Barnes, 2006; Lopez-Rojas et al., 2016). This supports the need for an expanded sample size when operating with tissue from adult or aged mice in future iterations of this experiment.

Likewise, our data demonstrates that when comparing between WT and *FMR1* KO animals, chronic AdipoRon administration at a dose of 20mg/Kg, no statistically significant difference, and thus no drug effect, is observed. Since both WT and *FMR1* KO conditions demonstrate a minor trend downwards in % LTP with vehicle administration it is worthwhile to consider the potential effect that injection stress may be having across a chronic treatment paradigm such as this one. It is well established that stress modulates synaptic plasticity (Ghilan et al., 2015). Indeed, the extent

to which vehicle administration decreases % LTP among *FMR1* KO mice is greater than in WT. This is a compelling detail that suggests cause for further probing this effect with particular awareness of effect size and sample size requirements, as well as with careful consideration of administration practices and other pharmacokinetic/pharmacodynamic details in future iterations of this experiment. While the absence of any statistical significance in these findings preclude concrete conclusions to be drawn from these data, this pilot experiment indicates that a drug effect of AdipoRon may indeed exist and that further electrophysiological exploration is necessary to better find and characterize it.

4.1.3 Chronic IP AdipoRon Administration at 20mg/Kg Does Not Significantly Impact the Measured Relative Abundance of Synaptic and Whole Tissue Proteins

In accordance with conducting a broad sampling of AdipoRon effects, this study examines multiple proteins in the hippocampal DG of WT and *FMR1* KO mice in their basal and phosphorylated states which have been identified as key biomarkers and therapeutic targets in the molecular etiology of FXS. Similarly, to electrophysiology, we do not note any significant effect of AdipoRon on relative protein abundance when compared to the vehicle administered group within the same condition across both synaptosomal and whole protein samples. Despite a lack of statistical significance in proteomics, we briefly discuss the observed trends in Western blotting and explore mechanistic considerations and future research.

As discussed in 1.2.2.5 of this thesis, SYN1 is an important contributor to presynaptic reserve pool vesicle docking and mobilization. We investigate relative SYN1 abundance in synaptosomal fractions to find virtually no change in whole homogenate but marginal increase in AdipoRon treated mice among both WT and *FMR1* KO mice compared to vehicle. Increased abundance, and

more specifically, increased activation of synapsins could present in the context of enhanced excitatory signaling as a metric of increased probability of neurotransmitter release. Given that SYN1 is a synaptic protein, it follows that it may be uniquely enriched in synaptosomal fractions and that a modulatory effect may be more likely to be observed there. Given its contribution to the stabilization of synaptic remodeling during generation of long-term potentiation, PSD95 is a biomarker of interest for the exploration of the proteomic infrastructure of the postsynaptic density across conditions.

In our approach, we observe a minor paradoxical effect where the AdipoRon treatment group is observed to exhibit slightly diminished protein abundance among WT mice but slightly increased protein abundance among *FMR1* KO animals. While this finding is contradictory, such a finding could potentially be attributed to an interaction between AdipoRon and genotype. That is to say that PSD95 is shown to be altered in *FMR1* KO mice and contributes to altered synaptic plasticity. This alteration may respond uniquely to an effector, like a therapeutic compound, when compared to vehicle administered mice as a form of drug-gene interaction. In whole protein westerns for PSD95, the decrease in relative protein abundance among AdipoRon treated mice is consistent across both WT and *FMR1* KO animals. This is a unique finding in the context of results from synaptosomal fractions but may potentially be explained by the synthesis and trafficking dynamics of PSD95 to its primary niche at the postsynaptic density. Findings from synaptosomal fraction may better reflect ready state PSD95 whereas whole protein findings may better reflect a decrease in total synthesis of PSD95 in the cytoplasm throughout the cell and may reflect a local decrease in protein translation in response to AdipoRon across conditions.

GluN2B is found to be nearly unchanged in whole protein Westerns and paradoxically increased in WT AdipoRon treated mice while decreased in *FMR1* KO AdipoRon treated animals. As an NMDA receptor subunit particularly documented to mediate bidirectional plasticity (Shipton & Paulsen, 2014), enrichment of GluN2B in synaptic fractions is logical compared to whole protein given the larger relative proportion of NMDA receptor subunits at the synapse specifically. Similarly, to findings of PSD95, these findings may be subject to a unique interaction between the unique location of a protein, genotype, and drug effect where AdipoRon may interact with GluN2 in *FMR1* KO mice uniquely compared to WT mice and especially so in synaptic fractions where this effect is more likely to be observed. AMPA receptor subunit GluA1 phosphorylation is regulated by neuronal activity and consequently contributes to membrane AMPA receptor expression. In synaptic fractions, both pGluA1 and total GluA relative protein abundance demonstrate a similar relationship where WT mice do not appear to exhibit any change in response to AdipoRon but WT mice exhibit decreased pGluA1 and total GluA1. This is contradictory to what might be expected of AdipoRon given that a therapeutic effect might be expected to increase the relative abundance of GluA1 subunits.

Together, these findings produce no significant change in the phosphorylated proportion of total GluA1 protein. A potential consideration here may be that perhaps AdipoRon and any potential effects it has on synaptic plasticity modulate either other AMPA subunits, or else target other mechanisms of plasticity all together. Similar findings exist in whole protein Westerns which demonstrate that AdipoRon does not appear to interact with AMPA receptor subunit GluA1.

Perhaps the most paradoxical result among the Western findings is the complete opposite effect that AdipoRon appears to have between synaptic and whole proteins among the phosphorylated proportion of total measured mTOR protein. In synaptic fractions the AdipoRon group appears to have a diminished phosphorylated ratio of mTOR while the inverse is true in the whole homogenate group. This finding is surprising given the ubiquitous role of mTOR in cell energy metabolism and points to an important area for future exploration given the localized effect that may be occurring in cell metabolic modulation and local protein translation within the neuron. Decreased total mTOR abundance among AdipoRon treated KO animals was the only statistically significant relationship observed among all whole homogenate protein measured. This is an intriguing finding given that phosphorylation of mTOR was not measured to be significantly different suggesting that the AdipoRon may decrease total mTOR expression but not activation. Further investigation of this relationship in particular is required because it is unclear whether these findings hold true or else if the methods in use are not adequately powered of sensitive enough to detect a change in mTOR phosphorylation which may legitimately exist.

Indeed, future inquiry should reprobe these relationships and venture to further probe this question in nuclear proteins. Synaptosomal fractions indicate a slight increase in the ratio of phosphorylated AMPK in the AdipoRon treated group of both WT and *FMR1* KO animals. Conversely, whole tissue Westerns indicate an increase in phosphorylated AMPK proportion among WT mice and a decrease in *FMR1* KO mice further supporting previous discussion for temporal heterogeneity among homeostatic proteins. Given AMPK's central role in energy metabolism, and since it represents a salient marker of cellular autophagy, this difference between synaptic and cytosolic AMPK phosphorylation is a unique finding. Total AMPK abundance was the only synaptosomal

fraction to show any significant differences. Future work should seek to replicate this findings and further investigate AMPK phosphorylation. Despite a significant result, it is largely premature to speculate on the relationship between AdipoRon treatment and AMPK expression because both AMPK phosphorylation and other protein abundances would be necessary to establish the exact effect of AdipoRon treatment more clearly.

Synaptosomal fractions of GSK3β shows increased phosphorylated protein proportion of total protein among AdipoRon treated mice in both WT and *FMR1* KO mice. Given that GSK3β is a constitutive protein, phosphorylation of GSK3β represents diminished GSK3β activity. Given that GSK3β inhibition is a contributor to cell autopsy and lysosomal acidification, GSK3β inhibition in "a step in the right direction" from a cell metabolic perspective when considering our inquiry into the role of adiponectin receptor modulation in autophagy as therapeutic target for functional alterations in FXS (Avrahami et al., 2020).

In whole protein evaluation, this relationship is conserved except that *FMR1* KO conversely shows a mild reduction in pGSK3β/GSK3β recapitulating previous variation is site specific proteomic observations. Similarly heterogenous results exist among pCREB/CREB ratios across synaptosomal fractions between WT and *FMR1* KO mice which both show an increase in phosphorylated (active) CREB compared to whole protein preparations which depict an increased pCREB/CREB in WT mice and a decrease in *FMR1* KO mice. It is reassuring to find increased phosphorylation in CREB in synaptosomal fractions because it indicates that there may be a local increase in the function of molecular modulators of protein synthesis-dependent long-term potentiation. This could be considered an advantageous finding in the context of diminished

hippocampal LTP, which while not directly demonstrated in this study, is well documented in literature, and discussed throughout this study.

Finally, ERK shows similar effects among synaptosomal fractions whereby AdipRon treated mice in both WT and *FMR1* KO groups have diminished phosphorylated ERK relative to vehicle administered mice and where whole protein preparations show a paradoxical relationship among these groups whereby pERK is diminished in *FMR1* KO mice and relatively unchanged in WT animals.

While none of the above discussed findings are statistically significant, they represent a broad look at the proteomic microenvironment of both whole homogenate lysate as well as synaptosomal fractions employed to look more specifically at the signaling landscape of the synaptic milieu across WT and *FMR1* KO in AdipoRon and vehicle treatment conditions. While the observed differences are merely speculations on trends, there is evidence that proteomic microenvironment and temporal distribution within the cell can differentially impact protein modifications, function, and localization (Heo et al., 2018). Such findings are especially relevant among synaptic proteins which can be expected to be particularly enriched, and post-translationally modified at their mechanistic niche that is the synapse relative to their cytosolic states where they may be synthesized or trafficked before localizing to their activity site. In the context of our inquiry, these findings demonstrate that further exploration of proteomics is necessary to better explore the role of adiponectin receptor modulation on the signaling environment and to better follow the molecular mechanisms of synaptic changes across protein targets in this approach to FXS therapy.

4.2. Future Work

This study sought to explore and build on existing research on the role of autophagy modulation in the FXS brain – from both within our lab and beyond. We sought to explore an uncharted niche of the FXS research field in the novel exploration of adiponectin receptor modulation with the exogenous compound AdipoRon as a potential therapeutic avenue in a mouse model of FXS. While the theory on which we build our assumptions is robust, rigorously described throughout this work, well documented in literature, and relied upon in current research and clinical trials, our research along this line of inquiry is in its infancy. Below we propose some important next considerations that need addressing in future iterations of this work.

4.2.1 Pharmacokinetics and Pharmacodynamics of AdipoRon

AdipoRon has been widely explored in a variety of applications from diabetes and metabolic syndrome to cancer and Alzheimer's disease. The pharmacology of AdipoRon is well studied but has never before been used in our application. Thus, future exploration of AdipoRon for FXS would benefit from a tailored pharmacological workup to center future work within established therapeutic and biologically relevant ranges.

First, it would be extremely beneficial to center future studies within inhouse-established neuroendocrine thresholds. Specifically, adiponectin is a ubiquitous protein hormone that participates in a broad variety of metabolic functions throughout the periphery. Indeed, it is found to fluctuate with a variety of factors including adiposity, circadian rhythm, and level of activity

(Becic et al., 2018; Swarbrick & Havel, 2008). By consequence, more work is needed to probe for both peripheral circulating adiponectin and CNS adiponectin abundance across time and within the FXS context. Likewise, it is not well established whether the exogenous modulation of adiponectin receptors alters endogenous adiponectin signaling in any capacity.

Both basal endogenous adiponectin measurements as well as measurements over the course of treatment would provide useful insights about the pharmacokinetics of endogenous and exogenous adiponectin dynamics. Likewise, the novel application of AdipoRon to FXS syndrome would benefit from a dose response curve to establish a therapeutic range within which AdipoRon exerts a beneficial effect.

Adiponectin signaling may be altered uniquely in FXS mice and thus may respond best to a unique dose to what is observed in literature in other pathologies. Basic tenets of pharmacokinetics and pharmacodynamics including dose, duration, route, delivery, and metabolism are important to examine given that dosages that are effective in other pathologies, may not be equally suitable for application to FXS without prior exploration. Indeed, some studies have found 50mg/Kg oral delivery to modulate AMPK activity while another study has found acute IP administration of 1mg/Kg for 7 weeks to be therapeutic in a mouse depression model (Fairaq et al., 2017; Khandelwal et al., 2022; Nicolas et al., 2018) . This highlights the pronounced need for tailored kinetic exploration of AdipoRon in the FXS context.

4.2.2 Experimental Design

Our approach in exploring the aims of this research was to broadly explore the effects of AdipoRon in an *FMR1* KO mouse model of FXS across molecular, functional, and behavioral metrics. Based on our findings, future studies looking to explore these questions would benefit from increased

sample size given that our pilot research indicates a small effect size at the current dose if one is present. Likewise, while Western blotting across whole tissue lysate of the hippocampal DG as well as in synaptosomal fractions revealed potential trends and temporal distribution and functions of relevant proteins, future studies would benefit from more sensitive proteomics which could include ELISA or else RNAseq. Given that proteomic differences in the proteins being used to evaluate drug effect in this study were too small to be significant, increased sensitivity would be very helpful here. Additionally, while we elected to approach our questions in the hippocampus of adult *FMR1* KO mouse because previous work from our group explored underlying relationships in adult mice, there is meaningful incentive to explore these questions across a variety of brain regions and across different developmental time points given the abundance of data that documents the evolving function and phenotypic findings in FXS across development (Razak et al., 2020).

CHAPTER 5. CONCLUSION

The objective of this thesis was the novel exploration of exogenous adiponectin receptor modulation with synthetic adiponectin receptor agonist AdipoRon. We proposed that through its role as a regulator of cell energy metabolism and autophagy, adiponectin and it's exogenous supplementation at 20mg/Kg delivered through IP injections for 15 days may act upon the molecular, functional, and behavioral alterations associated with FXS, including diminished hippocampal LTP in the MPP of the dentate gyrus, aberrant and dysregulated proteomic signaling including among mTOR, AMPK, CREB, EKR1/2/MAPK, and increased contextual and social-anxiety like behavior.

Our results address these aims by finding that chronic AdipoRon administration does not restore LTP in the hippocampal DG, does not impart significant changes in plasticity-related proteins through modulatory effects on cellular metabolism and autophagic flux in the hippocampal DG, and that genotype and treatment condition interact to produce a unique behavioral phenotype of anxiety-like behavior across treatments in adult male *FMR1* KO mice.

To our knowledge, this is the first interrogation of adiponectin receptor modulation as a therapeutic target for FXS and thus, while suffering from limitations including small sample size and limited proteomic sensitivity, provides a valuable pilot study of mechanistic, functional, and behavioral metrics of the application of AdipoRon to FXS pathophysiology. While our research trajectory along this line of questioning is in its infancy, the theoretical basis for autophagy modulation as a therapeutic approach to FXS syndrome is a popular approach with demonstrated efficacy.

Despite novel advances in pharmaceuticals and an ever-evolving toolbox of genetic therapy approaches, FXS remains a leading monogenic cause of ASDs and a prominent contributor to ID; for which approved therapies are limited to antipsychotics. This research represents a small scientific contribution to a profound and growing worldwide effort to develop better therapies, for improved clinical outcomes and for an increased quality of life for people with FXS and their families.

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