2-2018

Neonatal Stimulation of PKC Epsilon Signaling Normalizes Fragile X-Associated Deficits in PVN Oxytocin Expression and Later-Life Social and Anxiety Behavior

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by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The City University of New York

2018

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

Fragile X Syndrome (FXS) is an inherited developmental disorder characterized by disturbances in emotional and social behavior. Our studies have revealed suppressed hippocampal PKCε expression in *Fmr1* knockout (KO) mice, the leading model of FXS. To compensate for this deficiency, we stimulated PKCε in neonatal KO mice by administering a selective PKCε activator, dicyclopropyl-linoleic acid (DCP-LA), and studied its effect on ventral hippocampal neurons and a proximal target of the ventral hippocampus, the hypothalamus, which regulates social and emotional behavior. We observed that at postnatal day 18 (P18), vehicle-treated KO mice displayed increased surface localization of the 3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluR2 in the ventral CA1 region, indicative of increased neuronal excitability. Since the hippocampus is known to exert an inhibitory influence on the hypothalamus, we tested if this possible CA1 stimulation was associated with a suppression of oxytocin synthesis in the hypothalamus. Intriguingly, the number of oxytocin+ cells in the hypothalamic paraventricular nucleus (PVN) of P20 KO mice was sharply suppressed. However, both the increased surface localization of GluR2 and the suppression of PVN oxytocin+ cells in the KO mice were rescued by DCP-LA treatment from P6-14, to levels comparable to that in the wild-type controls. Moreover, this neonatal treatment regimen was able to fully rescue hyper-anxiety and social behavior deficits in adult (>P60) KO mice. Thus, we present a novel strategy to circumvent aberrant brain development in FXS and accompanying behavioral deficits, by activating PKCε signaling during neonatal development.

Acknowledgments

First and foremost, I cannot begin to express my profound gratitude to my mentor, Dr. Probal Banerjee, for his enduring support, patience, enthusiasm, and wisdom. Without his encouragement and guidance, this project never would have taken form. I could not have imagined a more phenomenal advisor for my doctoral thesis.

Besides my mentor, I would like to thank the rest of my advisory committee: Drs. Alejandra Alonso, Dan McCloskey, Carl Dobkin, Kathryn Chadman, and Raddy Ramos, for their insightful feedback, stimulating questions, and generosity with their time and expertise.

I am eternally grateful Drs. Abdeslem El Idrissi and William L'Amoreaux for introducing me to research, giving me a strong foundation in neuroscience and cell biology, and for encouraging me to apply to the Doctoral Program in Biology. I am also especially indebted to Dr. Lorenz Neuwirth, who took me under his wing when I was an undergraduate student, and was always extraordinarily generous with his time and advice.

I would next like to thank my dear lab-mates and friends with whom I have been privileged to work with over the years, especially Dan Kerr, Tatyana Budylin, Juliet Baidoo, Sumit Mukherjee, Kaushiki Chatterjee, Sreyashi Samaddar, Kristina Mastroianni, Ryan Schroder, Joseph Inigo, Sultana Begum, Narjes Baazaoui, Salim Bendaoud, Sara Guariglia, Francoise Sidime, Dinali Obeysekera, Kizzy Vasquez, Nechama Averick, Dr. Raja's lab, and Dr. Poget's lab. Thank you for your guidance, support, stimulating discussions, well-wishes, and for all the fun we've had over the years.

I am grateful to the many students that I have been blessed with over the years, but most of all, I would like to acknowledge the amazing Bishoy Gerges, Silvia Menkes, David Salame, Aheli Chatterjee, Rodina Sadek, Robert Candia, and Pranav Chanthrakumar, who gave their time and effort to assist me with my doctoral work. In truth, I have learned just as much from them as they learned from me. Thank

you for constantly inspiring me, and giving me a different kind of education.

Importantly, the completion of my thesis and success in the program was only possible thanks to the generous financial support from the CUNY Graduate Center and The College of Staten Island. Both the GC and CSI Biology Department staffs, as well as the CSI Center for Neuroscience and Developmental Disabilities, were an extremely helpful source of information in navigating the many steps of completing the thesis and graduating. I would also like to especially thank Dr. Laurel Eckhardt, Dr. Cathy Savage-Dunn, and Ms. Joan Reid for their help and support throughout the process. Additionally, my sincerest thanks goes to the CSI Animal Facility, especially Joanne, Ana, Rachel, and Jenna, for their unparalleled commitment in caring for the mice used for my studies. An immense thank you is also deserved for each and every mouse whose sacrifice contributed to this body of work. It is my deepest hope that their sacrifice increases our understanding of Fragile X Syndrome and may someday benefit those impacted by the disorder.

Last but not least, a special thank you to my family, especially my parents and my brother Daniel, for their endless love, support, and encouragement. I am eternally grateful of all that they have done for me over the years - without them, I would not be where I am today. Finally, I must thank my loving boyfriend Andrew Mancuso, who has supported me throughout the whole process. He has been an integral part of my journey: sharing all the stress, frustration, happiness, and excitement; and constantly encouraging me when tasks seemed insurmountable. Thank you for putting up with me, making me laugh, and inspiring me to “Work work work!" I could not have completed this thesis without your love and support.

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Abbreviations

5-HT_{1A}-R: Serotonin 1a receptor
AMPA-R: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ASD: Autism spectrum disorder
BNST: Bed nucleus of the stria terminalis
BrdU: Bromodeoxyuridine
C57: C57BL/6 mouse strain
CA1: Cornu ammonis 1
CA3: Cornu ammonis 2
Co-IP: Co-immunoprecipitation
DCP-LA: 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid
DCX: Doublecortin
DG: Dentate gyrus
EPM: Elevated plus maze
Fmr1: Fragile X mental retardation 1 gene
FMRP: Fragile X mental retardation protein
FVB/NJ: Friend Virus B NIH Jackson mouse strain
FXS: Fragile X syndrome
GluR2: Glutamate receptor 2 (AMPA receptor subunit)
GPCR: G-protein coupled receptor
Hoechst: Hoechst33342 dye
HPA axis: hypothalamic-pituitary-adrenal axis
i.p.: Intra-peritoneal injection
KO: Knockout
LD: Light-dark test
LTD: Long-term depression
LTP: Long-term potentiation
mPFC: Medial prefrontal cortex
NeuN:Neuronal nuclear protein
OF: Open field test
OXT: Oxytocin
OXTR: Oxytocin receptor
P#: Postnatal day #
Pde6b^{wt}: Rod Phosphodiesterase 6B, wild-type
Pde6b^{rd1}: Rod Phosphodiesterase 6B, retinal degeneration 1
PKC: Protein kinase C
PKCε: Protein kinase C epsilon isoform
PVN: Paraventricular nucleus of hypothalamus
SI: Social interaction test

I. Background and Significance

1.1. Fragile X Syndrome

1.1.1. Foreword

Fragile X Syndrome, I have found, is surprisingly a relatively little-known disorder among the general public. Indeed, while most people in recent years have at least heard of autism spectrum disorder, if not directly knowing a person affected by it, Fragile X Syndrome has received considerably less attention. As a case in point, whenever the topic of my work comes up in conversation with strangers, I have learned to preface myself with the question, "Have you heard of Fragile X Syndrome?" This disparity is largely due to the explosive surge in autism awareness that has taken place over the past few decades. However, despite its lack of notoriety compared to autism, Fragile X Syndrome is, in fact, the most common known monogenic cause of autism, as well as the most common cause of intellectual disability.[1] In spite of this, no single effective treatment for Fragile X Syndrome exists, and current treatments suffer from serious limitations and drawbacks. Many of these disadvantages stem from the fact that although Fragile X Syndrome is a disorder of brain development, current pharmaceutical treatment strategies tend to address symptoms only after they have already become permanent. In our studies, we address the possibility of early intervention during the neonatal period as a means to improve prognosis for Fragile X Syndrome in a permanent manner. In the process, we have identified a novel PKCε-dependent signaling pathway that appears to be disrupted in Fragile X Syndrome; and along these lines, demonstrate that pharmacologically targeting this pathway during early brain wiring is capable of significantly improving behavioral outcomes in a Fragile X mouse model.

1.1.2. What is Fragile X Syndrome?

In essence, Fragile X Syndrome (FXS) is a complex neurodevelopmental disorder that is marked by deficits in cognitive, sensory, emotional, motor, and peripheral functioning.[1] FXS is estimated to occur in approximately 1 in 3,600 to 4,000 men, and 1 in 4,000 to 6,000 women. As a matter of greater concern, however, genetic carriers of the disease are estimated to be among approximately 1 in 151 women (or about 1 million women in the United States); and 1 in 468 men (about 320,000 men in the United States). While carriers of the disorder do not necessarily have FXS, *per se*, they are at risk of developing symptoms associated with the disorder, and passing it on to their children.

First described in 1943 by James Purdon Martin and Julia Bell, FXS is fundamentally caused by a heritable mutation of the *fragile X mental retardation 1 (Fmr1)* gene on the X chromosome.[2] Fragile X Syndrome is in fact named based on the physical appearance of X chromosomes bearing the mutation: presence of the mutation causes the ends of the long (q) arms of the chromosome, where the mutation is located, to appear fractured under the microscope (Figure 1). This part of the chromosome is called the "fragile site" because when studied in the laboratory, the chromosome has the tendency to fracture at that location. Because of this characteristic appearance, an affected X chromosome is easily detectable by karyotyping.

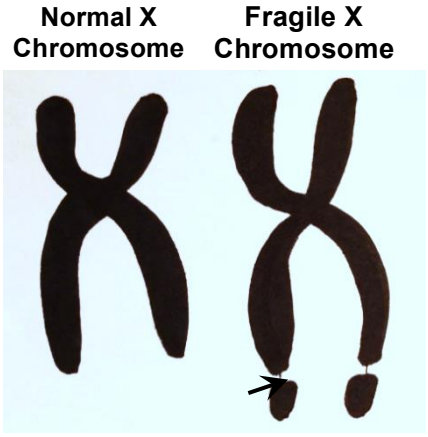


Figure 1. Representative karyotypes of a normal X chromosome (left) versus a FXS mutation-bearing X chromosome (right). Arrow indicates the fragile site. Image credit: McGill University, University of Edinburgh.

Individuals of all ethnic and racial backgrounds can be affected by FXS, as well as both sexes. However, it is important to note that the disorder is twice as prevalent in men as it is in

women. Moreover, females typically experience milder symptoms than men. This is primarily attributed to the fact that all males affected by the disease are homozygous, whereas the vast majority of affected females are heterozygous. Additionally, variability in random X-inactivation on the affected X chromosome can further reduce the severity of the symptoms in female patients.

Although FXS can significantly affect quality of life, carriers are often unaware that they harbor the mutation, since many carriers are either asymptomatic or display milder, associated symptoms (discussed in Section 1.1.3). Because of this, unless a family history of FXS is known, a carrier may not be aware they are at risk of having an affected child. Further complicating matters, infants born with FXS typically appear perfectly normal at birth and symptoms are not easily apparent during the first few years of life.[3] Parents usually begin to notice cause for concern at around 12 months of age in boys, and 16 months of age in girls. The most common signs that prompt a visit to the doctor are delayed attainment of developmental milestones, such as the onset of walking or first words, or failure to reach such milestones altogether. However, because many of the symptoms of FXS are behavioral and can be confused with those of other developmental disorders, a correct diagnosis typically does not occur until ~35-37 months in boys, and ~42 months in girls.[4] The disorder is typically diagnosed via polymerase chain reaction (PCR) or Southern blot analysis, both of which are sensitive to the primary mutation responsible for FXS (described in Section 1.1.3).

As it is a spectrum disorder, the symptoms of FXS vary widely in type and severity from patient to patient.[1] As the individual grows older, many physical abnormalities typically begin to emerge, including a narrowed face with an enlarged forehead and ears (Figure 2) flexible joints and fingers, macro-orchidism in males, heart defects, and/or reproductive issues in both males

and females.[1-3, 5-7] These abnormalities aside, FXS patients are usually otherwise physically healthy and generally have a normal life expectancy. Much more pressing than the physical features of FXS, however, are its behavioral and neurological symptoms.[1, 8-10] As the foremost cause of inherited cognitive impairment, FXS patients often have a lower than average IQ and may suffer from impairments in short-term and working memory, visuo-spatial abilities, and executive functioning. Also associated with FXS are numerous behavioral issues, including anxiety disorders, problems with speech and language, impairments in communication and social interaction, attention deficits, hyperactivity, perseverative behavior, and repetitive, stereotyped behaviors, such as self-injury, twirling, hand flapping, and rocking. Other neurological features of FXS include hypersensitivity to sensory stimuli, motor disturbances, epilepsy, and sleep disorders. Importantly, out of all of these symptoms, clinical anxiety ranks among the most common, present in 70% of male and 56% of female children with FXS,[8] and generally persisting throughout the lifespan.[11] At the neuronal level, the hallmark of FXS is the presence of abnormal dendritic spines with immature morphological characteristics.[12, 13] This in turn interferes with normal communication between neurons, leading to expansive, global alterations in brain functioning that are ultimately responsible for the neurological and behavioral symptoms associated with the disorder.

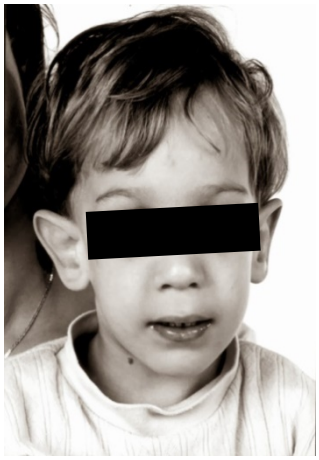


Figure 2. Crano-facial features of a child with FXS. Image credit: Fengler et. al, 2002.

Though great efforts have been made to shed light on the complex neurological processes underlying FXS, no direct cure for the disorder exists. Current treatment approaches generally involve a combination of behavioral therapy, as well as lifelong medication regimens aimed at

individual symptoms,[6] as further discussed in Section 1.6.1. However, the need for effective treatments for FXS remains imperative, not only due to its substantial impact on the quality of life for the patient, but also due to its great socioeconomic impact. Highlighting this point, a 1992 survey estimated that the average lifetime cost for the care of a single FXS patient could total to roughly $2 million USD (at the time of the study).[14] In 2002, this was calculated to translate to an out-of-pocket medical cost to families of $17,016 annually, when costs of hospitalizations, therapies, physician visits, and medications were taken into consideration. Compounding the problem, the majority of caregivers for FXS patients report experiencing employment problems due to the demands of caring for someone with the disability.[15] Perhaps most disheartening of all, only 9% of men and 44% of women with FXS are able to reach a high level of independence in adulthood, with the majority of patients requiring daily assistance for life.[8] When all of these difficulties are taken together, therefore, it is perhaps unsurprising that mothers of children with FXS have drastically elevated rates of mood disorders (43%) compared to mothers in the general population (13%).[16] Thus, although FXS lacks the attention received by many other neurological disorders, its tremendous impact on patients, families, and wider society cannot be denied, and the need for therapeutic advancements is high.

1.1.3. The Fragile X Mutation

At its core, FXS is characterized by a pervasive central synaptopathy, in turn manifesting as the broad range of neurological and behavioral symptoms overviewed in Section 1.1.2. In over 99% of cases, FXS is caused by a CGG repeat expansion mutation in the *Fmr1* gene on the X chromosome.[17] The number of CGG repeats determines whether an individual is classified as unaffected, a premutation carrier, or a full-mutation carrier. In unaffected individuals, the *Fmr1* gene contains 5-44 repeats of the CGG codon, located in the 5' untranslated region of the gene

(Figure 3). Here, the *Fmr1* alleles are stable upon transmission and are not methylated (and hereby silenced), except when located on an inactive X chromosome in females. When 55-200 CGG repeats are present, however, the allele is considered to be in the premutation form. Like normal alleles, FXS premutation alleles are not methylated and silenced, unless they are located on an inactive X chromosome. However, when transmitted maternally, the CGG repeat carries the risk of expanding in future generations, eventually becoming the FXS-causing full mutation. While a person carrying the premutation allele does not typically present the full range of symptoms of FXS, they have a heightened risk for associated disorders such as Fragile X-Associated Tremor/Ataxia syndrome and/or Primary Ovarian Insufficiency.[1] Lastly, individuals with over ~200 repeats are considered to possess the full mutation. While the mechanism is still not fully understood, this high number of CGG repeats leads to hyper- methylation of the repeats

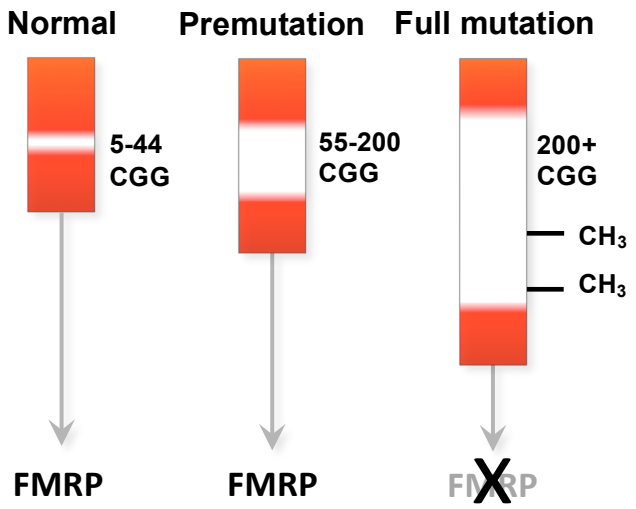


Figure 3. Schematic of variations in the Fmr1 gene. Left: In unaffected individuals, the Fmr1 gene contains 5-44 repeats of the CGG codon, located in the 5' untranslated region of the gene. Here, the Fmr1 alleles are stable upon transmission and are not methylated. Middle: When 55-200 CGG repeats are present, the allele is considered to be in the premutation form. Like the normal allele, the premutation is not methylated; however, it can expand upon maternal transmission. Right: Individuals with over 200 repeats are considered to possess the full mutation. This leads to extensive hyper-methylation of the CGG repeat, causing silencing of the gene. Image adapted from FRAXA.org.

and upstream CpG islands, resulting in silencing of the gene and loss of its product, Fragile X Mental Retardation Protein (FMRP).[18] FMRP is a neuronal RNA-binding protein believed to be involved in post-transcriptional regulation of gene expression. Downstream consequences of the loss of FMRP and its regulatory activities are generally accepted to underlie the pathophysiology of FXS. Bolstering this hypothesis, while the CGG expansion mutation accounts for the overwhelming majority of FXS cases, one case has been reported where a missense mutation was instead discovered in one of FMRP's RNA binding domains (the KH2 complex),[19] which has been found to be critical for stabilizing FMRP-RNA interactions.[20]

Significant efforts towards unraveling the mechanisms underlying *Fmr1* methylation and silencing have been made in recent years. To begin, we have learned that typically, in mammals, FMRP is most abundantly expressed in the central nervous system, as well as within the testes.[21, 22] In the mouse brain, expression levels of FMRP peak at around 1 - 2 weeks postnatally, before gradually declining.[23, 24] This period is believed to correspond to the infancy stage in humans. Methylation and silencing of the expanded *Fmr1* CGG repeat is a dynamic process that begins early in development. Studies of chorionic villi samples from human fetuses harboring the full mutation have revealed that the expanded repeat is methylated to an increasing degree as development progresses.[25] Southern blotting and MALDI-TOF studies have advanced as far as to pinpoint the main methylation site that is linked to blockade of FMRP expression: the FREE2 sequence, located within intron 1 of the gene.[26] Interestingly, mosaicism of CGG repeat length (and consequently, methylation status), is a well-described phenomenon in full-mutation carriers.[27-32] In most patients, variations in repeat length are apparent in all tissues. This phenomenon is especially pronounced in ~15% of patients, which are conveniently termed "mosaics." This mosaicism is believed to account for the wide variability in symptoms and

severity of the syndrome from one patient to the next. Moreover, interestingly, a subset of high-functioning patients have been found to express un-methylated premutation bands,[33] which presumably yield functional FMRP. However, these patients still express deficient levels of the protein overall compared to their neurotypical counterparts, resulting in the symptoms associated with FXS.

1.1.4. FMRP: Structure and Function

Elucidating the precise mechanisms of FMRP's function is undoubtedly key to deciphering its role in more complex processes at the level of synapses, circuits, and behavior. Correspondingly, the precise function of FMRP has been a subject of extensive study over recent decades. To begin, it has been found that within the central nervous system, FMRP is predominantly expressed in neurons, less so in glial cells.[21, 24, 34, 35] Within these neurons, FMRP is primarily localized within the cell body, dendrites, and synaptic compartments. While the latter especially points to a role in neuronal signaling, what exactly is FMRP's function?

The current hypothesis proposes a major role for FMRP in translational control. What evidence supports this idea? First, the majority of the protein (~85%) has been found to associate with actively-translating polyribosomes.[36] Second, FMRP contains three domains that mediate interactions with mRNA: two tandem K-homology (KH) domains, and one C-terminal RGG box.[37] While the KH2 domains of FMRP have been shown to bind to mRNA kissing complex motifs, the RGG box instead binds to G-quartet motifs. Additionally, it has been found that the RGG box of FMRP also binds to a novel motif found within Superoxide Dismutase 1 (Sod1) mRNA, referred to as SoSLIP.[38] (This particular motif consists of an arrangement of three independent, successive stem-loop structures, separated by short, single-stranded regions.) Taken together, these findings point to a role of FMRP in the translational regulation of a wide variety

of mRNAs, and that FMRP-mRNA recognition appears to depend largely on mRNA secondary and tertiary structure rather than nucleotide sequence alone.

The precise role of FMRP in translational regulation appears to be a fascinatingly complex one. Unfortunately, as a result, many of the molecular mechanisms behind FMRP's function still remain unclear. One prominent early theory of FMRP function was that FMRP shuttles newly-transcribed mRNAs from the nucleus to post-synaptic sites in the cell, presumably to facilitate translation. Supporting this hypothesis, FMRP has been found to not only contain a nuclear export signal (NES) and a Rev-like export signal,[39] but has also been demonstrated to be capable of shuttling between the nucleus and cytoplasm.[40]

FMRP also appears to be involved in mRNA trafficking along dendrites and axons. Within cells, mRNAs are co-transported with ribosomal subunits, translation factors, and other translation machinery within motile compartments called RNA granules. Studies have demonstrated that FMRP is found within such RNA granules, where it serves as a "molecular adaptor" between these granules and kinesin-II motor complexes, which transport the granules to their final destination in the cell.[41, 42]

Perhaps of greatest impact, however, recent ground-breaking work has suggested that FMRP may in fact be involved in translational inhibition as well (Figure 4). Specifically, it has been found that FMRP is capable of hampering mRNA translation by binding to polyribosomes, and consequently, blocking the elongation step of translation.[43] In this process, FMRP appears to interrupt ribosomal translocation - the synchronized movement of tRNA and its associated mRNA through the ribosome, thereby advancing the translational reading frame - by complexing with target mRNAs and the ribosomes associated with them. Thus, FMRP may, among other roles, function as a general repressor of translation. However, this role might be restricted to

specific mRNAs, and it has been suggested that FMRP may have opposing effects on translation depending on the mRNA target.

Nevertheless, many of the finer details regarding how FMRP regulates the translation of its target mRNAs have yet to be clarified. However, some important clues have been uncovered in recent years. It has been found that KH binding with mRNA kissing complexes, in addition to RGG binding with G-quartets, are generally associated with translational suppression,[20, 44-46] whereas RGG binding with SoSLIP is associated with translational enhancement.[38] Moreover, FMRP is subject to post-translational modifications that may influence the direction of translational regulation – phosphorylated FMRP is associated with stalled ribosomal translocation and inhibition of translation, whereas de-phosphorylation of FMRP is instead associated with the upregulation of translation.[47] FMRP phosphorylation, in turn, has been shown to be bi-directionally regulated in response to synaptic activity.[48, 49] Thus, the role of

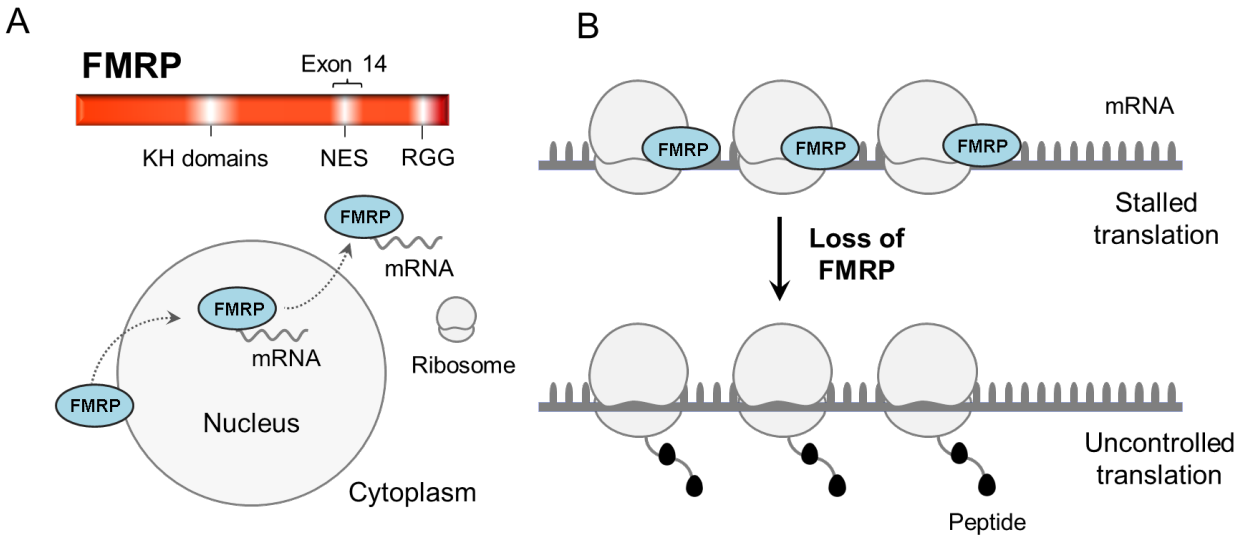


Figure 4. Schematic depicting mechanisms by which FMRP may regulate neuronal translation. (A) For some mRNA targets, FMRP may facilitate nuclear export to enhance translation. (B) For other mRNA targets, FMRP appears to interrupt ribosomal translocation complexing with its target mRNAs and associated ribosomes. Image credit: adapted from Fridell et. al. 1996; Darnell and Klann, 2015.

FMRP in translational regulation appears to be rather multi-faceted.

Regardless of this, due to the extensive evidence pointing to a regulatory role of FMRP in neuronal gene expression, a great deal of effort has been devoted to identifying the protein's specific mRNA binding partners. Invaluable immunoprecipitation studies have revealed that FMRP binds to the coding region of a diverse host of transcripts, encoding roughly 1,000 different proteins, many of which are pre- and post-synaptic (for a complete list, see Darnell et. al, 2011).[43] For instance, several of FMRP's target transcripts are associated with pathways involved in synaptic long term potentiation (LTP) and long term depression (LTD), through both glutamate and GABA receptor signaling. Further complicating the molecular mechanisms underlying FXS, signaling pathways associated with FMRP's targets are also involved in gene expression, regulation of the cell cycle, and programmed cell death – including, but not limited to CREB, calcium, PKA, PKC, G-protein coupled receptor (GPCR), RhoA, cAMP, and P13K/Akt signaling.

As one might imagine, given the broad range of proteins that are translationally regulated by FMRP, loss of FMRP can undoubtedly create cascading effects on larger neural networks, ultimately impacting cognition and behavior. The currently known mRNA binding partners of FMRP code for a multitude of intracellular signaling molecules that interact in elaborate and intricate ways that are still only beginning to be understood. Thus, despite being a single-gene disorder, the molecular underpinnings of FXS are extremely complex.

1.2. FXS is a Disorder of Brain Development

1.2.1. Synaptic Changes Associated with FXS

What exactly are the consequences of the FXS mutation and therefore, FMRP deficiency, in the brain? It is a simple question, but one that researchers have spent decades attempting to

answer. However, it has long been found that the major underlying cause of aberrant brain function in FXS is a pervasive central synaptopathy, marked by an overabundance of immature dendritic spines and aberrant synaptic transmission.[12, 13]

The dendritic spine is widely regarded as the cardinal anatomical substrate of synaptic plasticity, or the ability of synapses to modify their strength in response to changes in their activity.[50-53] Dendritic spines themselves are small (0.01 – 0.8 cu μm), membranous protrusions located on the dendrites of post-synaptic neurons. Nearly all dendritic spines possess a general "door-knob" like structure consisting of two compartments, termed the head and the neck. The head refers to the tip of the spine, upon which an incoming axon can terminate to form a synaptic contact. Localized within the head of the spine is the post-synaptic density: a complex membrane-associated structure containing neurotransmitter receptors, channels, and signaling proteins. The neck, in contrast, connects the head of the spine to the parental dendrite, extending up a few micrometers in length. Dendritic spines can be further classified by their shape as one of five conventional types: thin, stubby, mushroom, cup-shaped, or filopodia, which are widely believed to be spine precursors (Figure 5).[54] However, the prevailing theory is that dendritic spines actually exist as part of a continuum of these four types. Because the head of the spine is separated from the parent dendrite by the neck, each spine can form a local, synapse-specific compartment that is capable of segregating postsynaptic chemical responses, such as calcium

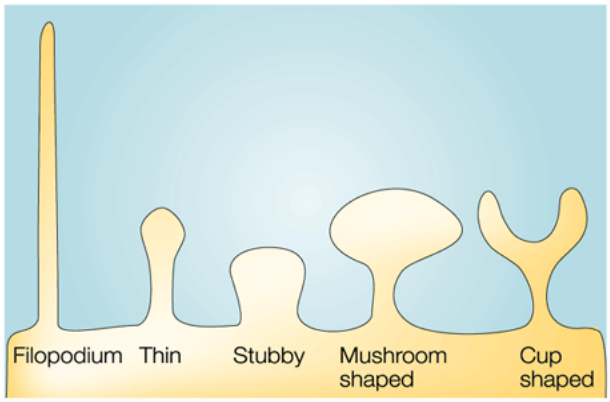


Figure 5. Dendritic spine classification. Dendritic spines are generally classified by their shape as one of five conventional types: thin, stubby, mushroom, cup-shaped, or filopodia (spine precursors). Image credit: Hering and Sheng, 2001.[52]

waves.[54-58] This isolation from the parent dendrite allows for input-specific synaptic plasticity restricted to independent, individual spine heads. Thus, dendritic spines act as functional integrative units within synapses, where synaptic inputs from each spine head can be integrated to collectively modulate the entire postsynaptic neuron's response to incoming signals. It has long been thought that through changes in their morphology, density, and distribution on a post-synaptic neuron, dendritic spines act as important modulators of neuronal responses to incoming stimuli.

Importantly, a spine's morphology is tightly linked to the functional properties of its synapse. Correspondingly, the postnatal maturation of the brain involves a dramatic shift in spine morphology profiles from immature to mature, as the brain adapts to its surroundings. However, these morphological profiles appear to vary greatly depending on the brain region. For example, the maturation of the cortex entails a shift in the distribution of spines from predominantly long and thin, to short and stubby.[59-62] In pyramidal neurons of the hippocampus, however, the opposite appears to hold true - young animals have an abundance of stubby and mushroom-shaped spines, whereas adults seem to predominantly display long, thin ones.[63]

FMRP appears to play an essential role in modulating dendritic spine architecture, and therefore, synaptic function and plasticity. First, FMRP is normally enriched within dendritic spines.[35] Secondly, FMRP appears to regulate the translation of a multitude of post-synaptic proteins, including receptors, channels, scaffolding proteins, and synaptic adhesion proteins, as well as a variety of intracellular signaling proteins.[43] Lastly, FMRP itself is dynamically regulated by synaptic activity – FMRP itself is translated, degraded, and post-translationally modified in response to synaptic inputs.[35] FMRP and associated mRNAs are also co-transported into dendrites both under basal conditions, as well as in response to synaptic activity.[64-66]

Moreover, FMRP's synthesis coincides with the time-scale of the induction of stable synaptic plasticity (10 – 30 minutes).[67]

Dendritic spine dysmorphogenesis is considered the hallmark neuroanatomical feature of FXS.[13] Most intriguingly, dendritic spine aberrations observed in FXS patients are reminiscent of the developing brain. For instance, post-mortem analyses of human tissue from FXS patients have revealed an increased density of cortical dendritic spines compared to neurotypical controls.[68] This observation is reminiscent of the early postnatal brain, prior to synaptic pruning. Moreover, an elevated proportion of these spines assume the long, thin morphology that is associated with spine immaturity within the cortex (Figure 6),[68-70] and these findings have been paralleled in *Fmr1* knockout mice[71, 72] Similar observations have also been made within the hippocampus – *Fmr1* KO pyramidal neurons display a heightened proportion of immature spines; however, here the total spine density is unchanged.[73]

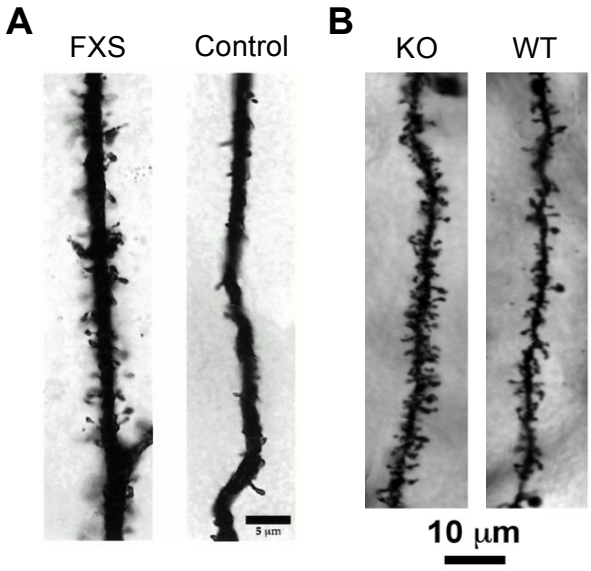


Figure 6. Representative photo-micrographs of cortical dendritic spines obtained from (A) human FXS patient compared to neurotypical control; (B) Fmr1 knockout mouse (KO) versus wild-type control (WT). Irwin et. al, 2000,[10] Hayashi, 2007.[261]

Based on these findings, the general consensus is that FXS results from the uncontrolled expression of dosage-sensitive genes that are required for normal dendritic spine development, remodeling, and transmission. Concisely put, in patients with the full-blown *Fmr1* mutation, FMRP is under-expressed, and as a result, mRNA translation of proteins essential for normal synaptic plasticity becomes dysregulated. As a result, the net global effect appears to be one where proteins required for

normal synaptic function may be under-expressed, whereas other proteins may be overexpressed when they are not required.

What, then, are the functional consequences of FMRP deficiency within synapses? Dendritic spine morphology is exceptionally plastic, and closely associated with a spine's functional properties. Plastic changes in dendritic spines are thought to adjust synapse function and strength, subsequently governing information storage and processing in the brain.[50-54, 60] As touched on in the previous section, one major identified consequence of FMRP silencing is that it perturbs the processes of long-term potentiation and long-term depression,[74] two major mechanisms of synaptic plasticity that are widely believed to be the molecular correlates of cognition and emotion. Long-term potentiation (LTP) refers to a persisting increase in synaptic strength that occurs with repeated, high frequency stimulation, capable of lasting for hours or longer. In contrast, long-term depression (LTD) is recognized as the weakening of synaptic connections, typically in response to low frequency stimulation. Together, LTP and LTD work in concert to permit bimodal, activity-dependent adjustments in synaptic strength. Accordingly, memories are believed to be encoded by modifications in synaptic strength through the coordination of both LTP and LTD.[75] In essence, LTP is thought to provide a mechanism through which new memory traces can be formed and retained in the brain. On the other hand, LTD appears to selectively weaken synapses so that cells can make practical use of LTP - if all synapses were permitted to continue increasing in strength, they would eventually reach a ceiling level of efficiency, which would prevent the encoding of new information. At the molecular level, LTP is believed to result in part from the activation of calcium-sensitive kinases that phosphorylate various protein targets to ultimately increase sensitivity of the post-synaptic site to incoming excitatory signals (for example, CAMKII). Oppositely, LTD is thought to arise from

the activation of calcium-sensitive phosphatases that may instead dephosphorylate these targets.[76]

Widespread changes in LTP and LTD have been associated with FXS. In *Fmr1* KO mice, marked deficits of LTP have been reported in the cortex, including the prefrontal, somatosensory, and visual cortices.[77-81] Decreases in LTP magnitude have also been reported in the hippocampus.[82, 83] On the other hand, metabotropic glutamate receptor-dependent LTD (mGluR-LTD) has been found to be enhanced in the hippocampus and cerebellum in the absence of FMRP[84-86]. The latter phenomenon in particular has gained a great deal of attention in recent years due to the potential of group I mGluR antagonists as a therapeutic strategy for FXS. Unfortunately, however, this strategy has not yet demonstrated meaningful improvements in the human patients, compared to placebos.[87] Nevertheless, en masse, these observations suggest that FMRP plays an essential role in dendritic spine maturation, stabilization, and plasticity; and absence of FMRP results in stunted spine maturation and altered synaptic signaling.

1.2.2. FXS Arises From Changes in Synaptic Function During Brain Wiring

Considering that FMRP expression peaks during the early postnatal period, one of its most important functions is presumably to direct the development and function of young synapses as they undergo dynamic changes during postnatal brain maturation. Importantly, alterations in synaptic plasticity and transmission during early life may have an especially deleterious effect on the brain. Synaptic function must be tightly controlled to ensure appropriate levels of neuronal signaling during the early stages of brain wiring.. Subsequently, FXS is believed to stem from disturbances in normal brain maturation, due to inherent changes in synaptic functioning. However, while the anatomical and functional abnormalities of synapses in FXS are well described, less is known about circuit-level alterations that ultimately lead to the

behavioral symptoms of FXS. As of yet, it is still unclear how FXS-related changes in LTP and LTD may alter the activity of larger neuronal networks. However, it is known that the activity of a circuit is influenced by the balance of net excitatory and inhibitory synaptic activity within that circuit. This balance of excitation and inhibition is a delicate one, and is crucial for precise nervous system development and functioning.

Importantly in the context of FXS, the functions of neuronal circuits are greatly shaped by their activity during critical, or sensitive, periods during development. Critical periods are restricted time windows in which a brain region or circuit is most receptive for a developmental stage to occur, often in response to a stimulus - for instance, an environmental influence. The presence of the stimulus triggers the brain region or circuit to undergo the developmental changes, and thus become altered in a profound and often permanent way. However, the absence of the stimulus during this period of time leads to aberrant development, or even a lack of development altogether. Moreover, after the window closes, the developmental stage is usually no longer able to occur. A very well-studied example is the development of the visual cortex, first described in the ground-breaking work of Wiesel and Hubel in 1963.[88] In their studies, Wiesel and Hubel discovered that neurons in the cat visual cortex were most responsive to visual inputs at an early age, and that this responsiveness disappeared after the cat reached maturity. They further demonstrated that if cats had one eye deprived of light from birth to three months old, they only developed vision in their un-deprived eye. After the three months had elapsed, the cats were no longer able to see from the deprived eye at all.

In the case of FXS, it is believed that synaptic plasticity is altered during critical periods of brain wiring in regions that contribute to cognition, behavior, and sensory processing; such as connections within and between the cortex, hippocampus, amygdala, and hypothalamus. In this

scenario, stimuli needed to trigger developmental changes in the brain may not necessarily be missing, but the synaptic machinery required to respond to such stimuli are impaired, or may respond inappropriately to a stimulus. Importantly, once a few of such improper connections have formed, they may lead to further inappropriate connections during early learning, thereby amplifying the defect. Moreover, these effects on the initial wiring of the brain may be to a large extent irreversible upon reaching adulthood, highlighting the need for early intervention strategies that target the root of the malady as opposed to the symptoms.

1.2.3. Circuit-Level Hyper-Excitability is Associated with FXS

As described, FXS is marked by the over-abundance of immature synapses across multiple brain regions. As a result, the FXS brain is considered reminiscent of the developing brain. Importantly, a wealth of studies have shown that the immature brain is much more excitable than the mature brain; with much of this work focusing on the hippocampus and cortex as model systems.[89, 90] However, whether this increase in excitability involves mainly excitatory or inhibitory neurons or synapses, remains unclear. In the first few days of life in rodents, the neurotransmitter GABA exerts an excitatory effect due to high intracellular chloride levels established by the Na-K-Cl cotransporter NKCC.[91] However, studies in which fast GABAergic transmission is blocked have demonstrated an important role of glutamatergic AMPA receptors in this hyper-excitability, as opposed to GABA receptors alone.[92] Supporting this hypothesis, during the first few weeks of life, there is an over-abundance of AMPA receptors in the brain.[93, 94] The resulting increase in circuit-level excitability in the immature brain, relative to the mature brain, is believed to be the reason why the early postnatal brain is far more susceptible to seizures than the adult brain.[95, 96]

This observation is especially interesting in the context of FXS because increasing

evidence has pointed to the possibility of circuit hyper-excitability in the Fragile X brain. At first glance, many of the disorder's symptoms, such as hyperactivity, sensory hypersensitivity, hyperarousal, and enhanced seizure susceptibility, appear to reflect elevated excitability of different brain regions. Interestingly, studies on the *Fmr1* knockout (KO) mouse model of FXS have provided evidence that this notion is indeed the case. For one, *Fmr1* KO mice demonstrate higher synchrony of cortical network activity, as determined by calcium imaging studies,[97] and higher hippocampal excitability, as determined by electrophysiological studies.[85] Secondly, *Fmr1* KO mice exhibit longer spontaneously-evoked Up states, brief periods of local network activity marked by tonic, synchronous firing between groups of neighboring neurons.[98] Third, *Fmr1* KO cortical neurons demonstrate higher firing rates during these Up states.[97, 99]

Unfortunately, human studies in this area are still few and far between. However, a recent EEG study has reported that FXS patients exhibited greater power in the cortical gamma frequency band,[100] indicating heightened neuronal activity.[101] Interestingly, this phenomenon was shown to correlate with difficulties in sensory and social processing in the patients. This data is consistent with mouse studies reporting reduced excitatory drive to inputs to fast-spiking inhibitory interneurons which normally synchronize and regulate gamma band neural activity.[102, 103] Additionally, FXS patients have also been shown to demonstrate heightened theta rhythms,[104, 105] which have been associated with working memory,[106] and might explain information processing deficits in these individuals.[107]

The hyper-excitable state of the Fragile X brain unquestionably has consequences on brain function, but these consequences are still only beginning to be understood. The most clear manifestation of this phenomenon is the high occurrence of seizures in FXS patients. Seizures are thought to arise from excessive electrical activity in the brain, and it is estimated that at least

20% of FXS patients experience them.[108, 109] Interestingly, the majority of FXS patients with a seizure disorder display an EEG pattern characterized by centro-temporal spikes, in a similar manner to those observed in Benign Rolandic Epilepsy of Childhood (BREC).[110, 111] However, while nearly all children with BREC outgrow the disorder, FXS patients often do not, supporting the idea that the FXS brain remains in an immature state throughout the lifespan.

Another likely consequence of circuit hyper-excitability in FXS is sensory hyper-sensitivity. Specifically, this feature of FXS is believed to result from abnormally increased cortical responses to sensory stimuli. For instance, a host of studies have found that FXS patients display abnormally heightened EEG responses to sound (see Rotschafer and Razak, 2014 for a review).[112] Consistent with human data, *Fmr1* KO mice reliably demonstrated enhanced auditory startle responses,[113-115] paired-pulse-inhibition,[116, 117] and a heightened propensity for audiogenic seizures.[113, 118]

Lastly, the concept of hyper-excitability in the FXS brain is an interesting one because it may also potentially contribute to the exaggerated anxiety levels associated with the syndrome. In this scenario, one likely culprit may be hippocampal over-excitation. As will be described in the upcoming sections of this literature review, the hippocampus is an important regulator of the stress response and anxiety levels through its inputs to the hypothalamus. While this regulation is quite multifaceted, it has been shown that the hippocampus sends inhibitory projections to the parvocellular subdivision of the paraventricular nucleus (PVN), which contains centrally projecting, oxytocin producing neurons.[119, 120, 164] Therefore, if activity of the hippocampus were increased in FXS, this could result in excess inhibition of these PVN neurons, and consequently, suppression of oxytocin production. Given the importance of oxytocin in social behavior and anxiety (discussed further in Section 1.5.2), hyper-activity of the hippocampus could potentially

result in abnormalities in both of these behavioral domains in FXS. Moreover, as will be described in the next few sections, many other symptoms of FXS are attributed to excess anxiety, and this is feature of FXS differentiates its behavioral phenotype from that idiopathic autism. Thus, targeting hippocampal hyper-excitability may prove effective in rescuing a significant portion of the FXS behavioral phenotype.

1.3. FXS vs. Idiopathic Autism

Before continuing, at this point it is important to address the connection between FXS and idiopathic autism. Most intriguingly, approximately 46% of male and 15% of female FXS patients are co-diagnosed with an autism spectrum disorder (ASD).[1] Moreover, FXS is the most common monogenic cause of ASD. As a result, FXS has attracted considerable interest for its potential value in uncovering some of the neurobiological underpinnings of idiopathic autism.

On the surface, FXS and idiopathic autism appear to share many similarities. Numerous symptoms of FXS seem to overlap with the symptoms of idiopathic autism, including the core deficits in sociability, communication, and the presence of stereotyped behaviors.[1, 8] A considerable overlap also exists between the secondary symptoms, including anxiety, hyper-activity, sleep disorders, and seizure susceptibility. However, a growing body of evidence suggests that FXS and idiopathic autism may not be as comparable as they first seem.

To begin, the first and foremost difference between FXS and idiopathic autism lies in the etiology of the two disorders. At present, idiopathic autism is believed to be due to multiple, varied causes – some genetic, some environmental, and others unknown – that lead to similar changes in behavioral outcomes.[121, 122] Thus, ASD relies primarily on a behavioral diagnosis based on observations by parents, caretakers and clinicians. On the other hand, FXS relies on a medical diagnosis as all cases stem from a single, identifiable cause – mutation of the *Fmr1*

gene.

Nevertheless, the behavioral symptoms of FXS and idiopathic autism may appear identical at first glance. Could this mean that mutations in ASD-associated genes lead to similar outcomes as the FXS mutation? While further study is still needed to answer this question, growing evidence seems to suggest that the behavioral symptoms of the two disorders may, for a large portion of the patients, actually originate from rather distinct biopsychosocial pathways. For example, let us consider the shared core deficit in social interaction. Classically, individuals with autism are described to live in their own world, unaware of or indifferent to the social situations surrounding them, and hence may rarely seek out social interaction.[121-123] Of course, this is certainly not the case for *all* individuals with ASD, especially high-functioning patients. Nevertheless, this phenomenon is considered the general underlying cause for the social deficits associated with ASD. On the other hand, many patients with FXS are generally not only socially alert, but in fact often yearn for interaction with others.[124, 125] Despite this, however, FXS patients are often so plagued by severe social anxiety that they tend to avoid social situations altogether.[1]

Gaze avoidance is yet another shared feature of both disorders that may stem from distinct origins. Many patients with idiopathic ASD exhibit deficits in eye contact because they are indifferent to social cues,[121-123] while others may find eye-contact to be over-stimulating and therefore, aversive. On the other hand, patients with FXS typically avoid eye contact as a direct result of their social anxiety.[1] A major study illustrating this difference examined eye movements in patients with either FXS or idiopathic ASD.[126] It was found that when the ASD patients were presented with images of human faces, their gaze path was for the most part, random. On the other hand, when FXS patients were presented with the same images, they would initially glance at the eyes of the faces, but then quickly look away – a strong indicator of social anxiety.

Along these lines, patients with autism often have deficits in theory of mind – the ability to infer what another person is thinking or feeling, in order to see things from their point of view.[121-123, 127] However, this is usually not the case for patients with FXS. Highlighting this point, in the aforementioned gaze-tracking study, FXS and ASD patients displayed striking differences in brain activation when presented with images of emotional faces. Interestingly, it was revealed that the FXS group, compared to the ASD group, displayed elevated activity within the hippocampus, superior temporal gyrus, insula, and post-central gyrus, all areas associated with social and/or emotional processing.

Lastly, let us consider the shared core symptom of repetitive and stereotyped movements, such as rocking or hand-flapping. Because these actions are self-stimulating, engagement in these behaviors are colloquially referred to as "stimming." For patients with idiopathic autism, stimming behavior can be attributed to multiple, varied reasons.[128] One theory is that such behaviors promote the release of beta endorphins within the nervous system, producing a calming effect. Another explanation is that for some patients, stimming may instead help to regulate sensory input. For example, stimming may provide sensory stimulation for patients who feel under-stimulated, or alternatively, can soothe patients who feel over-stimulated. In other cases, stimming can also take on the form of self-injurious behavior. This form of stimming may, at times, be communicative in nature. For instance, a patient may engage in head-banging or other forms of self -injury because they are frustrated and unable to express what they are thinking or feeling. In individuals with FXS, on the other hand, stereotypic behaviors are almost always provoked by anxiety, hyper-arousal, or hyper-sensitivity to sensory stimuli.[1] In these cases, stimming helps to calm the patient and/or reduce sensory overload. For example, a FXS patient may stim because he/she perceives their environment to be too loud or too crowded, or

may feel overwhelmed by a stressful social encounter.

Taken together, these findings suggest that while, on the surface, individuals with FXS and ASD may display similar behavioral profiles, on closer inspection, it is apparent that these behaviors stem from substantially different psycho-emotional and motivational factors. Again, let it be noted that this is not the case for all individuals with FXS or ASD: as a counter-point, one study reported that 33% of children with FXS between the ages of 21 to 48 months were indistinguishable from children of the same age with idiopathic autism.[129] However, at this young age, it may be difficult to discern the underlying causes of behavioral issues such as gaze aversion or stereotypies.

The extent of the overlap between FXS and idiopathic ASD, at the neurobiological level, still remain unclear. Studies comparing FXS and ASD are uncommon, and therefore, results must be interpreted cautiously. However, it is known that a few key differences generally exist between FXS and ASD brains, both in structure and function. Structurally, children with FXS display greater volumes of white matter in the temporal lobe, grey matter in the cerebellum and caudate nucleus, and reduced volume in the amygdala, compared to neurotypical controls.[130] On the other hand, children with ASD instead display cortical enlargement compared to controls. Functionally, it has been found through EEG studies that ASD patients have reduced long-range activity between the frontal lobe and the rest of the cortex[131,132] as well as potentially reduced local connectivity within the cortex.[133] In a different vein, FXS patients display increased long and short ranged connectivity.[104]

Thus, FXS and idiopathic ASD are not entirely discrete, nor are they entirely disconnected. Many genes that are regulated by FMRP may overlap with genes altered in ASD.[43] Additionally, FMRP may regulate proteins with similar regulatory functions as those affected in

ASD. Thus, different neurological etiologies may result in a similar developmental course for some patients. Conversely, patients may have different behavioral outcomes depending on the genes and pathways affected, as well as the individual's environmental experiences. Nevertheless, the general consensus is that most behavioral symptoms associated with FXS are traceable to excessive anxiety and hyper-arousal, whereas the pathology of ASD is much more varied and multi-faceted. Consequently, such distinctions are important to bear in mind while developing therapeutic approaches geared specifically toward either disorder.

1.4. Anxiety, a Prominent Feature of FXS

1.4.1. What is Anxiety?

One of the most prominent features of FXS is the presence of excessive anxiety. It is estimated that anxiety disorders occur in 70% of male FXS patients and 56% of female patients.[1] Moreover, in many cases, numerous behavioral symptoms of FXS – such as social deficits, gaze aversion, and stereotypies, for instance – are believed to be, at their core, anxiety-driven.

Anxiety is a general term for the emotional state characterized by feelings of persistent worry, tension, and unease.[134] Like most emotions, these feelings may range from mild to severe in their intensity. For healthy individuals, anxiety is a normal reaction to stimuli that may prove threatening or dangerous. Thus, anxiety is useful from an evolutionary standpoint in that it serves a protective function, motivating an organism to escape or address the source of the anxiety. Importantly, a distinction must be made between anxiety and stress. Stress generally refers to the emotional and physiological response to the presence of a stress-causing factor ("stressor"). While stress typically disappears after the stressful situation is over, anxiety is a sustained state that can persist even long after the stressor is gone.

No single person is free from anxiety, yet how it affects an individual is relative. Anxiety

is considered pathological when it is persistent, disproportionate to the cause, and/or disruptive to the extent where it may interfere with daily functioning (such as performance at work, or interpersonal relationships). In many cases, anxiety may manifest even in the absence of an identifiable threat. These conditions are collectively termed anxiety disorders, and altogether, are the most common mental illnesses in the United States, with 40 million Americans affected.[135] Among the most prevalent anxiety disorders are generalized anxiety disorder and social anxiety disorder. Generalized anxiety disorder is characterized by persistent, excessive, and exaggerated worry that may not necessarily be specific to one particular event or issue.[136] On the other hand, social anxiety disorder refers to the specific, pervasive fear of social situations.[137] For instance, a person with social anxiety disorder may feel highly anxious about being embarrassed, judged, or rejected by others; and as a result,, tend to avoid social situations whenever possible, even if doing so negatively impacts the person's ability to lead a normal life. Both generalized and social anxiety are frequently associated with FXS.[1]

1.4.2. Anxiety Detection in Rodents

Recent years have witnessed an explosion of interest in anxiety disorders and their neurological correlates. Many of the resulting studies have employed the use of rodent models to identify anxiety's molecular underpinnings. One would be correct in thinking that anxiety detection in rodents would likely be a challenging task – how can one truly peer into the mind of a mouse? In truth, it is impossible to know what a rodent is thinking without either being one, or being able to communicate with one. However, the manipulation of specific circuit components in model organisms is necessary to gain a mechanistic understanding of the neurobiological underpinnings of anxiety. As a result, quantitative measurements of rodent behaviors that meet the criteria for validity – namely face, predictive, and construct validity – have been historically

well-used and accepted in the study of anxiety disorders.[138]

In general, anxiety assays for rodents are based on approach-avoidance conflict tasks.[138] These tasks capitalize on the conflicting drives of rodents to explore novel environments, yet at the same time, avoid open spaces where they may be more vulnerable to danger (for example, predation). The gold standard for the detection of anxiety phenotypes in rodents is the elevated plus maze (Figure 7).[139, 140] This test employs the use of a plus-shaped apparatus, consisting of two oppositely-positioned, precipice-like open arms, and two closed (walled) arms, elevated several feet above the floor. From the center of the maze, the rodent has access to all four arms of the maze. During the test, the rodent is placed within the center and allowed to explore the maze over a set interval of time, usually five to ten minutes. Time spent in and entries to the

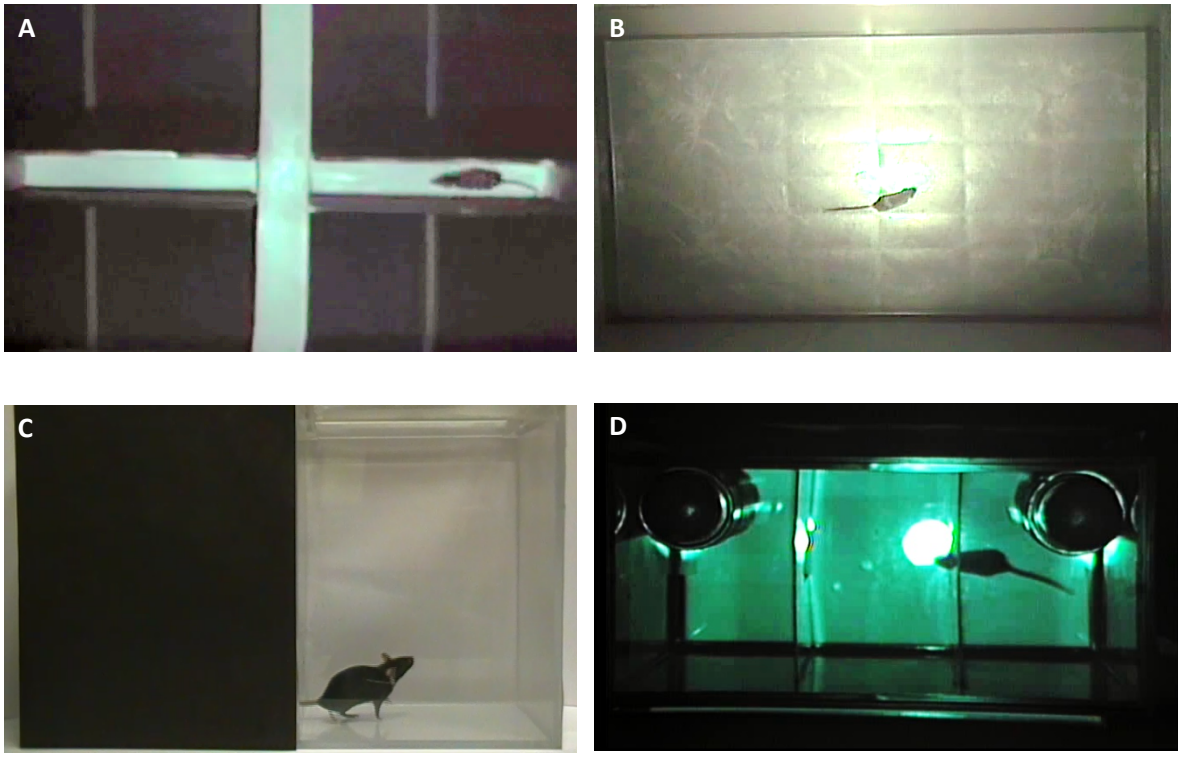


Figure 7. Common behavioral assays of anxiety in mice. (A) Elevated Plus Maze, (B) Light-Dark Box, (C) Open Field, (D)Three-Chambered Social Interaction Test, in which reduced sociability may be an indicator social anxiety, under careful interpretation.

open arms are then calculated. Rodents predisposed to anxiety will spend more time huddled within the safety of the closed arms, as opposed to venturing out into the open arms, where there is a risk of falling. This test is especially attractive to behaviorists because of its high degree of face validity, as patients with anxiety disorders tend to avoid situations deemed hazardous or risky (such as skydiving). Performance in the elevated plus maze is also sensitive to pharmacological manipulation, indicating a high degree of predictive validity. For instance, time spent in the open arms is improved by many anxiolytic drugs, such as benzodiazepine[141]; while it is reduced by anxiogenic drugs, such as caffeine,[142] phenylethylamine, and amphetamine.[143] Lastly, plasma corticosterone, a biological marker of stress, is elevated with open arm exposure.[144]

Other commonly used assays of anxiety in rodents are the open field and light-dark box.[138] Both of these tests are similar to the elevated plus maze in that they, too, capitalize on approach-avoidance conflicts. In the open field test, the rodent is allowed to explore a large, brightly-lit, enclosed arena. In such a scenario, anxious animals will spend more time against the walls of the apparatus compared to the center, where they would be more exposed to danger (such as a potential predator).[145] The light-dark box is similar in concept in that it is based on intrinsic motivation to escape a brightly illuminated environment, in which the rodent may be vulnerable or exposed, into a dark compartment.[146] Here, the rodent is placed into a smaller apparatus that consists of a brightly lit chamber and a pitch black chamber, separated by a window. The rodent is allowed to move freely between the two chambers, and time spent in either chamber is recorded. In this particular assay, anxiety-like behavior is measured by time spent in the dark chamber, where the rodent is better hidden from predators (or pesky human experimenters). Like the elevated plus maze, both the open field and light dark test have a high

degree of face and predictive validity. However, an important caveat of all these tests is that they are all dependent on normal locomotor functioning. Therefore, animal models predisposed to motor deficits or hyper-activity, cannot be accurately assessed for anxiety-like behaviors in these tests. In these cases, alternative measures of anxiety that depend less heavily on movement may be used, such as assays of neophagia (fear of novel objects or foods), or novelty-suppressed feeding, in which exposure to a new environment suppresses food consumption.

Lastly to be considered are measures of social anxiety. Unfortunately, distinguishing between autistic-like impairments in social cognition (i.e. indifference or failure to recognize social cues) versus social anxiety in rodents remains a difficult challenge. Caution must be taken to avoid jumping to the conclusion of socially-provoked anxiety in rodents without a strong basis. However, reduced sociability could potentially reflect socially-evoked anxiety in models that already demonstrate hyper-anxiety in non-social tasks. The three-chambered social interaction test is considered the gold-standard for assessing sociability in rodents.[147] In this test, the subject is placed within an apparatus divided into three accessible compartments: left, right, and center. On one far end of the apparatus, an empty wire cage is placed ("novel object"); whereas on the opposite end, a wire cage holding captive an unfamiliar mouse ("novel mouse") is placed. The subject is allowed to freely roam the apparatus, and time spent investigating either the novel object or the novel mouse is recorded. Social preference can then be assessed by calculating the ratio of time spent with the novel mouse versus the novel object. Avoidance of the novel mouse may be considered an indicator of social anxiety, although additional measures may be helpful supporting this claim, such as whether this avoidance disappears after repeated exposure to the novel mouse.

1.4.3. Neurological Basis of Anxiety

A host of studies, human and animal alike, have identified several key brain regions in the regulation of anxiety (Figure 8).[148] While our knowledge of these circuits is still limited, the advent of optogenetic techniques will likely vastly improve our understanding of anxiety disorders and their neurobiological underpinnings within the near future. Based on the current knowledge, it has been postulated that anxiety arises from complex interactions between the amygdala, bed nucleus of the stria terminalis (BNST), medial prefrontal cortex (mPFC), and ventral hippocampus.[148-151] These structures all highly interconnected, and together, register emotionally salient stimuli and coordinate both physiological and behavioral responses to them through several downstream effectors. Remarkably, these circuits are highly conserved between rodents and humans.

The basolateral amygdala (BLA) is considered the master orchestrator of the anxiety response.[152] Primarily, the BLA is believed to be involved in 'threat assessment' and the subsequent initiation of appropriate behavioral responses.[148] First, sensory inputs from the thalamus and sensory association areas are received by the BLA and processed to determine their relative level of threat. Here, these incoming sensory cues are associated, *via* Hebbian mechanisms, with predicted outcomes of either a negative or positive emotional weight. In this way, the stimulus is appraised as threatening or rewarding, thereby determining whether fear or reward pathways should be subsequently recruited. This information is then relayed to a number of different downstream pathways to direct the anxiety response, when appropriate. Threat assessment within the BLA is further refined through the BLA's dense reciprocal connections

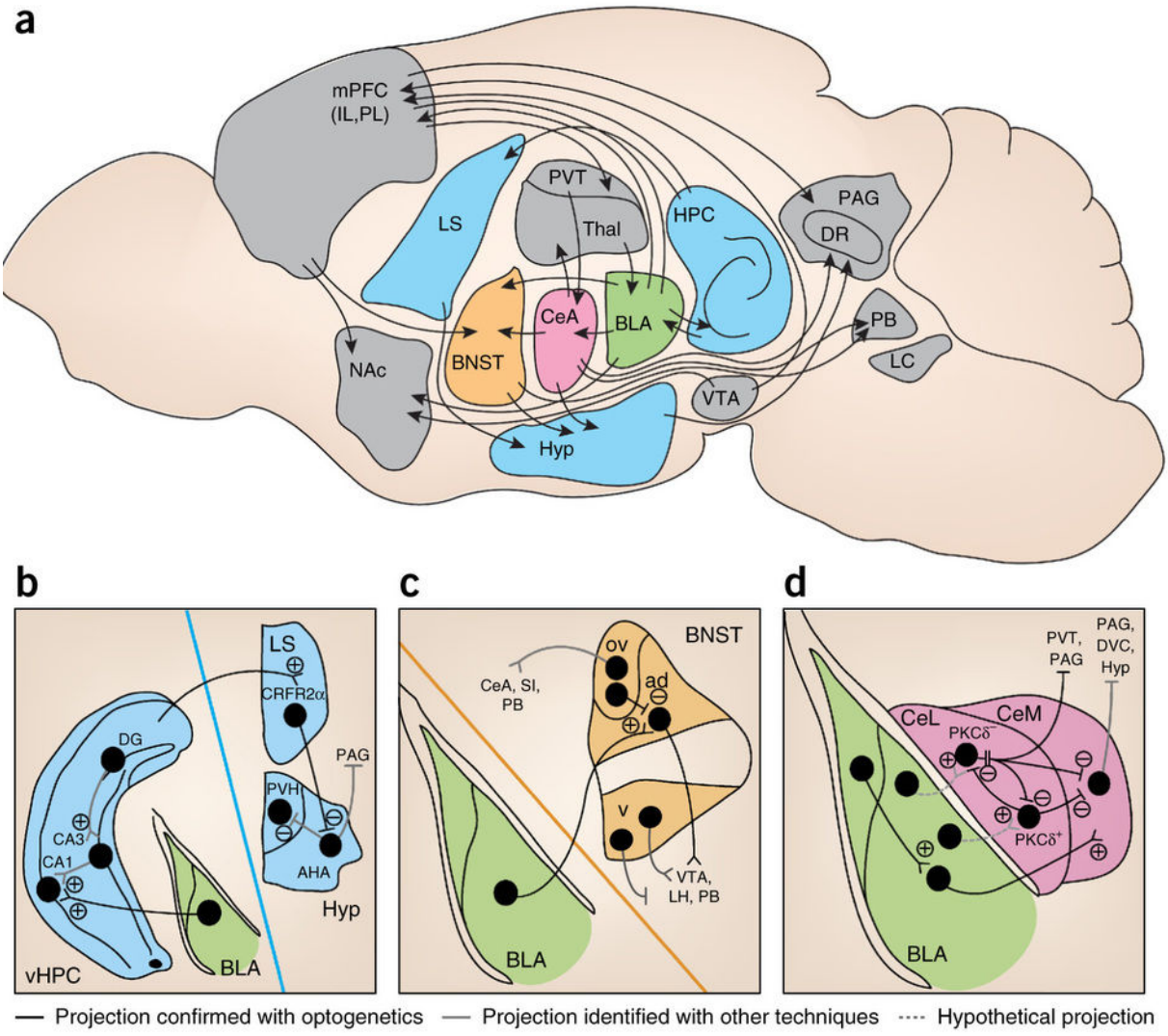


Figure 8. Major circuits involved in anxiety generation. BLA: basolateral amygdala, BNST: bed nucleus of the stria terminals, BS: brainstem, CeA: central amygdala, Hyp: hypothalamus, mPFC: medial prefrontal cortex, PAG: periaqueductal grey, vHPC: ventral hippocampus. (Image credit: Calhoon and Tye, 2015.)

with both the mPFC and ventral hippocampus. The ventral hippocampus further aids in this process *via* its own efferents to the mPFC.

In the event that a stimulus is deemed sufficiently threatening, the BLA orchestrates the anxiety response through the recruitment of several brain regions. The first of these is the local central nucleus of the amygdala, the amygdala's major output site. The central amygdala is associated with two major downstream effector pathways. First, the central amygdala projects to the brainstem to orchestrate the autonomic responses associated with anxiety, such as changes in respiration, heart rate, blood pressure, and respiration. Second, the central amygdala is speculated to be involved in conscious perception of anxiety *via* the ventral amygdalofugal pathway to the anterior cingulate, orbitofrontal, and prefrontal cortices. The other major efferent target of the BLA is the BNST. This area instead acts as a relay site to the hypothalamic-pituitary-adrenal (HPA) axis, which governs the body's neuroendocrine responses to stress.[153] Activation of the HPA axis ultimately leads to release of cortisol into the bloodstream, which prepares the body for energy expenditure and physical activity by inundating the circulation with glucose, thereby supplying an immediate energy source to the muscles needed for flight (or to fight). Importantly, the activity of the BLA is shaped not only by sensory inputs, but also through afferents from the mPFC and ventral hippocampus.[148] Projections from the mPFC are believed to influence the likelihood of a threat appraisal through fear-memory retrieval. On the other hand, projections from the ventral hippocampus appear to target fear-encoding neurons in the BLA to provoke fear renewal after extinction has taken place.[154]

Lastly, the BLA itself promotes anxiety-associated responses through direct activation of the ventral hippocampus. Specifically, excitatory projections emanating from the BLA have been found to activate pyramidal neurons in the CA1 region of the ventral hippocampus;[155] and

activation of these neurons has been shown to produce anxiety-like behavior in rodents in both elevated plus maze and open field tests.[155, 156] Similarly, lesions to the ventral, but not dorsal, hippocampus produce an anxiolytic effect.[157, 158]

1.5. The Paraventricular Nucleus is an Important Modulator of Anxiety and Social Behavior

1.5.1. The Paraventricular Nucleus of the Hypothalamus

What are the downstream effectors that drive these behavioral responses? One major player is the paraventricular nucleus (PVN) of the hypothalamus. Among other roles, the PVN is a vital component of the HPA axis that initiates the endocrine and autonomic responses to stress,[153] and thus may be of particular relevance to the pathology of FXS. Consisting of a butterfly-shaped pair of nuclei that flank either side of the third ventricle, the PVN has been recognized in recent years as one of the most important autonomic control centers of the central nervous system.[159]

The PVN is densely innervated by glutamatergic inputs from the amygdala (Figure 9).[160] In response to incoming stress signals, the medial parvocellular division of the PVN synthesizes and secretes corticotropin releasing hormone (CRH) into the median eminence, the anatomical link between the hypothalamus and the anterior pituitary gland. In response, the anterior pituitary gland secretes adrenocorticotropic hormone (ACTH) into the bloodstream, which ultimately stimulates the secretion of glucocorticoid steroid hormones from the adrenal gland. Among their many physiological roles, the glucocorticoids are the primary mediators of the body's peripheral and central responses to stress. Peripherally, glucocorticoids prepare the body for stress by mobilizing glucose to the bloodstream, increasing muscle tone, and delaying parasympathetic processes that are non-essential during a crisis, such as feeding and digestion. Centrally,

glucocorticoids act on the hippocampus, amygdala, and frontal lobe in the short-term to enhance the formation of emotional memories. However, the long-term effects of chronic glucocorticoid exposure appear to exact the opposite effect, leading to the atrophy of hippocampal synapses and ultimately, hippocampal neurons themselves, reducing cognitive function.[161]

Concomitantly, the PVN also putatively serves to eventually blunt the anxiety response after exposure to a stressor. In mammals, the PVN is the body's main supplier of the peptide hormone oxytocin, which, among other roles, functions centrally as regulator of anxiety (discussed in greater detail in the following section). In brief, anxiogenic and stressful stimuli significantly boost oxytocin synthesis within the PVN and stimulate central oxytocin release.[162-166] In turn, centrally-released oxytocin has widely been shown to generate an over-arching anxiolytic effect (discussed further in Section 1.5.2). Taken together, these findings suggest that PVN-derived oxytocin may be especially important in tempering anxiety after activation of the stress response.

Importantly, PVN activity is highly regulated by the hippocampus. Primarily, stimulation of the hippocampus produces a net inhibitory effect on the parvocellular division of the PVN.[119, 120, 167] Explaining this, projections from the ventral portion of the subiculum (a major source of hippocampal efferents to the hypothalamus) contact GABA-ergic neurons in the BNST, medial pre-optic area, and hypothalamic nuclei, which in turn project directly to the PVN.[168] It has been hypothesized that the ventral hippocampus may serve to inhibit HPA axis activity following exposure to a stressor.[169] Significantly, the parvocellular division of the PVN is not only responsible for synthesizing CRH to initiate the HPA axis, but also produces and releases oxytocin centrally. Therefore, it is possible that over-activation of the ventral hippocampus could additionally lead to an inhibitory effect on the central oxytocin system.

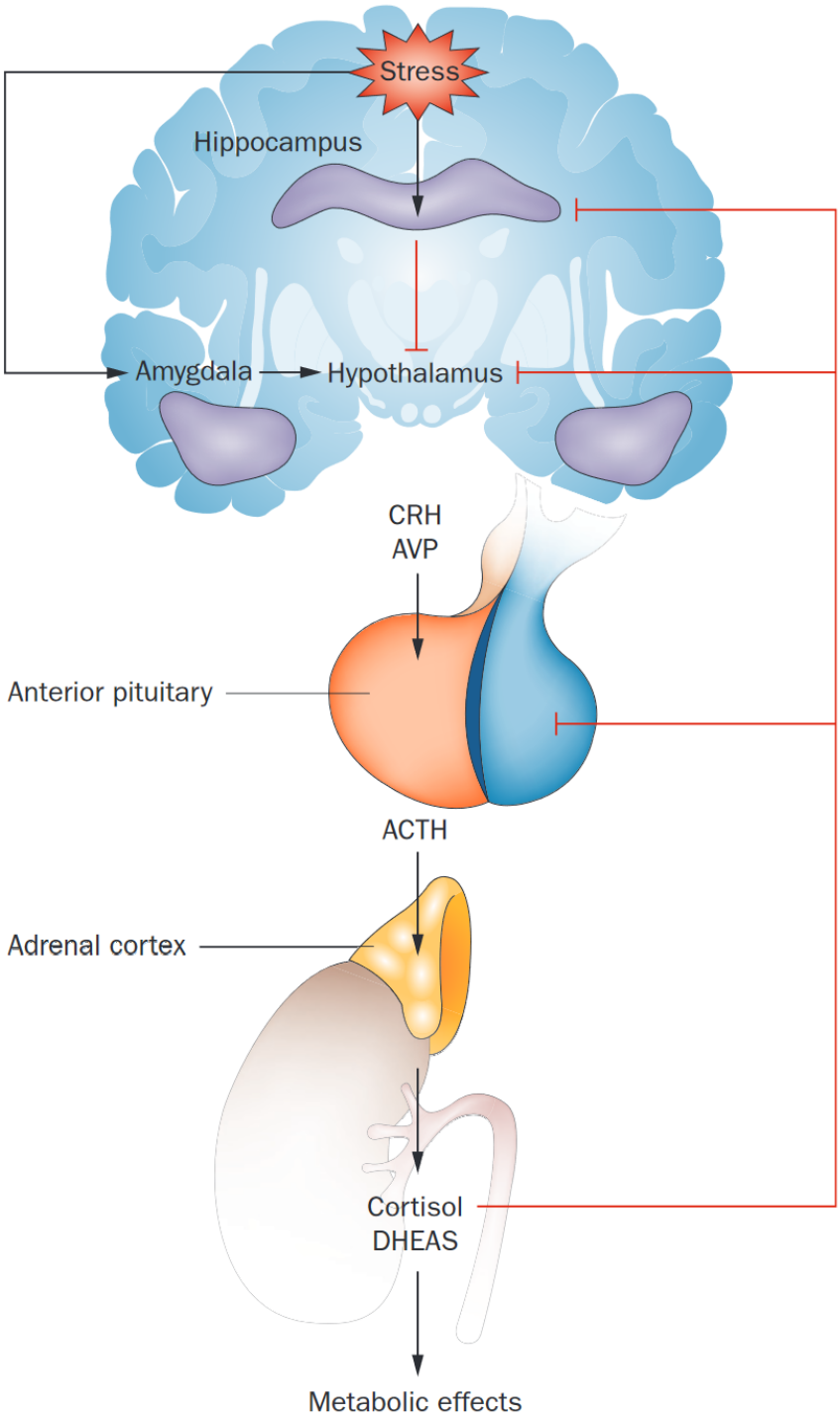


Figure 9. Schematic of the HPA axis. Image credit: Papadopoulos and Cleare, 2012.

1.5.2. Oxytocin Mediates Sociability and Tempers Anxiety

What are the potential consequences of suppressed oxytocin synthesis on behavior? Oxytocin is a mammalian peptide hormone that is associated with diverse peripheral and central effects.[170-176] The mature, active form of oxytocin consists of nine amino acids with an amidated C-terminus (cysteine-tyrosine-isoleucine-glutamine-asparagine-cysteine-proline-arginine-glycine-amide), and a disulfide bridge formed between the two cysteines at positions 1 and 6 (Figure 10). Oxytocin's structure is remarkably similar to that of arginine vasopressin, which is also a nonapeptide containing a sulfur bridge. However, the two differ at residues 3 and 8. Oxytocin is colloquially referred to as the "love" hormone, and indeed, appears to play a significant role in a number of pro-social behaviors, in addition to anxiety- and stress-coping.[171-174, 177]

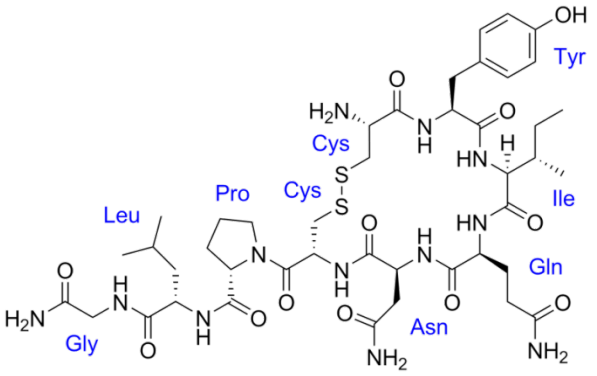


Figure 10. Structure of oxytocin. Oxytocin is a nonapeptide hormone and neurotransmitter involved in a wide range of physiological and behavioral functions.

Endogenous oxytocin primarily originates from the paraventricular nucleus (PVN) of the hypothalamus.[174] Additionally, it is produced in the adjacent supra-optic nucleus (SON) and medial preoptic area (MPA). Oxytocin-expressing neurons in the SON project mainly to the posterior pituitary and are responsible for oxytocin release into the peripheral bloodstream.[177] Oxytocin produced within the MPA, on the other hand, is predominantly associated with male and female sexual behavior.[178, 179]

Two classes of neurons within the PVN synthesize and secrete oxytocin: magnocellular and parvocellular. Magnocellular oxytocin-producing neurons project to the posterior pituitary,

where oxytocin is released into the bloodstream to exert peripheral effects.[177] However, oxytocin also possesses several important functions centrally as a neurotransmitter.[174, 176] For this purpose, oxytocin is instead synthesized within parvocellular neurons of the PVN that project to the amygdala, BNST, nucleus accumbens, prelimbic cortex, and other regions of the brain to mediate a wide variety of behavioral effects.

Due to its diverse peripheral and central actions, oxytocin synthesis has been well characterized.[174] Oxytocin is encoded by the OXT gene and synthesized from an inactive precursor protein that is enzymatically hydrolyzed into smaller fragments to yield the mature peptide. Additionally, the OXT gene encodes an additional oxytocin carrier protein, neurophysin I, which is co-synthesized with oxytocin. Upon synthesis, oxytocin is stored within Herring bodies and transported to axon terminals for release.[180] In magnocellular neurons, these axons project to the posterior pituitary gland and secrete oxytocin into the blood of the neurohypophysis to exert peripheral (hormonal) effects.[174, 177] On the other hand, the parvocellular subset of neurons within the PVN are responsible for producing centrally-acting oxytocin.[174] Central targets of the PVN are known to include the brainstem, spinal cord, and structures of the hypothalamus and limbic system.

Oxytocin is associated with a single receptor, conveniently referred to as the oxytocin receptor or OXTR.[181, 182] The OXTR belongs to the G-protein coupled-receptor (GPCR) superfamily, and is specifically associated with the G-protein Gq, which activates the canonical phosphatidylinositol-calcium second messenger system to exert a variety of intracellular effects. The OXTR is widely expressed throughout the body, including the male and female reproductive tracts (uterus, ovaries, testes, and prostate gland), mammary glands, kidneys, and brain. Peripherally, oxytocin and its receptor are well studied for their roles in lactation and parturition.

Several reports have also pointed to a role for oxytocin in the estrous cycle, male reproductive system function, and bone formation.

Within the brain, oxytocin acts as a neurotransmitter and is known to mediate a wide spectrum of behavioral functions,[170-177] as well as processes related to energy homeostasis, including thermoregulation and food intake.[183-185] More pertinent to FXS, however, oxytocin is strongly linked to a variety of pro-social behaviors[186] in both males and females.[171, 173, 174, 177] These behaviors include, but are not limited to social attachment, affiliative behaviors, pair-bonding, sexual receptivity, parental care, social preference, social recognition, and social memory.[187]

The circuits through which oxytocin mediates social behavior are still a subject of much research. However, it has been found that the extended amygdala is receptive to socio-sexual stimuli, and in response, elicits release of both oxytocin and arginine vasopressin from the PVN.[188] In turn, the PVN is known to radiate oxytocinergic projections to the nucleus accumbens of the ventral striatum, a key component of the brain's reward circuitry. Here, OXTR activation and D2-like dopamine receptor co-activation are thought to be involved in assigning motivational value to social stimuli.[189]

Given its crucial role in social behavior, the oxytocinergic system has attracted great interest as a potential target in the treatment of ASD. For instance, recent work has demonstrated that both exogenous and evoked oxytocin can ameliorate social deficits in the Cntnap2 knockout mouse, a widely used model of autism.[190] Importantly, these studies are not limited to mouse models - for instance, oxytocin delivery via intranasal spray has shown some success in improving face processing,[191] emotion recognition,[192] social interaction,[193] and repetitive behaviors[194] in autistic individuals. On the other hand, like most drugs, exogenous oxytocin is

not without unintended side effects, and has been linked to hyperactivity and aggression in some patients.[195] Since most side effects are due to non-specific actions of therapeutics, correcting abnormalities within the endogenous oxytocinergic system may prove to be a better therapeutic strategy than exogenous administration, as shall be discussed in more detail in the next section.

In addition to its facilitative effects on social behavior, oxytocin is also believed to be important regulator of anxiety and stress coping.[173, 196, 197] In rodents, both peripheral and central administrations of oxytocin have been shown to exert anxiolytic effects.[198-201] This phenomenon has also been observed in humans administered oxytocin via intranasal spray.[202-204] Endogenous oxytocin has also been found to have anxiolytic actions during the post-partum period[205, 206] and in males post-mating.[207] Moreover, oxytocin-deficient female mice display enhanced anxiety-related behavior, and this is reversed by central administration of oxytocin.[208]

How does oxytocin serve to temper anxiety? One current model proposes a role for oxytocin in the modulation of serotonin release. It has long been recognized that the neurotransmitter serotonin plays important roles in the regulation of emotional behavior.[209] Centrally, serotonin is primarily synthesized in the raphe nuclei of the midbrain, pons, and medulla.[210] Importantly, about half of serotonergic neurons within the raphe nuclei have been found to express the oxytocin receptor, and local oxytocin infusion enhances serotonin release within the median raphe nucleus (MRN),[211] which supplies serotonin to brain areas relevant to anxiety, including the cortex, septal region, hippocampus, and amygdala. Furthermore, this local infusion of oxytocin to the MRN has been shown to reduce anxiety-associated behaviors in the open field in mice in 5-$HT_{2A/2C}$ receptor dependent manner.[211] These results may have important implications for FXS, as serotonin dysregulation is speculated to occur in FXS patients.[212]

Oxytocin may also mediate anxiety behaviors through actions in the medial prefrontal

cortex (mPFC). Supporting this hypothesis, the pre-limbic region of the mPFC both expresses oxytocin receptors[213], and receives axonal projections from oxytocin-producing neurons in the hypothalamus.[214, 215] Furthermore, it has been found that oxytocin infusion to the mPFC reduces anxiety-like behavior in rodents independent of sex.[216] However, the mechanisms by which endogenous oxytocin may regulate anxiety in the mPFC remain unclear. Infusion of an oxytocin receptor antagonist was found to produce no effect on anxiety-behavior in the aforementioned study, yet oxytocin receptor antagonists have been shown to block the anxiolytic properties of oxytocin in other studies in contexts where endogenous oxytocin is elevated.[207, 217] As such, further studies are needed to address this discrepancy.

1.5.3. Early Organizational Effects of Oxytocin

Lastly, some work has demonstrated that early postnatal oxytocin plays a fundamental role in shaping later life behavior. While this is still a new and exciting area of research, manipulation of the oxytocinergic system in rodents has provided some insight into how oxytocin may be especially important in establishing adult social behavior. In one study, it was found that female mice treated with an oxytocin receptor antagonist on postnatal day zero make a reduced number of social approaches in a three-chambered social interaction apparatus as adults.[218] Interestingly, oxytocin-treated male mice in this study did not differ significantly from controls. However, another group found that male rats given oxytocin treatments during the adolescent period, they were more socially interactive during adulthood.[219] Taken together, these studies indicate that oxytocin appears to perform some kind of organization role during the development of circuits mediating social behavior in both males and females, although the timing of its effects appears to be sex-dependent. Based on these studies, it is possible that in male mice, oxytocin might be more influential in establishing social behavior during later stages

of development; whereas in female mice, oxytocin appears to exert its effects earlier on.

Interestingly, the effects of oxytocin during brain maturation are not limited to the development of social behavior. A growing body of work suggests that at a young age, oxytocin also appears to be involved in establishing adult responses to stress. For instance, rats administered daily oxytocin injections in the pubertal period were shown to be more exploratory in a novel open field environment, which was interpreted as an anxiolytic effect.[219] Supporting this case, it was found in a separate study that female prairie voles administered oxytocin on postnatal day 1 have lower baseline levels of plasma corticosterone by postnatal day 8.[220]

How does oxytocin impact the development of these behaviors, mechanistically? Unfortunately, this is also a relatively new and untouched area of research. On the other hand, some existing work has shown that oxytocin also seems to direct the organization of the serotonergic system. In one study, it was shown that male prairie voles treated with oxytocin daily displayed greater serotonin innervation in the hypothalamus and cortical amygdala at P21.[221] Given the importance of serotonin in anxiety and mood, any disruption to the early oxytocinergic system would potentially have a huge impact on later-life emotional functioning. Alternatively, it is also possible that early oxytocin levels help direct the development of the oxytocinergic system itself. In this case, disturbances to the oxytocin system during early life would impact both social and emotional behavior. Moreover, it is possible that during the postnatal period, oxytocin may regulate the development of circuits or brain regions that are less or non-responsive to oxytocin later in life, if expression of the OXTR in those regions is transient. Since both social behavior and anxiety are affected in FXS, it is likely that early defects in the oxytocin system could contribute to long-term changes in socio-emotional functioning.

As described in Section 1.5.1, stimulation of the hippocampus produces a net inhibitory effect on the parvocellular division of the PVN, where centrally-released oxytocin is primarily synthesized.[119, 120] This inhibition has been attributed to the ventral hippocampus, which activates inhibitory interneurons within the BNST which project to the PVN.[168, 222] Based on this knowledge, it is possible that over-excitation of the ventral hippocampus in FXS may lead to exaggerated suppression of PVN oxytocin synthesis. Given the likely role of oxytocin in establishing later life emotional and social behavior, we speculate that this reduction in oxytocin levels during early circuit formation may be a key culprit in anomalies in these behaviors in FXS.

1.6. Significance and Objectives of the Current Study

1.6.1. Shortcomings of Current Approaches in Treating FXS

Taken together, the aforementioned findings provide compelling evidence that oxytocin is essential for normal social functioning and anxiety levels. Considering that social deficits and hyper-anxiety are hallmark characteristics of FXS, it is highly likely that the oxytocinergic system may be compromised in the disorder, particularly during brain development. If this is indeed the case, then targeting the oxytocinergic system and its upstream/downstream effectors may prove a useful strategy in correcting many FXS-associated symptoms. However, to our knowledge, studies investigating the integrity of oxytocinergic system in FXS or taking advantage of this promising avenue of treatment are astonishingly lacking.

Presently, treatments that directly counteract the effects of the FXS mutation remain elusive, and those currently in use are aimed at secondary symptoms of the disorder. As a result, many patients with FXS take must take multiple medications simultaneously in order to manage their symptoms. These may include anti-convulsants, anti-anxiety medications, drugs to manage

irritability, attention problems, and/or hyper-activity. It is not unheard of for a patient to have as many as four prescriptions at once. This is disadvantageous not only because of the potential for side effects and the risk of drug interactions, but also because not enough is known about how long-term exposure to such psychiatric medications may affect developing brains when treating children. As of yet, there is no one single treatment approach for FXS. Recently, it was reported that although group 1 metabotropic glutamate receptor antagonists demonstrated promise in early stages of research, they ultimately failed to improve symptoms in double-blinded clinical trials beyond placebos,[87] a major setback for FXS research. Exogenous administration of oxytocin has shown some promise in patients with autism,[191-193, 195] but to our knowledge, however, only one study has examined its effects on FXS patients.[223] Here, it was reported that intranasally-applied oxytocin improved eye-gaze frequency and reduced salivary levels of cortisol in the short-term (20 minutes post-administration). However, unfortunately, these are rather indirect measures of an individual's emotional state, and more salient effects were not reported.

1.6.2. Exogenous Oxytocin Administration is Not an Ideal Therapeutic Strategy for FXS

While exogenous oxytocin administration may therefore hold some promise for FXS patients, based on data from studies on ASD patients, it is certainly not without its shortcomings. In humans, oxytocin is generally administered *via* one of two main routes: intravenously and intranasally. Intravenous administration is primarily utilized to achieve peripheral effects, such as the stimulation of uterine contractions during labor. In contrast, intranasal administration is primarily utilized to achieve behavioral effects.

The first problem with exogenous oxytocin administration to achieve behavioral effects is that the half-life of oxytocin is quite short. Human studies have estimated a half-life of approximately 3-8 minutes in the plasma in rodents[224] and humans[225] although it is longer-lasting

in the cerebrospinal fluid: approximately ~19 minutes in rats.[226] Oxytocin is believed to be cleared from the cerebrospinal fluid (CSF) via two mechanisms: (1) pooling into the subarachnoid space, where it is ultimately absorbed into the venous blood through the arachnoid villi;[227] and (2) active transportation into the blood by peptide transport system 1.[228]

The second problem regarding exogenous oxytocin administration is its efficiency. Oxytocin administered intravenously does not cross the blood brain barrier in meaningful amounts; therefore, intranasal administration must be employed to achieve central effects.[229] There has been much controversy regarding the efficacy of oxytocin penetration into the brain following intranasal administration. Based on human and animal studies, it has been estimated that only 0.002-0.005% of intranasally administered oxytocin reaches the CSF within 1 hour, even when doses greatly exceeding the pituitary content are administered.[229]

However, one might argue that CSF concentration is an indirect measure that does not necessarily reflect availability in brain tissue. Circumventing this issue, one recent study explored intranasal oxytocin penetration via arterial spin labeling, a non-invasive pharmacodynamic technique that quantitatively measures regional cerebral blood flow (rCBF). In this study, 40 IU of oxytocin was administered to healthy male subjects, and this dose was found to be sufficient to increase rCBF in areas part of the social brain network within 78 min.[230] It is prudent to note that this dose greatly exceeds human pituitary oxytocin content, which has been estimated *via* bioassay to be approximately 14 IU (28 ug).[231] In the aforementioned study on FXS patients, moreover, doses of 24 and 48 IU were necessary to improve eye-gaze frequency and reduce salivary cortisol, respectively.

Therefore, on account of oxytocin's poor brain penetrance and rapid clearance, relatively high doses of the peptide are required to achieve appreciable effects on behavior. Moreover, it is

likely that larger amounts of exogenous oxytocin are required to produce behavioral effects, compared to peripheral effects. This is because the oxytocin receptor (OXTR), like most G-protein coupled receptors, is de-sensitized upon stimulation.[232] Since basal concentrations of oxytocin are higher in the brain than in the plasma (~1-10 pg/mL),[233] higher doses are therefore likely needed to elicit meaningful effects on behavior. However, the usage of supra-physiological doses of oxytocin to achieve central effects is problematic for many reasons. First, intranasal application has been found to achieve higher concentrations of the peptide in plasma than in the CSF. It is estimated that 1% of intranasally-administered oxytocin enters the systemic circulation.[234] This is precarious because much lower amounts of oxytocin are required to produce peripheral effects than central ones. Basal concentrations of oxytocin are much lower in the plasma than in the brain, and thus, peripheral OXTRs are normally exposed to far lower concentrations of oxytocin than central OXTRs. Because of this, peripheral OXTRs are much more sensitive to stimulation and it likely that they would be inadvertently affected by intranasal oxytocin administration. Moreover, peripheral targets of oxytocin are widespread. These include, among others, the kidneys, where oxytocin is involved in water homeostasis; the pancreas, where oxytocin increases glucagon and insulin secretion; and the heart, where oxytocin has been found to raise heart rate variability.[235]

Furthermore, the usage of supra-normal doses to achieve central effects is problematic for economic reasons. The production of synthetic oxytocin relies on solid-state synthesis with modifications for disulfide bond formation and amidation of oxytocin's C-terminus. This is a multi-step process that is relatively expensive and time-consuming compared to the synthesis of many non-peptide drugs. Moreover, since oxytocin penetration is thought to be relatively poor, and its clearance from the body is relatively swift, the majority of the oxytocin administered is

wasted.

It is also important to consider the practicality of the treatment from a clinical standpoint. The effects of exogenously-administered oxytocin are fleeting, lasting only about 45 minutes post-administration in humans.[236] Therefore, repeated administration over the long term would be required to achieve normal functioning in many patients. However, human data on outcomes following chronic treatment are still lacking. In fact, there is some evidence that extended treatment with oxytocin may be counter-productive. In male prairie voles, long-term administration, beginning in youth through adulthood, in fact was discovered to lead to deficits in social bond formation.[237]

Last, but not least, exogenous oxytocin has also been reported to carry the potential for undesirable behavioral side effects. For instance, intranasal oxytocin has been found to cause hyperactivity and aggression in a subset of ASD males.[195] On the other hand, some reports have found that oxytocin has no effect at all in some patients. For instance, one study detected no benefits of oxytocin in autistic youths that were given 12 or 24 IU intranasal oxytocin once daily for four consecutive days.[238] Thus, intranasal administration of oxytocin, though better than other methods of delivery, may not be an ideal solution for a significant proportion of patients.

1.6.3. Targeting Defects in Brain Development: A New Angle for Approaching FXS

Current approaches in treating FXS are limited and are fraught with many disadvantages. In light of these shortcomings, we propose that identifying and correcting FXS-associated changes within the endogenous oxytocinergic system may be a worthwhile new direction to pursue. In the current study, we have honed in on FXS-associated changes in the ventral hippocampus, as a major upstream regulator of the hypothalamus. The ventral hippocampus is potentially among the most relevant brain structures to the pathogenesis of FXS, as it modulates

the activity of the cortex, amygdala and hypothalamus to coordinate emotional and social behavior.[148, 155, 239, 240] Hence, the hippocampus is likely to play a central role in the socio-emotional make-up of a human being.

When envisaging such strategies, however, it is once again important to remember that FXS is a developmental disorder – that is to say, FXS arises from intrinsic changes in the normal trajectory of brain development, leading to lifelong consequences; as opposed to a condition that can be caused by intrinsic or extrinsic factors at any age, which may or may not be life-long. Given the role of the ventral hippocampus in social and emotional behavior, we postulate that any insult to its development during early life may potentially leave a profound impact on the behavior of an individual, as clearly appears to be the case in FXS. Since FMRP functions as a widespread neuronal translational regulator, FXS is considered to result from global, uncontrolled expression of dosage-sensitive genes that are required for normal dendritic spine development, remodeling, and function. Such alterations in synaptic plasticity during early stages of developmental wiring may in turn lead to defects in the formation of many cognitively and behaviorally relevant neurological circuits, such as connections within and between the hippocampus, hypothalamus, and amygdala. Moreover, these effects on the initial wiring of the brain may be to a large extent irreversible upon reaching adulthood, highlighting the need for early intervention strategies that target the root of the malady, as opposed to the symptoms. Importantly, even a few aberrant connections made during brain development may be capable of resulting in developmental disorders: once a few of such improper connections are formed, it is likely that they may lead to further inappropriate connections during early circuit formation, thereby amplifying the defect into a syndrome that the affected individual would have to live with their entire life.

Despite this point, most therapeutic strategies to ameliorate FXS-associated dysfunctions have avoided treatment during brain development. In contrast, in our efforts toward a therapeutic strategy, we have focused on the early stages of postnatal brain development in FXS. Thus, unlike the majority of studies conducted in this area, we have adopted a novel strategy of early intervention to prevent or reduce the onset of the socio-emotional symptoms associated with FXS. In other words, instead of treating symptoms after they have already developed, our strategy aims to guide brain developmental back onto the correct trajectory before the symptoms become permanent.

Of course, we recognize that it is desirable in the clinic to avoid therapy during the early stages of life. However, most clinical scientists agree that aberrant brain connections formed during the early brain development are likely to be causal to the FXS-associated behavioral deficits. The impression that mouse studies do not translate to human therapy is often based on treatments that are offered beyond the point critical development, when the brain can still be nudged to form the right connections. Therefore, it is highly important to conduct preclinical studies during early brain development. Such studies would have a greater likelihood of eventually translating into successful human clinical trials.

1.6.4. Objectives of the Current Study

For a number of years, our team has been interested in elucidating hippocampal serotonin 1A receptor- mediated signaling pathways linked to anxiety and depression. Our mechanistic studies have identified protein kinase C epsilon (PKCε) as an important signaling molecule during neonatal brain development, with an integral role in early hippocampal neurogenesis.[241] These findings have inspired us to study the role of PKCε in other disorders of brain development, such as FXS. However, because of the fact that PKCε signaling appears to be key

to proper brain development, we speculate that targeting PKCε during adulthood may prove far less effective in treating the symptoms of FXS. Thus, the objectives of the current study were two-fold. First, we sought to examine the integrity of early postnatal PKCε signaling in FXS. Second, we investigated whether early intervention, by stimulating PKCε signaling during early postnatal development, could permanently, yet safely, prevent or reduce the onset of FXS-associated socio-emotional disturbances.

In this thesis, we report that PKCε expression is stunted in the developing *Fmr1* KO mouse brain, and that this is linked to heightened GluR2-containing AMPA receptor expression in hippocampal glutamatergic synapses. This may provide a mechanism by which hippocampal excitability may become exaggerated in the syndrome. The ventral hippocampus is a major regulator of the HPA axis and stress response through its inhibitory projections to the paraventricular nucleus of the hypothalamus (PVN). Accordingly, over-activity of the ventral hippocampus might lead to inappropriate inhibition of neuroendocrine cells within the PVN. We have found that during the early postnatal stage, the *Fmr1* KO PVN displays a reduction in the number of oxytocin positive cells. This is an important finding not only because of the role of oxytocin in pro-social behavior, but because oxytocin itself may help to organize the development of neuronal circuits relevant to behaviors altered in FXS, including social interaction and anxiety. Based on these findings, we questioned whether targeting PKCε signaling in the neonatal *Fmr1* KO hippocampus might serve to correct oxytocin levels in the downstream PVN at this age. By targeting this pathway early in life, we speculated it might be possible to mitigate the effects of the *Fmr1* mutation on later-life social behavior and anxiety. True to our hypothesis, we report that neonatal administration of a PKCε-specific activator is sufficient to normalize changes in GluR2- AMPA receptor density in neonatal ventral

hippocampal synapses, oxytocin levels in the PVN, and significantly reduce the severity of FXS-associated changes in social and anxiety-like behavior in *Fmr1* KO mice during adulthood. Thus, in the current study we (1) have uncovered a previously-unidentified signaling pathway implicated in FXS; and (2) present a novel strategy to circumvent aberrant brain development in FXS and accompanying socio-emotional disturbances, by augmenting PKCε signaling during neonatal development.

1.6.5. PKCε is an Important Signaling Molecule in Postnatal Brain Development

Before moving forward, it may be prudent to review the current knowledge of the role of PKCε in brain development. Protein Kinase C (PKC) is a family of serine and threonine protein kinases which play important roles in numerous signal transduction cascades within and outside of the nervous system.[242] PKCs are categorized into three subfamilies based on sequence homology and activator requirements: conventional (α, β and γ), novel (δ, ε, ζ, μ, and ε), and atypical (λ and δ). Conventional PKCs require calcium, diacylglycerol (DAG), and a phospholipid such as phosphatidylserine to be activated. On the other hand, novel PKCs, such as PKCε, require DAG, but not calcium. Thus, they both are activated through similar signal transduction pathways, such as phospholipase C (PLC). Lastly, atypical PKCs require neither calcium nor DAG.

Protein kinase C epsilon (PKCε) is a novel PKC isotype.[243] While calcium independent, it is phorbol ester and DAG sensitive. The responsiveness of PKCε to second messengers requires phosphorylation at three conserved sites: Thr-556 in the activation loop, Ser-729 in the C-terminal hydrophobic site, and Thr-710 at an autophosphorylation site. Neuronal PKCε is activated by a variety of different second messengers, though primarily DAG and phosphatidylinositol 3,4,5,-triphosphate (PIP_3). Importantly, PKCs are known for their long-term

activation: they remain catalytically active in the cell after the original activation signal and/or calcium wave has subsided. The intracellular localization of PKCε, following its activation, is dependent on which second messenger is bound to its C1 domain. For instance, PKCε is translocated to the plasma membrane and/or cytoskeleton in response to DAG, whereas it instead translocates to Golgi-networks in response to arachidonic acids and linoleic acids. Enhancing the spatial and temporal organization of PKCε are adaptor proteins, collectively called RACKS (receptors for activated C kinase), which anchor PKC near its substrates to optimize the speed and precision of the response. One such adaptor protein responsible for PKCε translocation that has been identified is RACK2, a Golgi membrane protein associated with vesicular release.[244]

Though it is expressed throughout the entire body, PKCε is predominantly localized in the brain.[245, 246] Highest levels of expression are found in the hippocampus, islands of Cajella, and olfactory tubercule; but moderate expression also occurs in the frontal cortex, striatum, nucleus accumbens, lateral septal nuclei, and caudate putamen. The known actions of PKCε within neurons are numerous and diverse. *In vitro*, activation of PKCε has been demonstrated to promote the outgrowth of neurites during neuronal differentiation via stabilization of actin filaments.[247] When bound to actin, PKCε becomes anchored to the cytoskeletal matrix and remains catalytically active. One important functional consequence of this in the nerve terminal appears to be the facilitation of glutamate release, and thus, may participate in regulating excitatory neurotransmission and plasticity.

We have previously reported that the PKC isoform epsilon (PKCε) is highly enriched in the mouse hippocampus at postnatal day 6 (P6), the onset of postnatal hippocampal development (Figure 11).[248] During this time, PKCε orchestrates neurogenesis within the dentate gyrus downstream of the serotonin 1a receptor (5-HT_{1A}-R) (Figure 12). Moreover, PKCε's

upregulation further increases up to the event of eye-opening at P15,[248] suggesting that it may be essential in guiding early postnatal hippocampal synaptogenesis in response to incoming visual stimuli.

Most intriguingly, Darnell and colleagues have recently demonstrated that PKCε mRNA is one of the many targets of FMRP-mediated translational regulation, raising the possibility that its expression may be dysregulated in FXS.[43] Supporting this hypothesis, recent work by Sun and colleagues demonstrate that juvenile administration of bryostatin-1, a non-selective PKCε activator, is able to rescue hippocampal synaptic structural abnormalities in FXS knockout (KO) mice, as well as deficits in spatial memory.[249] Thus, it is very likely that PKCε-mediated signaling may guide some of the processes underlying synaptic maturation that are impaired in FXS. As discussed in Section 1.6.3, insults to synaptic transmission during hippocampal development may have dire consequences on the formation of downstream circuits involved in cognition, mood, and behavior. We have obtained data that demonstrates hippocampal PKCε signaling may be an especially important early mediator of these functions.

In a set of studies conducted prior to this thesis, we have found that neonatal intraperitoneal injection of 3 mg/Kg DCP-LA (i.p.) is able to permanently rescue elevated anxiety-like behavior in adult serotonin 1a receptor knockout mice, a frequently-used model of mood and anxiety disorders (Figure 13). This occurs in conjunction with a partial rescue of hippocampal neuroblast proliferation at P6 in these mice (Figure 14).

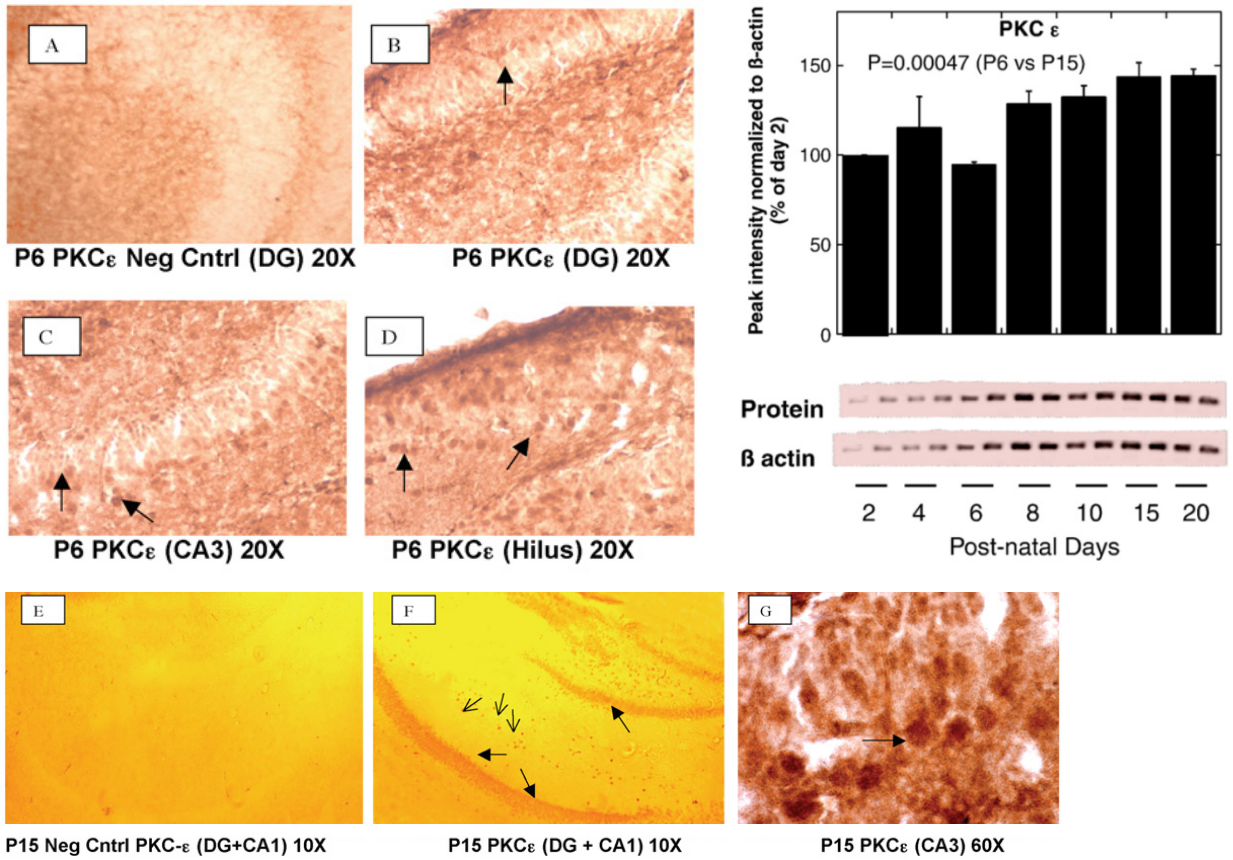


Figure 11. PKCε is highly expressed in the hippocampus during neonatal development, its levels steadily increasing from P6 through P15. Solid black arrows: cell bodies, thin black arrows: stratum radiatum. (Purkayastha et. al, 2009).

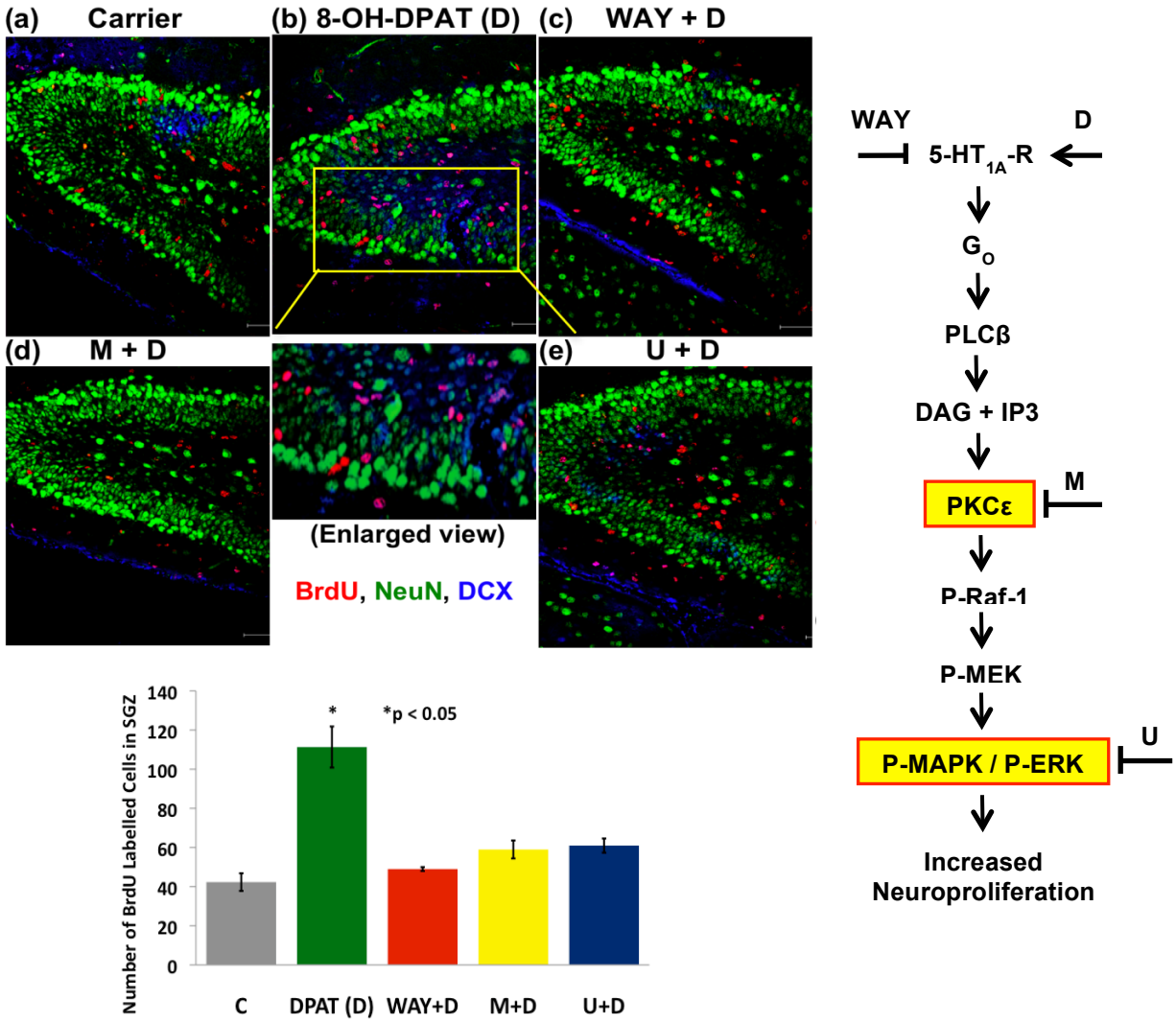


Figure 12. PKCε directs early postnatal hippocampal neurogenesis downstream of the serotonin 1a receptor (5-HT_{1A}-R) .C57/BL6 mice, aged P6, were injected with BrdU 2h before intra-hippocampal infusion of 8-OH-DPAT in the presence and absence of agonists and inhibitors. Immunolabeling of newly divided cells with BrdU (red), mature neurons labeled with NeuN (green) and neuronal progenitor cells marked with doublecortin (DCX, blue) was performed. D= 8-OH-DPAT, WAY = WAY100635, M = Myr-εV1/2, U = U1026. Data presented as mean ± S.E.M. (Samaddar et. al.2013.)

Taken together, these findings suggest that PKCε-mediated signaling may contribute to the establishment of normal anxiety behavior during adulthood, one possible mechanism being regulation of early postnatal hippocampal neurogenesis. Indeed, mounting evidence has suggested that activation of the upstream serotonin 1a receptor by serotonin, agonists of the receptor, and mechanisms elicited by antidepressant drugs can augment the production of newborn granule cells in the hippocampus, and that a fine balance of this process is necessary to maintain normal mood and anxiety levels.[250, 251] During early development, this process is furthermore putatively involved in the normal establishment of hippocampal mossy fibers, and likely the downstream hippocampal circuitry. However, the extent to which these processes are impacted during early brain development in FXS remains unknown. A fine balance of these processes during the postnatal period is likely necessary for the precise formation of fundamental circuits involved in learning, memory, and emotion, and disruption of this balance could presumably lead to profound effects on the FXS brain, manifesting as behavioral deficits in these areas.

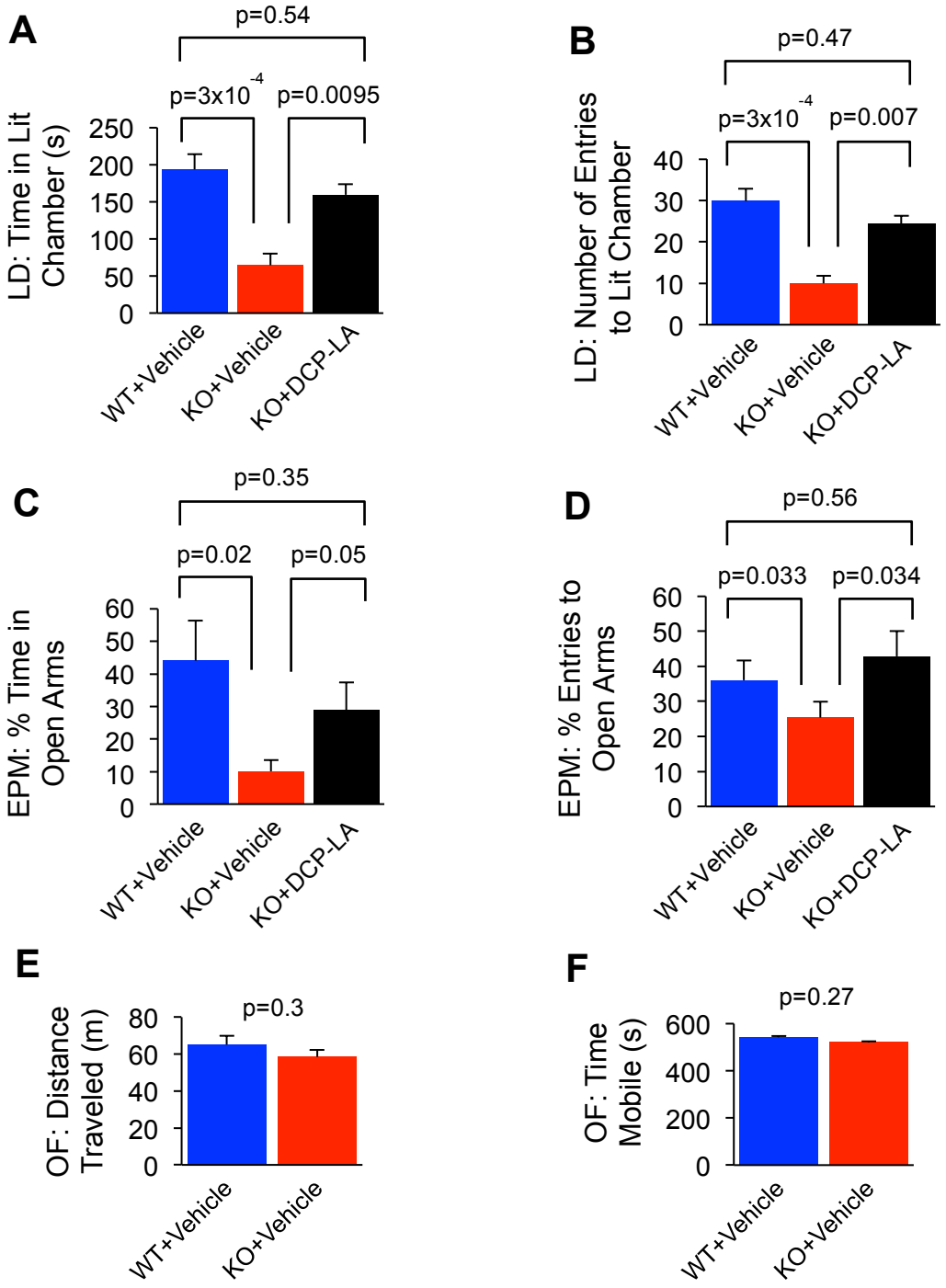
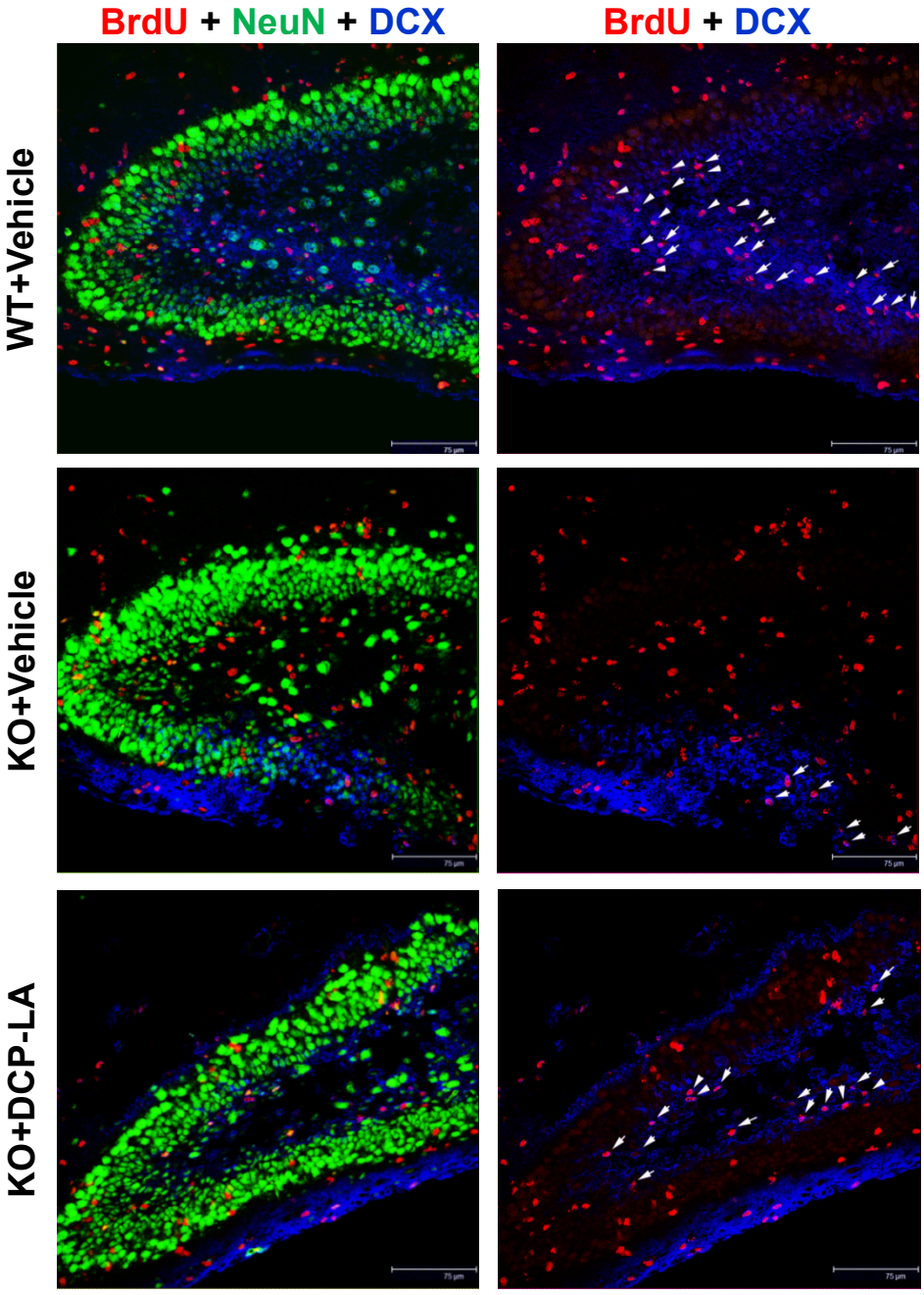


Figure 13. Peripheral administration of PKCε activator DCP-LA (3 mg/kg, i.p.) at P6, P10, and P14 rescues anxiety-like behavior in female 5-HT_{1A}-R knockout (KO) mice the light-dark test (LD, A and B) and elevated plus maze (EPM, C and D). Compared to wild-type mice (WT), the knock-out mice (KO) displayed significantly decreased number of entries (A) and time spent (B) in the lit chamber, indicating heightened anxiety. Similarly, KO mice spent less time in, and made fewer entries to the open arms of the EPM. All of these anxiety-like behaviors were fully corrected in KO females that were neonatally treated with DCP-LA. No significant difference was observed among WT and KO mice in locomotion in the open field test (OF, E, F) indicating that the results in the LD test were not influenced by differences in motor capability. In our studies, male KO mice did not demonstrate anxiety-like behavior (data not shown).



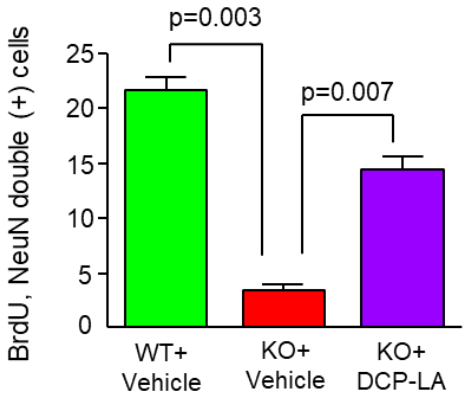


Figure 14. Stimulation of PKCε boosts neuroproliferation in 5-HT_{1A}-R KO female mice at P6. As shown earlier, PKCε mediates 5-HT_{1A}-R-linked activation of Erk1/2 in the P6 hippocampus to regulate neuroblast proliferation (Figure 12). While female 5-HT_{1A}-R KO mice demonstrate reduced neuroblast proliferation at P6 compared to wild-type (WT) controls, this suppression was partially corrected following intrahippocampal infusion of DCP-LA 24 hrs prior. In contrast to the females, no differences were observed in male P6 KO pups (data not shown). Green: NeuN, Red: BrdU, Blue, doublecortin (DCX). Scale bars=75 µm. Data represents mean ± S.E.M. (Samaddar et. al, 2013.)[241]

1.6. 6. The AMPA Receptor – A Potential Target of PKCε-Mediated Signaling

Although we have discovered that PKCε is an important mediator of early postnatal hippocampal neurogenesis and the establishment of normal anxiety behavior (see Section 1.6.4), it is prudent to bear in mind that PKCε may also guide hippocampal development through additional, independent pathways. Moreover, abnormalities in early postnatal neurogenesis may not easily explain deficits in synaptic functioning that persist in FXS throughout the lifespan. In our attempts to uncover additional potential PKCε-mediated pathways that may be altered in FXS, we have focused on hippocampal AMPA receptors, which largely influence synaptic function and plasticity in this region during the early postnatal period.

The AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor is a transmembrane receptor of glutamate, and the most common receptor in the brain.[252] An ionotropic receptor permeable to cations, the AMPA receptor is primarily responsible for fast, excitatory synaptic transmission within central synapses. The AMPA receptor consists of a ligand-gated ion channel composed of combinations of four discrete subunits (Figure 15), termed GluR1-4.[253] Most AMPA receptors are assembled in the endoplasmic reticulum as dimers, which are later joined together to form tetramers. Each subunit contains an

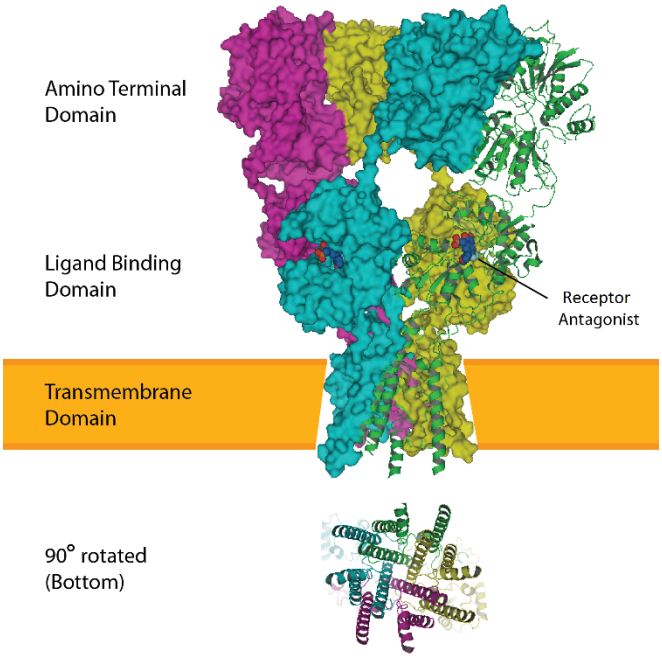


Figure 15. Structure of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor with an antagonist bound to the glutamate binding site. Image credit: Curtis Neveu.

agonist binding site at the N-terminal. When two of these sites are occupied, the ion channel pore opens, with current increasing as more binding sites become occupied. Remarkably, the channel can open and close quite quickly, at a speed of 1 ms.[254] The AMPA receptor is highly expressed in the hippocampus throughout the lifespan, including development.[255] Within the hippocampus, most AMPA receptors contain both GluR1 and GluR2.[256]

AMPA receptor function is largely dependent on its subunit composition. Much evidence has suggested that the GluR2 subunit is particularly important in this regard.[257] First, GluR2 is responsible for determining the permeability of the channel to specific ions. Primarily, AMPA receptors lacking GluR2 are permeable to sodium, potassium, and calcium; whereas receptors containing the unit are impermeable to calcium. This is because the GluR2 subunit can be post-transcriptionally modified so that uncharged glutamine within AMPA-R's ion channel lining (residue 607) is converted into the positively charged arginine, which prevents calcium from passing through the pore.[258] Almost all GluR2 subunits in mature neurons are edited in this fashion.[257] In addition to cation permeability, the GluR2 receptor has also been shown to be required for normal long-term synaptic strengthening (LTP)[259] and long-term synaptic weakening (LTD)[260] in the hippocampus. Lastly, the GluR2 subunit also appears to be responsible for AMPA receptor trafficking. For instance, it has been found that PKC-dependent phosphorylation of the intracellular serine 880 (Ser880) residue triggers internalization of the receptor from the synapse to dampen the neuron's response to incoming excitatory signals.[261, 262] Specifically, GluR2 is stabilized at the synapse through its interactions with the PDZ proteins PICK1 and GRIP; but phosphorylation of Ser880, located within the PDZ-binding domain of GluR2, destabilizes this interaction.

Given its function in mediating fast excitatory synaptic transmission, AMPA receptors

are integral to normal synaptic plasticity. Changes in the number and composition of synaptic AMPA receptors have been shown to mediate both LTP and LTD.[255] Along these lines, changes in AMPA receptor dynamics would certainly impact not only hippocampal development, but also the foundational wiring of hippocampal-mediated macro-circuits that are involved in cognition, behavior, and mood. Since LTP and LTD are both disrupted in FXS,[74] we therefore sought to identify possible changes in AMPA receptor dynamics during hippocampal development that may underlie this phenomenon. Given that PKCε is abundantly expressed in the hippocampus during its postnatal development,[248] we investigated whether PKCε may potentially play a role in AMPA receptor dynamics during this time.

1.6.7. DCP-LA, A Selective Activator of PKCε

PKC activators have been widely used in the treatment of various medical conditions.[263] In our attempts to correct altered PKCε signaling in the *Fmr1* KO brain, we have employed a highly selective PKCε activator, the linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (dicyclopropyl-linoleic acid, or DCP-LA).[264] The derivative is structurally identical to linoleic acid, except that it contains cyclopropane rings instead of *cis*-double bonds (Figure 16). Importantly, DCP-LA does not occur in nature, but is synthesized from linoleic acid.[265] In brief, linoleic acid methyl esters are cyclopropanated using a modified Simmons-Smith reaction[265] and purified by column chromatography.

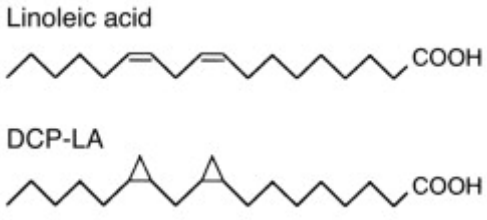


Figure 16. Chemical structure of 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) compared to linoleic acid.

An essential fatty acid, linoleic acid is abundant in nuts, fatty seeds, and vegetable oils, including palm and olive oil. Linoleic acid is

known to participate in a wide range of biological activities. For instance, it is the major dietary precursor of arachidonic acid,[266] which is required for the synthesis of prostaglandins and thromboxanes; and it is also a regulator of PLC[267] and PKC[268] signaling. In contrast to linoleic acid, however, the biological activity of DCP-LA is much more restricted, acting primarily as a selective activator of PKCε. DCP-LA has shown to exhibit >7-fold specificity for activation of PKCε versus other PKC isozymes, by binding to PKCε's phosphatidylserine binding site.[264, 269] This is a significant advantage of DCP-LA as a therapeutic because as a phosphatidylserine-binding PKC activator, unlike diacylglycerol-binding activators, seeming to produce little or no downregulation of PKC.[270] Moreover, DCP-LA is quite potent, demonstrating activity at concentrations as low as 10nM, but with maximal effects at 100 nm.[264]

Since its recent development, DCP-LA has gained growing interest due to the many and diverse roles of PKCε in both normal physiology and in disease. The therapeutic potential of DCP-LA has been particularly investigated in the context of Alzheimer's disease (AD). In one study, for instance, it was found that treatment with 3 mg/Kg of DCP-LA twice a week for 12 weeks was able to prevent synaptic loss, accumulation of amyloid plaques, and cognitive deficits in the rapidly-progressing 5XFAD transgenic mouse model of AD.[271] Similarly, it was independently found that acute intra-peritoneal injection of 1 mg/Kg DCP-LA was able to improve learning deficits imparted by amyloid-β1−40 peptide, which is linked to synaptic disorder and cognitive function; as well as improve deficits imparted by scopolamine, a muscarinic ACh receptor inhibitor that is also linked to cognitive impairment.[272] Additionally, DCP-LA has been shown to improve age-related learning impairments in accelerated-senescence-prone mice (SAMP8) treated with this same dose.[273] Possibly explaining these findings, it has been found that DCP-LA promotes the translocation of presynaptic α7

acetylcholine (ACh) receptors to the synapse, which in turn leads to enhanced glutamate release and long-lasting facilitation of hippocampal synaptic transmission.[274]

Interestingly, DCP-LA has also been evaluated as a potential therapeutic in the context of depression, albeit to a lesser degree than AD. Intriguingly, it was found that DCP-LA promoted translocation of the serotonin 1a receptor to hypothalamic post-synaptic membranes of mice subjected to restraint stress.[275] This was associated with increased serotonin release and inactivation of glycogen synthase kinase 3β (GSK-3β), which has been linked to serotonin-sensitive anxiety and social behavior.[276-278] Remarkably, even oral administration of 1 mg/Kg DCP-LA treatment was able to rescue depressive-like behaviors in these mice.

DCP-LA has several attractive advantages as a therapeutic agent. First, the precursor of DCP-LA, linoleic acid, is naturally abundant. Second, as an unsaturated fatty acid, linoleic acid readily crosses the blood brain barrier, most likely by simple diffusion.[279] Numerous studies have shown that DCP-LA is also capable of crossing the blood brain barrier,[271-273, 275] and peripheral injection of doses as low as 1/mg kg produce appreciable effects on behavior.[272, 273] As mentioned above, DCP-LA also appears to produces therapeutic effects on behavior when this same dose is administered orally.[275] DCP-LA is also superior to many alternatives due to its relatively high biovailability: due to the presence of its cyclopropane rings, DCP-LA resists metabolism in vivo.[280] DCP-LA's effects are also relatively long-lasting, compared to other PKC activators: it has been found that PKC exposed to DCP-LA remains activated for at least four hours.[281] Lastly, using appropriate drug delivery methods, DCP-LA can also be targeted to specific brain regions, reducing the risk of potential behavioral and peripheral side effects. For instance, DCP-LA-containing nanoparticles decorated with antibodies directed towards hippocampal-specific proteins could be delivered intranasally, bypassing the blood brain barrier,

as well as non-specific brain regions. Although oxytocin could arguably be delivered in a similar fashion, one must still contend with its abysmally short half-life. Furthermore, nanoparticles which could be used for slow, chronic release of oxytocin (which are usually, polylactide, poly-glycolide, and or poly-lactide-co-glycolide), are incompatible with antibody-directed targeting, since these have no functional groups to graft the antibodies onto, such as hydroxyl, amine, or thiol groups. Thus, DCP-LA has the advantage. Additionally, preparation of DCP-LA and an appropriate delivery system would likely be more economical than mass-production of oxytocin, considering the relative simplicity of the reaction and the abundance of linoleic acid in nature.

In sum, although many questions regarding the therapeutic potential of DCP-LA still remain to be answered, it has shown great promise thus far, and has several advantages as a therapeutic agent for FXS. Based on these advantages, the use of DCP-LA and its methyl esters have been already been patented for the treatment of neurodegenerative diseases,[280, 281] and undoubtedly will be the focus of much study in the near future.

However, it is important to note that DCP-LA has also been reported to demonstrate some activity that is possibly independent of PKCε stimulation. In one study, it was found that DCP-LA may inhibit protein-phosphatase 1 (PP1),[282] a serine/threonine phosphatase involved in neuronal signaling, glycogen metabolism, muscle contraction, and protein synthesis, among other roles. In the aforementioned study, this effect was found to indirectly augment levels of phosphorylated (activated) Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a downstream target of PP1. Therefore, to confirm whether DCP-LA corrects FXS-associated changes *via* activation of PKCε as opposed to independent pathways, we have conducted a subset of our studies using DCP-LA in conjunction with a myristoylated form of the selective PKCε peptide inhibitor, εV1/2 (EAVSLKPT). This widely-used PKCε inhibitor specifically

exerts its effects by blocking the translocation of the kinase.[283, 284] Like DCP-LA, Myr-εV1/2 has been used in a variety of applications,[283, 285, 286] and, like DCP-LA, appears to be capable of crossing the blood brain barrier, due to the presence of the myristoyl moiety.

1.6.8. The *Fmr1* Knockout Mouse Model of FXS

The use of appropriate models is critical in elucidating the pathogenesis of human disorders, and developing treatments for them. The inbred laboratory mouse has been used as a model organism to study inherited human conditions over the past century. Much of what is known about FXS is thanks to *in vivo* studies performed on mice. In particular, the *Fmr1* knockout mouse has been an invaluable tool in unraveling the molecular mechanisms underlying FXS.[287-289] Knock-out (KO) mice are those in which investigators have inactivated ("knocked out") a pre-existing gene, typically by either replacing the gene or inserting an artificial piece of DNA into the gene's sequence, thereby disrupting the function of the protein product.[290] Briefly, such knock outs are generated *in vitro* in pluripotent stem cell lines *via* homologous recombination, in which process the normal copy of the gene or portion of the gene is exchanged for the KO version. Cells containing the KO gene subsequently then cloned and micro-injected into a host blastocyst, which are then implanted into pseudo-pregnant recipient females. The resulting offspring, which will be chimeras for the KO gene, can then be bred to eventually produce a homozygous KO line, in which the gene has been knocked out in all tissues.

In many cases, the loss of the gene's activity results in phenotypic changes that mimic those observed in the human condition. Such is the case for *Fmr1* inactivation, as the gene is particularly well-conserved from mouse to human: the genomic structure of the mouse and human *Fmr1* genes share 95% sequence identity, 97% amino acid identity (including a conserved serine residue that is believed to tune the activity of FMRP on the translation of the

mRNA it is bound to), and lastly, exceptional structural conservation.[291] Thus, although the *Fmr1* KO model does not emulate the mutation found in patients, it is a fitting model for analyzing the consequences of the loss of function of FMRP.

An immeasurable number of studies have established that the *Fmr1* KO mouse model demonstrates exceptional face validity, or the degree of descriptive similarity between the model and human patients (for a review, see Kazdoba et al, 2014).[288] In other words, *Fmr1* KO mice share many molecular, morphological, and behavioral abnormalities with human patients with FXS, and respond well to treatments that are effective in humans. For example, some phenotypes exhibited by the model that are analogous to human features of FXS include macro-orchidism, heightened seizure susceptibility, heightened sensitivity to sensory stimuli, deficits in pre-pulse inhibition, perseverative and repetitive behaviors, hyper-anxiety, reduced social interaction, and cognitive deficits, although mixed results across laboratories have been obtained for the latter.

Microscopic analysis of post-mortem brain tissue from both human patients and *Fmr1* KO mice also reveal similarities at the synaptic level. Both the mouse model and human patients share the presence of immature dendritic spines in the cortex.[71, 292] Moreover, the model replicates the elevated cortical spine density that is observed in human patients, corresponding to periods of early development prior to synaptic pruning.[293, 294] This is especially important considering that this synaptopathy is central to the symptoms of FXS. As a result, because of these similarities, the *Fmr1* KO mouse model is well accepted for its usefulness in studying synaptic changes that occur in FXS, and consequently for illuminating the repercussions of these changes on early brain development.

Despite the usefulness of the *Fmr1* KO mouse as a tool for studying FXS, it must be noted that some strain-dependent phenotypic variability has been reported. Several reports have

demonstrated the importance of genetic background on behavioral features of *Fmr1* KO mice (for a review, see Spencer et al, 2011).[295] Two of the most commonly used genetic backgrounds for producing *Fmr1* mutants are the C57/BL6 (C57) and FVB/NJ (FVB) strains. However, numerous behavioral phenotypes appear to be dependent on which of these two strains is used. For instance, *Fmr1* KO mice appear to demonstrate reduced sociability[296] and impaired learning[297] on the FVB background, but not the C57 background Nonetheless, both strains replicate the immature spine phenotype observed in the patients,[71, 294, 298] suggesting that the variability between strains, and possibly between individuals with FXS, may be due (in part) to contributions by background genes, although this area requires further exploration.

In our preliminary studies, we have used *Fmr1* KO mice on the FVB/NJ strain, selected for their behavioral similarity to human FXS patients. This model was originally generated *via* insertional mutagenesis of the mouse *Fmr1* gene.[299] Briefly, exon 5 of the gene was interrupted by insertion of the neomycin resistance gene (Neo). This artificial sequence was then introduced, *via* homologous recombination, to embryonic day 14 (E14) stem cells derived from the 129/Ola strain. A FVB/N-129 hybrid mouse line carrying the mutation was then bred, and later repeatedly backcrossed to the FVB/NJ strain (Jackson labs).[300, 301] The resulting *Fmr1* KO mice and their WT counterparts were generously gifted to us by Drs. Carl Dobkin and Abdeslem El Idrissi.

Importantly, it must be noted that the FVB/NJ strain has one major drawback: they are homozygous for a retinal degeneration-causing mutation in the *Pde6b* gene ("retinal degeneration 1" or "rd1"), which normally encodes the rod CGMP-phosphodiesterase beta-subunit required for transmission of visual stimuli. As a result, all FVB/NJ mice are near blind by the time they are weaned.[302] This is arguably a significant confound because many behavioral assays in mice rely at least partially on visual perception (the elevated plus maze being a good

example). To counter this problem, we have conducted the majority of our studies on transgenic FVB/NJ mice (*Fmr1* KO and control) that are homozygous for the wild-type *Pde6b* gene ($Pde6b^{WT}$), and therefore have intact vision. (Jackson labs).[303, 304] Consequently, results obtained from behavioral assays performed on this sighted transgenic strain are more reliable than those obtained from the original, blind FVB/NJ strain ($Pde6b^{rd1}$).

1.6.9. Summary

Fragile X Syndrome (FXS) is an inherited developmental disorder characterized by disturbances in emotional and social behavior. FXS primarily arises from a silencing mutation of the *Fmr1* gene on the X chromosome, and subsequent loss of the neuronal translational regulator protein FMRP. Decades of studies have established that FMRP is a prerequisite for normal synaptic plasticity and transmission. However, alterations in synaptic plasticity during early stages of developmental wiring may in turn lead to defects in the formation of many cognitively and behaviorally relevant neurological circuits, such as connections within and between the hippocampus, hypothalamus, and amygdala. Moreover, these effects on the initial wiring of the brain may be to a large extent irreversible upon reaching adulthood.

Current treatments for FXS are greatly limited, have many drawbacks, and, despite the fact that FXS is a disorder of brain development, avoid intervention while the brain is still maturing. Moreover, although FXS is characterized by socio-emotional disturbances, and thus it is very likely that the oxytocinergic system is compromised in FXS, the majority of studies have surprisingly overlooked this line of research. In our efforts toward a therapeutic strategy, we have focused on correcting inherent changes in the oxytocinergic system, during the early stages of postnatal brain development. To do this, we have targeted the ventral hippocampus as a major regulator of the HPA axis. Recent findings have indicated that the expression of a key signaling

molecule during hippocampal development, PKCε, may be dysregulated in FXS. We therefore investigated the integrity of PKCε signaling in the *Fmr1* KO mouse brain, and potential consequences of disrupted PKCε signaling on ventral hippocampal regulation of the oxytocinergic system. Lastly, we evaluated whether early postnatal stimulation of PKCε could be a viable therapeutic strategy for FXS. To answer this question, we employed the use of a selective PKCε activator, dicyclopropyl-linoleic acid (DCP-LA), which has several advantages as a therapeutic agent and has already shown promise in the treatment of neurological conditions.

II. Materials and Methods

2.1. Subjects

All mice were housed in the College of Staten Island (CSI) Animal Care Facility and handled following a protocol approved by the CSI Institutional Animal Care Committee. All mice were housed in a temperature-controlled room (20±2°C) under a standard light-dark cycle of 12:12 h, with free access to food and water. All mice were housed with at least one partner, and one enrichment object, in the same home cage. Blind (*Pde6b*^{rd1}) FVB/NJ mice used in preliminary studies were generously provided by Drs. Carl Dobkin and Abdeslem El Idrissi. Sighted (*Pde6b*^{WT}) FVB/NJ mice were obtained from The Jackson Laboratory (*Fmr1* KO: strain #004624; WT controls: strain #004828). Unless otherwise stated, all mice used in the study were males, since men are more severely affected by the FXS mutation than women. Female KO mice used were homozygous for the *Fmr1* mutation. *Fmr1* KO mice were backcrossed after every second generation, and no generational differences were detectable in any of the groups. For all drug treatments, litter-mates were randomized into experimental or control groups, using at least one pup per litter per treatment group, in order to account for potential litter effects.

2.2. Drug Treatments

2.2.1. DCP-LA and Vehicle Treatments

DCP-LA was obtained from R&D systems. *Fmr1* KO littermates were injected intra-peritoneally with 3 mg/Kg DCP-LA or vehicle at P6, P10, and P14. These time points were chosen because they coincide with the time-frame of postnatal hippocampal development. In particular, P6 and P14 are crucial time-points in this process: P6, because it corresponds to the

timing of when GABAergic signaling switches from excitatory to inhibitory;[91] and P14, because it coincides with eye-opening and an immediately-following peak in synaptogenesis in response to visual inputs.[305] Moreover, PKCε expression in the hippocampus begins to increase at P6, and peaks around P14. In humans, P6 corresponds to the third trimester, and P14 corresponds to roughly 2 years old, at which point symptoms have begun to emerge in the patients.

A 1 mg/ml DCP-LA solution in 95% PBS, 5% DMSO was dissolved in sterile PBS to obtain a working solution. The working solution was injected intra-peritoneally (i.p.) into each pup to achieve a final concentration of 3 mg/Kg of body weight for DCP-LA, with a final systemic DMSO concentration of <1%. WT and KO controls were similarly injected with vehicle (sterile PBS containing the same concentration of DMSO) at the same time points. Hippocampal infusion *via* stereotactic injection was avoided since (1) previous studies have found that systemic DCP-LA administration is sufficient to correct behavior (see Section 1.6.6.), (2) this technique is highly invasive and likely to damage the structures targeted, as well as surrounding tissues, and (3) PKCε is highly expressed in the hippocampus, relative to most other central and peripheral tissues.[306]

2.2.2. Myr-εV1/2 Treatments

For studies involving the use of the selective PKCε inhibitor, Myr-εV1/2 (N-Myr-EAVSLKPT), the peptide was prepared by solid-phase synthesis as previously described.[283, 307, 308] The peptide was then dissolved in the aforementioned DCP-LA working stock solution to achieve a final systemic dosage of 50 μg/Kg, in accordance with previously-published studies.[309] The inhibitor was then injected (i.p.) simultaneously with 3 mg/Kg DCP-LA at P6, P10, and P14. WT and KO controls were similarly injected with vehicle at the same time points.

To ensure the inhibitor would enter the brain, we additionally delivered Myr-εV1/2

intranasally at the aforementioned time-points. For each time point, we administered an intra-brain concentration of 1 μM, assuming an efficiency of 10% transfer across the olfactory epithelium. Brain volumes at P6, P10, and P14 were estimated, using water-displacement in a graduated cylinder, to be ~100, ~150, and ~200 μl, respectively. For these intranasal administrations, a 1 mg/ml stock solution of the inhibitor was prepared, then diluted in sterile PBS to create a working solution of 4 μl total volume per pup. The pups were anesthetized *via* i.p. injection of ketamine (60 mg/Kg) and xylazine (6 mg/Kg), then placed in the supine position on a warm heating pad. For each pup, the 4 μl volume of inhibitor working solution was applied to each nostril, alternately, in 0.25 μl aliquots. An interval of 3 minutes was allowed in between each application, to avoid suffocating the pups. To allow enough time for the inhibitor to reach the hippocampus and take effect, the pups were given the previously-described i.p. injections of inhibitor and DCP-LA approximately 30 minutes after the intranasal application of the inhibitor. Importantly, for all experiments in which the inhibitor was used, control WT and KO pups were also anesthetized as described above and administered an equal volume of sterile PBS intranasally.

2.3. Immunohistochemistry

2.3.1. Immunolabeling Procedure

Mice were anesthetized *via* i.p. injection of ketamine (60 mg/Kg) and xylazine (6 mg/Kg), then perfused trans-cardially with sterile PBS, followed by 4% paraformaldehyde (PFA) in sterile PBS. The brains were fixed by overnight immersion in 4% PFA in PBS, then dehydrated overnight in 30% sucrose in PBS. Serial coronal slices of 30 μm thickness were obtained by cryosectioning, and blocked overnight in 0.1% Triton X-100 and 10% serum obtained from the species used to raise the secondary antibody, in PBS. This was followed by

treatment with the primary antibody in the same blocking solution overnight. The slices were then washed 3x in PBS (10 minutes each wash) and incubated overnight in blocking solution containing secondary antibody covalently linked to ether Alexa Fluor 488 (green), 568 (Red), or 633 (Far-Red). The slices were then washed again 3x in PBS, then incubated in a solution of 25 ug/ml of Hoechst33342 in PBS for 25 minutes, and washed again as described. Lastly, the slices were mounted onto glass slides using ProLong Gold-Antifade Mountant. To obtain a complete representation of the structures along the anterior-posterior axis, every 6th slice was used. For all immunohistochemical procedures, control slices were stained omitting primary antibody, to confirm specificity of binding. For hippocampal studies, slices were taken exclusively from the ventral hippocampus, which has been demonstrated to be involved in the regulation of emotional behavior.[310, 311]

2.3.2. NeuN Immunolabeling

For NeuN staining, the following antigen retrieval steps preceded blocking: 2N HCl at 37°C for 25 min, 1N HCl at 0°C for 10 min, neutralization with 0.1 M boric acid for 10 min, and then 3 rinses with 1x PBS buffer (10 min each). Slices were then processed as described in Section 2.3.1.

2.3.3. Surface GluR2 Immunolabeling

To study surface levels of GluR2, primary antibody targeting the extracellular N-terminus was used in conjunction with non-permeabilizing conditions (Triton X-100 and antigen retrieval steps omitted). For all studies of surface levels of GluR2, control slices were stained under permeabilizing conditions (including Triton X-100), allowing us to analyze total levels of protein (cytosolic vs. membrane-bound protein).

2.3.4. BrdU Immunolabeling

For neuroproliferation studies, P6 pups were injected with 100 mg/Kg of 5-bromo-2'-deoxyuridine (BrdU, Sigma) intra-peritoneally. The pups were sacrificed 24 h later, and proliferating neuroblasts were then detected with antibodies against BrdU, and counter-probed with antibodies against doublecortin and NeuN.

2.3.5. Antibodies

The following primary antibodies were used: Mouse-anti NeuN, Millipore (1: 500); Goat-anti GluR2 (N-19), Santa Cruz (1:1,000); Rabbit-anti Oxytocin, Immunostar (1:1,000); Rabbit-anti PKCε (C-15), Santa Cruz (1:1,000); Mouse-anti BrdU, Sigma (1:250); and Goat-anti DCX, Santa Cruz (1:500). Secondary antibodies used were Goat-anti mouse Alexa-Fluor 488, Goat-anti Rabbit Alexa-Fluor 568, Rabbit-anti Goat Alexa-Fluor 568, and Rabbit-anti Goat Alexa-Fluor 633, Santa Cruz (1:500).

2.3.6. Imaging and Analysis

All images were acquired using a Leica SP2 AOBS confocal microscope at a 1024 x 1024 pixel resolution. Z-stacks were acquired along the height of each slice at 20x and 63x magnification, with an interval of 1 μm between each z-position. Z-stacks were carefully examined to ensure even penentration of the antibody throughout the entirety of each slice. For each experiment, laser settings were kept constant from group to group and slice to slice. For all experiments, at least 3 sections each from at least 3 different animals were studied. Moreover, at least 3 such sets of experiments were performed for proper statistical analysis.

For studies where relative levels of PKCε or GluR2 were compared, the immunofluorescence intensity of each channel (red, green, and/or blue) per stack was measured with Adobe Photoshop after subtracting background levels of fluorescence present in a

secondary antibody control, although these were minimal. For cell quantification studies, cells were counted with IMARIS 7.6. Briefly, three-dimensional volumes of the stacks were rendered to view the exact positions of positive cells in the slice. For these quantification studies, all Z-stacks used were of the same thickness (30 μm). Cells were then automatically counted using the counter tool and double-checked for accuracy by a blind observer. Data obtained from Photoshop or IMARIS 7.6 was plotted using Excel to obtain column graphs, and statistical analysis was subsequently performed in Statistica 10. All data were presented as mean ± S.E.M. Statistical significance was defined as $p<0.05$.

2.4. Western Blotting

2.4.1. Western Blotting Procedure

Hippocampal and hypothalamic lysate were acutely acquired from mice as follows: mice were fully anesthetized *via* i.p. injection of ketamine (60 mg/Kg) and xylazine (6 mg/Kg), then sacrificed by cervical dislocation. Brains were harvested and immediately submerged in oxygenated, ice-cold artificial cerebrospinal fluid (ACSF) containing 0.25 M sucrose. The brains were then sectioned into 300 μm thick slices using a Leica vibratome and incubated in the above solution. A 1.5mm diameter biopsy hole-puncher was used to isolate the DG, CA3, and CA1 subregions of the ventral hippocampus, and paraventricular region of the hypothalamus. A dissection microscope was used in order to fully discern each of these regions. The tissue samples were then immediately homogenized in RIPA buffer containing a complete protease inhibitor cocktail.

Protein concentration from each sample was determined using the DC Protein Assay (Bio-Rad) in a micro-plate, then scanned using the SoftmaxPro software version 2.6 (Molecular Devices). Aliquots containing 30 ug of total protein were then resolved on a 10% SDS PAGE

gel. The proteins were then transferred to a PVDF membrane, blocked in 3% BSA (for phosphorylated proteins) or 5% NFDM (for non-phosphorylated proteins) for 2 h at room temperature. The blots were then incubated with primary antibodies in the same blocking solution overnight at 4°C and washed 3X with 1x TBS containing % Tween (TTBS). Lastly, the blots were incubated with secondary antibodies for 2 h at RT, washed 3X with TTBS, and then developed using SuperSignal West Pico Chemiluminescent Substrate for non-phosphorylated proteins or SuperSignal West Dura Extended Duration Substrate for phosphorylated proteins (Thermo Fisher). Lastly, resulting chemiluminescent bands were imaged using an Alpha Innotech Fluorochem FC2 version 1307 scanner, using the accompanying Alpha View software.

2.4.2. Antibodies

The following primary antibodies were used for western blotting: Rabbit-anti PKCε (C-15), Santa Cruz (1:1,000); Goat-anti GluR2 (N-19), Santa Cruz (1:1,000); Goat-anti phospho-GluR2 (Ser 880), Santa Cruz (1:1,000); Rabbit Anti-ERK1/2, Santa Cruz (1:1,000); Mouse anti-Phospho-T202, Y204-ERK, Cell Signaling (1:1,000); Rabbit anti GluR1, Immunostar (1:1,000); Goat anti phospho-GluR1 (Ser 831), Santa Cruz (1:750); and Mouse-anti β-actin, Sigma (1:10,000). Secondary antibodies used were Goat anti-Rabbit IgG-HRP (1:50,000); Rabbit anti-Goat IgG HRP (1:20,000) and Goat anti-Mouse IgG-HRP (1:50,000), Santa Cruz.

2.4.3. Analysis

Densitometric analysis was performed using Image J software. The intensity of each protein band was quantified on unadjusted images. To account for potential loading artifacts, all protein bands were normalized to the intensity of corresponding β-actin bands, which served as internal controls. For all experiments, at least 3 brains were processed per group, and at least 3 such sets of experiments were performed for proper statistical analysis. Data were presented as

mean ± S.E.M. Statistical significance was defined as $p<0.05$.

2.5. Co-Immunoprecipitation

2.5.1. Co-Immunoprecipitation Procedure

Hippocampal lysate was acutely acquired from P18 mice as described above. A 200-ug aliquot of total protein lysate was incubated with 2-ug of primary antibody for 24 hours, and then this mixture was incubated with Protein A/G sepharose beads (Santa Cruz) for an additional 24h to pull down the antibodies, target protein, and linked protein complexes. The samples were then resolved *via* SDS-PAGE and subjected to western blotting as described in Section 2.4. For the input samples, 25-ug aliquots of total protein lysate that did not undergo co-immunoprecipitation were loaded. For all experiments, at least 3 brains were processed per group, and at least 3 such sets of experiments were performed for proper statistical analysis. Data were presented as mean ± S.E.M. Statistical significance was defined as $p<0.05$.

2.6. Behavioral Testing

2.6.1. Subject Handling

Behavior testing was conducted in age- and sex- matched mice between the ages of P60 – P80. All behavior testing was conducted during the light phase. Mice were subjected to only one behavior test per day starting at P60, and were tested every other day. Because the mice were housed on a standard 12:12 light:dark schedule, on each testing day, the mice were transported to a quiet, adjacent anteroom to the behavior testing room and were acclimated in the dark for 1 hr prior to testing, allowing sufficient time for the animals to become active and alert. During testing, the animals were recorded using an overhead camera. No experimenters remained in the room during testing. Additionally, a white noise generator was used to block out any extraneous

noises that might cause distraction. After each trial, the apparatus was thoroughly cleaned with 70% ethanol in order to prevent odor cues left behind by the previous mouse. Importantly, the person conducting the test was always the same female. We make this acknowledgement in light of recent findings that female experimenters may induce less stress in mice than male experiment,[312] as well as to address the possibility that mice may react differently to different experimenters in general. At all times, the mice were handled very quietly and gently to avoid causing undue stress, and were transported to and from the behavioral testing room on a comfortable soft platform to which they were acclimated previously.

2.6.2. Elevated-Plus Maze (EPM)

The EPM apparatus used consisted of two opposing open arms (36 cm x 7 cm) and two closed arms (36 cm x 7 cm), joined at a square central area (5 cm × 5 cm). The apparatus was constructed of wood with the maze floor covered with white-painted vinyl, which allowed sufficient traction for the mice to explore the maze comfortably while also providing sufficient contrast to detect their movements. The entire apparatus was elevated to a height of 50 cm above the floor by four supports beneath each arm. The open arms were illuminated at 70 lux and the closed arms were illuminated at ~10 lux by a central, overhead light. This was done to avoid the potential of a ceiling effect on anxiety measures that could be induced by stressful bright lighting. Mice were initially placed in the center of the maze, and the number of entries to and time spent in the open arms were subsequently measured over a period of ten minutes.

2.6.3. Light-Dark Test (LD)

Designed based on the innate tendency of mice to hide in a dark chamber, the light-dark test was conducted according to published protocols with minor modifications.[313] The light-dark test apparatus consisted of a plastic box consisting of a 50 cm x 25 cm rectangular arena with 40

cm high walls, which was divided into two 25-cm compartments. The dark compartment was unlit (<5 lux), painted black with an opaque ceiling and walls, and the light compartment was transparent and illuminated by an overhead fluorescent tube (150 lux at cage floor). During the test, each mouse was placed in the center of the lit chamber and subsequently monitored for 10 min as it moved freely between the two chambers. Lastly, the total time spent in each chamber was recorded as an anxiety measure.

2.6.4. Three-Chambered Social Interaction Test (SI)

Testing was conducted based on previously-published protocol.[147] Testing took place over three sessions within a three-chambered box of standard dimensions,[147] with openings between the chambers. The apparatus was illuminated to 7 lux as opposed to normal overhead lighting to avoid provoking anxiety in the subjects. After habituation to the empty box for 10 min, the subject was presented with the choice to investigate an unfamiliar age- and sex-matched mouse under one pencil cup (target mouse) on one side of the apparatus, versus an empty pencil cup (novel object) on the opposite side of the apparatus, for 10 min. The time spent investigating each pencil cup and the number of approaches to each cup was measured. Social approaches were defined as instances where the subject mouse sniffed or engaged in active, close contact with the novel mouse. Sociability was defined as the subject mouse spending more time investigating the novel mouse than in the chamber containing the inanimate novel object. Lastly, a social preference index was calculated for each mouse as follows: social preference index = time spent with novel mouse / (time spent with novel mouse + time spent with novel object). An index above 0.5 indicated that more time was spent investigating the novel mouse versus the novel object; an index below 0.5 indicated more time was spent with the novel object versus the novel mouse; and an index of 0.5 indicated that the subject spent an equal amount of time with

the novel mouse and novel object. Lastly, to avoid any potential biases towards one particular chamber, the positions of the target and novel object were alternated between subjects.

2.6.5. Innate Olfaction Test

To address the possibility that differences in social interaction could be attributed to differences in olfactory perception, we first performed an innate olfaction test prior to conducting the social interaction test. Mice were familiarized to a highly palatable treat (Nabisco Teddy Grahams) every day for one week, then were food-deprived for 18 hours prior to the test. The mice were placed in a clean, empty cage and the latency to detect a Teddy Graham (buried 3 cm under the bedding) was scored using a stopwatch. Latency to discover the treat was then measured using a stopwatch. Mice were allowed to eat their treat and then returned to the home-cage. However, no differences were found in olfactory sensitivity between our WT and KO mice, in accordance with previous reports.[314]

2.6.6. Open Field (OF)

This test was conducted according to a previously published report with minor modifications.[145] In this test, each mouse was placed in a large, rectangular arena (92 cm x 62 cm, illuminated at 400 lux) from which escape was prevented by high (23 cm) surrounding walls. In such a situation, mice generally prefer the periphery of the apparatus to the center of the open field, as open spaces correspond to areas where, in the wild, predators may easily spot the mice. Animals were individually placed in the center of the arena (covering one-fourth of the total area of the arena, i.e. 46 cm x 30 cm), and their movements video recorded for ten minutes by an overhead camera positioned above the center of the arena. To characterize spontaneous locomotor activity of each mouse, the total distance traveled, time spent mobile, and average speed were recorded. Additionally, time spent in and entries to the center of the arena were used

to assess anxiety.

2.6.7. Analysis

Captured videos were subsequently analyzed using ANY-Maze motion-tracking software. For behaviors that could not be detected by ANY-Maze, the videos were scored manually while blinded to the treatment groups. Statistical analyses were carried out using Statistica 10, using unpaired, two-tailed Student's t-test or analysis of variance (ANOVA) whenever appropriate. Significant main effects or interactions were followed up with Tukey's HSD (honestly significant difference) post hoc test. For all experiments, at least 4 mice were tested per group, and at least two such sets of experiments were conducted for proper statistical analysis. Data were presented as mean ± S.E.M. Statistical significance was defined as $p<0.05$.

III. Results

3.1. Pharmacological stimulation of PKCε during the neonatal stage rescues behavioral abnormalities in adult male *Fmr1* KO, *Pde6b*^{rd1} mice.

In our earlier studies, we have found that neonatal administration of a selective PKCε activator, dicyclopropyl-linoleic acid (DCP-LA), rescues anxiety-like behavior in 5-HT_{1A} receptor KO mice upon adulthood (Figure 13). Since many symptoms of FXS are attributed to hyper-anxiety, we sought to investigate whether this treatment regimen could similarly rescue anxiety-like behavior in *Fmr1* KO mice, the leading model of FXS. In a preliminary study, male WT and *Fmr1* KO pups on the FVB/NJ, *Pde6b*^{rd1} (retinal degeneration 1) background were administered i.p. injections of 3 mg/Kg DCP-LA or vehicle (PBS containing <1% DMSO) at ages P6, P10, and P14, corresponding to the time frame of postnatal hippocampal maturation. The pups were then allowed to reach adulthood (P60), at which time they were subjected to behavioral testing (Figure 17). In these preliminary experiments, only male mice were studied because men are generally more severely affected by the FXS mutation than women.

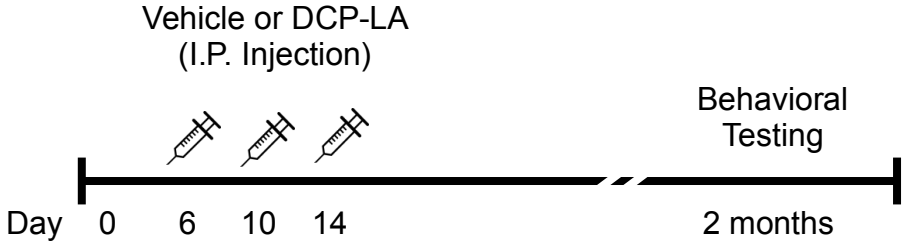


Figure 17. Treatment scheme for behavioral studies.

3.1.1. Anxiety-like Behavior

In accordance with previous reports, we observed that our *Fmr1* KO mice that were treated with inert vehicle as pups displayed significantly heightened anxiety-like behavior in the elevated plus maze (EPM) at P60, compared to vehicle-treated WT controls. We observed that the vehicle-treated KO mice spent approximately 4x less time in the open arms (Figure 18A), and made half as many entries as to the open arms (Figure 18B) as their WT counterparts. The vehicle-treated KO mice were also less exploratory in the EPM compared to the WT group, as measured by a drastically reduced number of head dips, a risk-assessment behavior in which the mouse peers over the edge of the open arm towards the floor ($p=0.007$) (Figure 18C).

As expected, the hyper-anxiety phenotype displayed by the vehicle-treated KO mice was nearly fully reversed in KO mice treated with DCP-LA as pups. DCP-LA treated KO mice were statistically comparable to the WT group in both time spent in the open arms and entries to the open arms ($p=0.30$ and 0.79, respectively), and these improvements were on the cusp of significance when compared to the vehicle-treated KOs ($p=0.051$ and 0.052, respectively) (Figure 18A and B). The KO+DCP-LA group was also significantly more exploratory in the EPM than the vehicle-treated KO group, as measured by the number of head dips ($p=0.022$), and were marginally comparable to controls ($p=0.11$) (Figure 18C). Nevertheless, taken together, these findings suggest that neonatal DCP-LA administration appears to considerably diminish anxiety-like behaviors in the EPM during adulthood, in *Fmr1* KO mice on the classical FVB/NJ background.

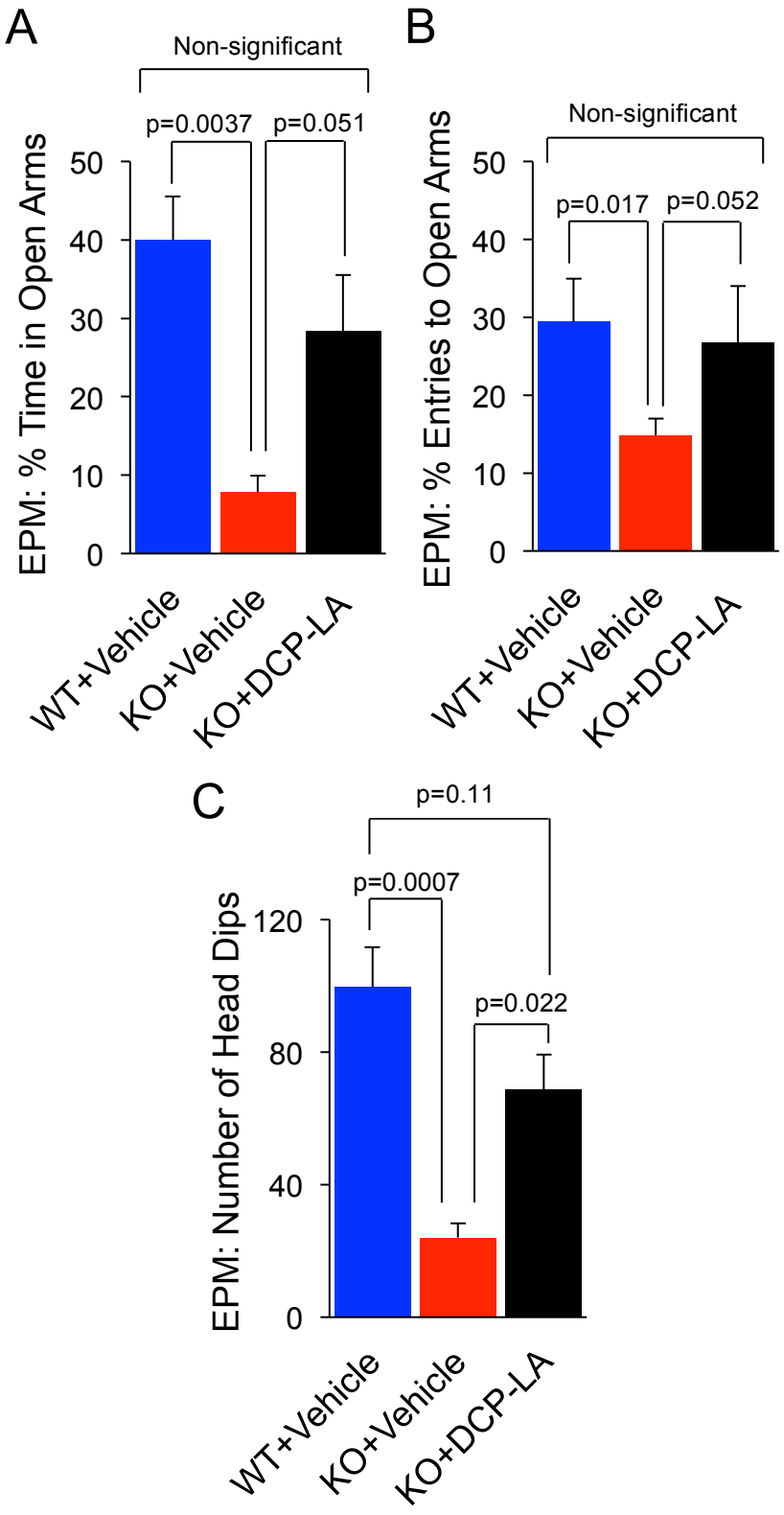


Figure 18. Neonatal DCP-LA treatment rescues anxiety-like behavior in the elevated plus maze (EPM) in male FVB/NJ *Fmr1* KO mice. Group n's: 4-5 per group.

3.1.2. Social Interaction

Encouraged by the above results, we then investigated whether social behavior was altered in the *Fmr1* KO mice, and moreover, whether our neonatal DCP-LA treatment regimen could correct any apparent social deficits. In accordance with independent studies, we found a marked reduction in social investigation in our vehicle-treated KO mice compared to WT controls in the three-chambered social interaction test (Figure 19). When given the choice between investigating a novel mouse versus a novel inanimate object, we found that the vehicle-treated KO mice did not display a preference for either stimulus, spending roughly an equivalent percentage of time investigating the novel mouse and object (Figure 19A). On the other hand, we observed that the WT mice displayed a trend towards a preference for the novel mouse over the object ($p=0.12$). In sharp contrast, DCP-LA-treated KO mice significantly preferred the novel mouse to the object ($p=8.1x10^{-5}$).

Overall, we observed that vehicle-treated KO mice spent a lower percentage of time investigating the novel mouse compared to WT mice, with the difference only slightly missing the threshold of statistical significance ($p=0.067$) (Figure 19B). On the other hand, the DCP-LA treated KO mice were roughly comparable to the WT mice in time investigating the novel mouse ($p=0.19$). It is important to note that our observed results that were on the border of significance may be attributed to the small group sizes used in these preliminary studies (4-5 mice per group). Nevertheless, when we examined the number of social approaches to the novel mouse per group, we found that the vehicle-treated KO mice made significantly less approaches than the WT mice, despite the small group size tested ($p=0.046$) (Figure 19C). Consistent with our previous findings, this deficit was fully rescued in the DCP-LA treated KO mice when compared to the WT controls ($p=0.023$). Collectively, these results point to a deficit in social interaction in *Fmr1* KO mice on the FVB/NJ strain, which appears to be rescued with neonatal DCP-LA treatment.

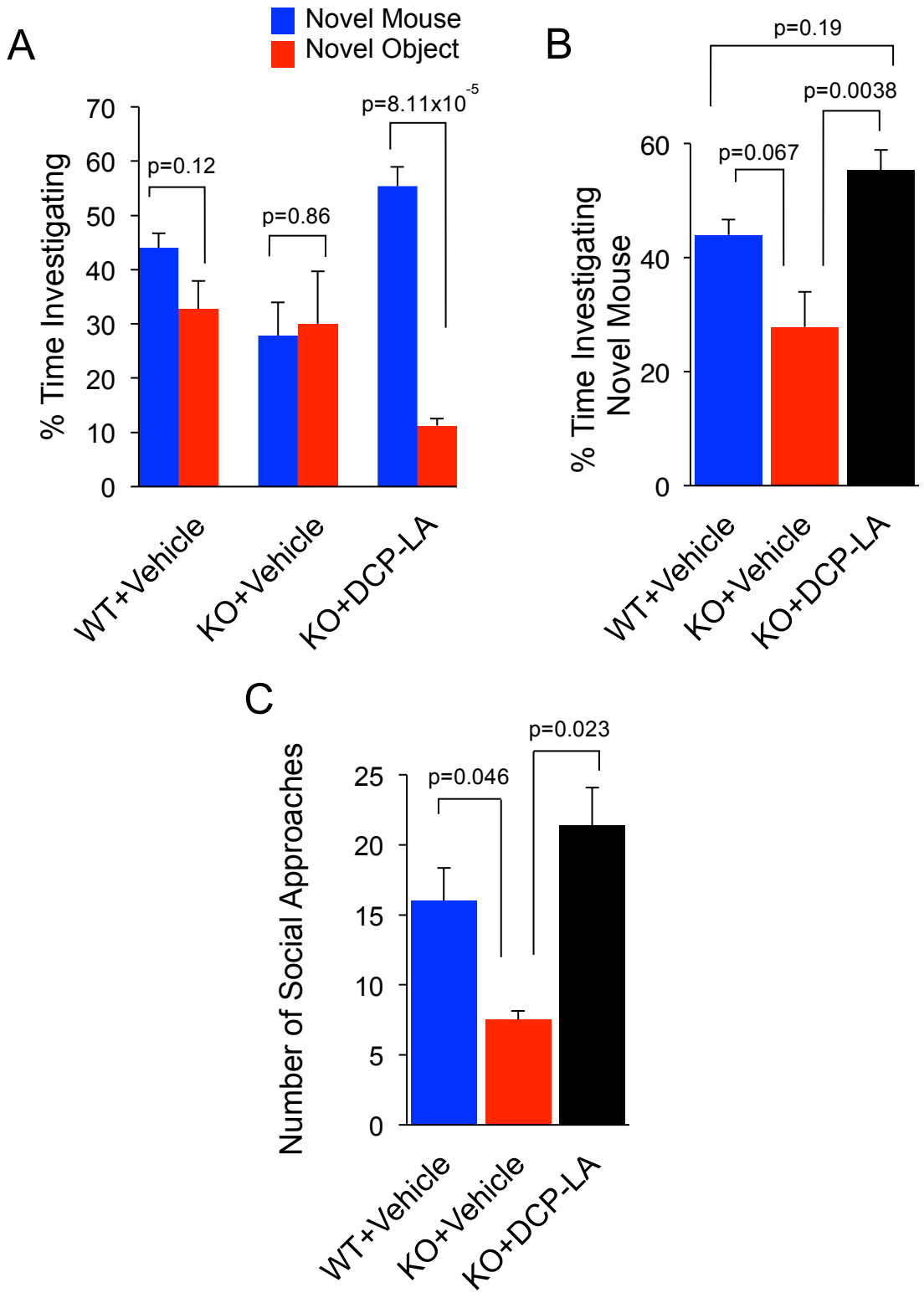


Figure 19. Social behavior deficits in male FVB/NJ *Fmr1* KO mice are corrected by neonatal DCP-LA treatment. Group n's: 4-5 per group.

3.1.3. Locomotor Behavior

To address the possibility that the previously observed differences in the EPM and SI tests were influenced by differences in locomotor behavior, we then performed the open field test (OF). Although we noted that the vehicle-treated KO mice spent less time mobile and traveled less distance at the beginning of the test, they were comparable to their WT counterparts by the final two minutes of the test in both measures (Figure 20, panels A and B, respectively). Based on this, it is possible that the hypoactivity observed in the vehicle-treated KO mice at the beginning of the test could likely have been due to a delay in acclimation to the unfamiliar testing environment. In other words, though the KO mice appeared to refrain from exploring the apparatus initially, they eventually appear to adjust, behaving similarly to the WT mice by the end of the test (Figure 21). Considering that the test was relatively short in duration (10 min), it is likely that this observation reflects reactions to novelty rather than motor impairment.

In addition, we observed distinct hypoactive phenotype in the DCP-LA treated KO males throughout the test, as measured by both time mobile and distance traveled (Figure 20 A and B). However, this seems unlikely to be due to anxiety, if our results from the EPM test are any indication. Based on this observation, is possible that DCP-LA may exert an inhibitory effect on locomotor activity in this strain of mice, through currently unknown mechanisms.

We then examined whether the *Fmr1* KO mice demonstrated reduced exploration of the center zone of the OF, which can be interpreted as an indicator of anxiety, since anxiolytic drugs often increase this measure. However, we detected no differences between vehicle-treated KO mice and WT controls in time mobile in the center zone (Figure 22). This is not wholly surprising since the open field is considered to be better suited for measuring locomotor behavior rather than anxiety-like behavior. On the other hand, we observed robust differences between the

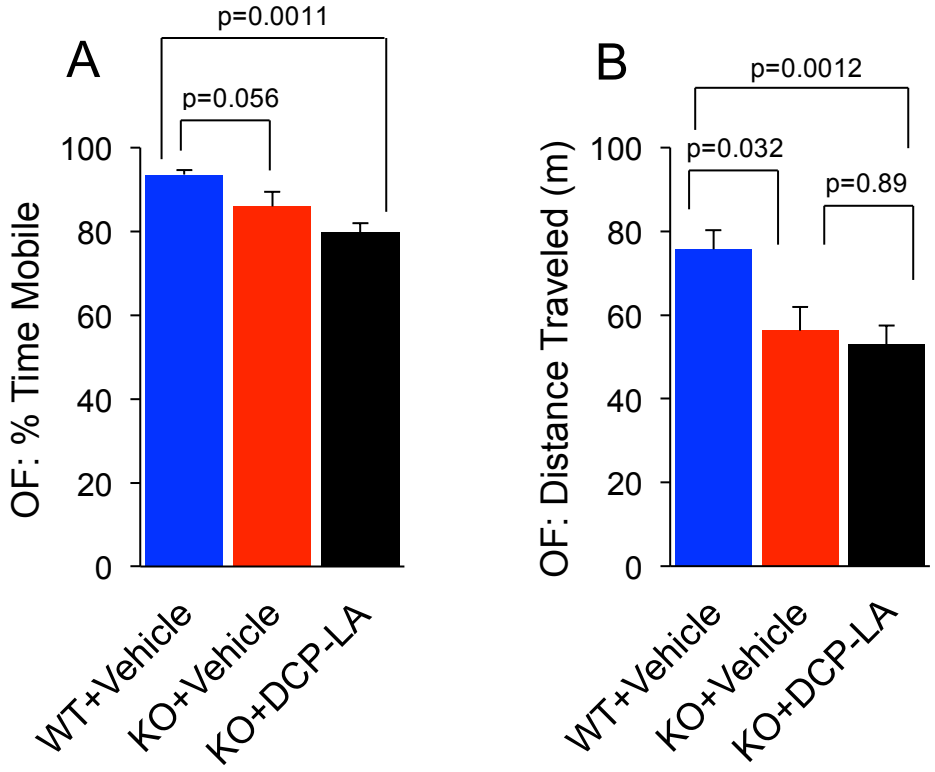


Figure 20. FVB/NJ *Fmr1* KO mice display hypo-activity in the open field compared to WT mice. Neonatal treatment with DCP-LA did not rescue this hypo-activity. Group n's: 4-5 per group.

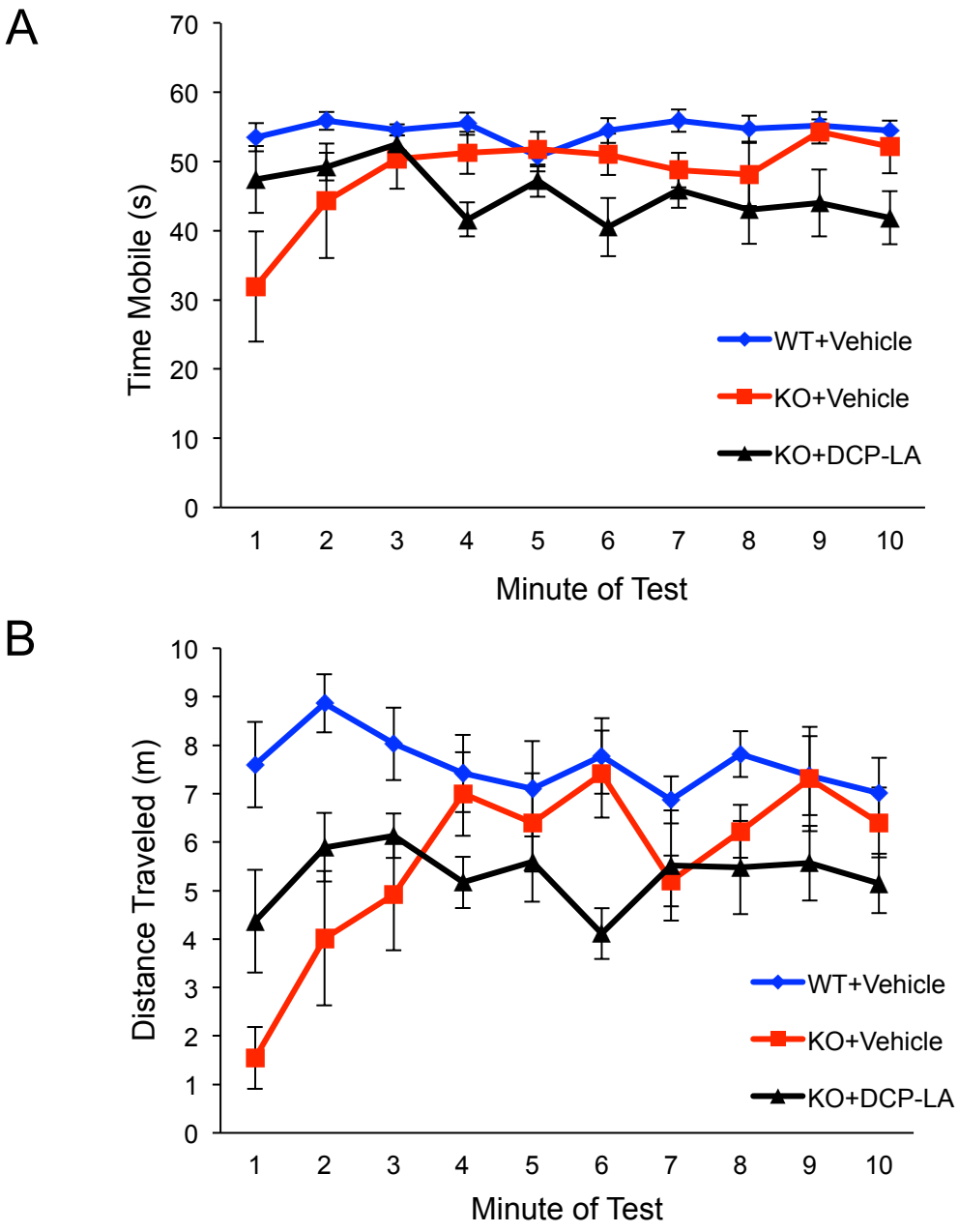


Figure 21. Minute-by-minute time courses of locomotor activity in the open field. Group n's: 4-5 per group.

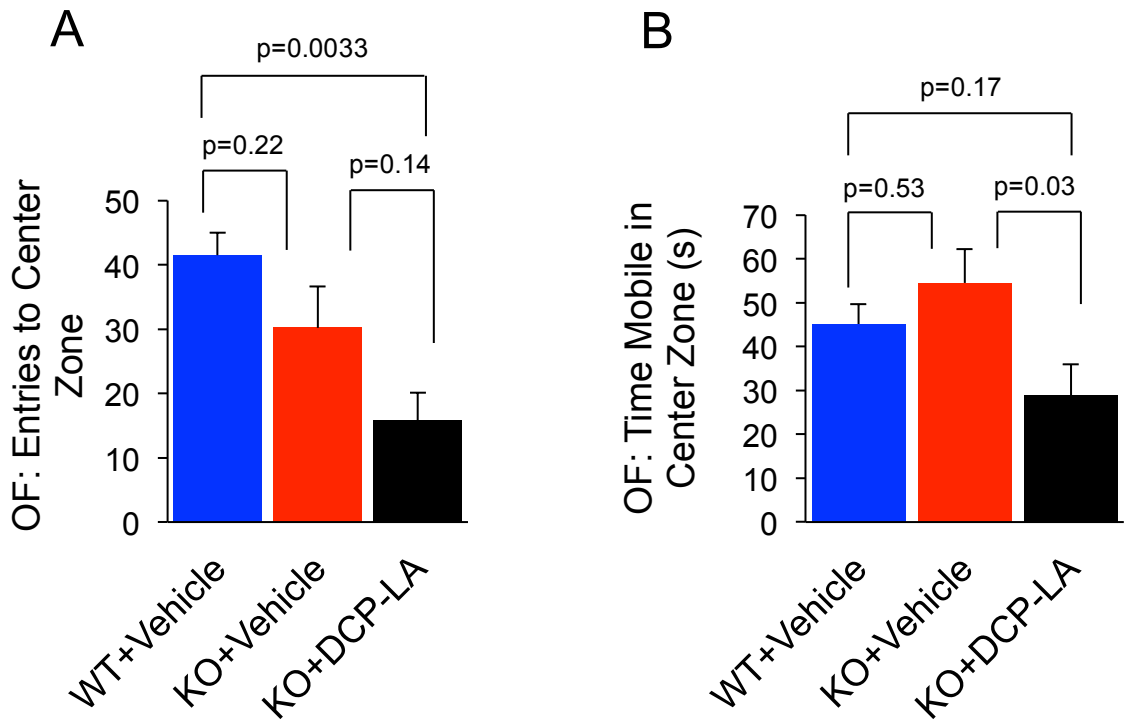


Figure 22. Effects of neonatal DCP-LA treatment on open field (OF) center-zone behavior in FVB/NJ *Fmr1* KO mice. Group n's: 4-5 per group.

vehicle treated WT and KO groups in the EPM, as described in 3.1.1 (Figure 18), which is considered the gold standard for assessing anxiety-like behavior. Thus, the OF may not have been anxiogenic enough to produce a discernable effect on exploration in the center zone.

Lastly, we observed a pattern of reduced exploration of the center zone in the DCP-LA treated KO mice (Figure 22). However, this observation may not automatically indicate heightened anxiety: these results are in fact consistent with our earlier observations that DCP-LA-treated KO mice appear hypoactive in the OF (Figure 20). Moreover, our DCP-LA-treated mice appear to display reduced anxiety-like behavior in the EPM (Figure 18), which is generally accepted to be a better assessment of anxiety phenotypes.

It is important to note that the mice used in these preliminary studies harbored the retinal degeneration 1 mutation in the *Pde6b* gene (rod cGMP phosphodiesterase), which leads to near blindness by weaning age. This may present a significant confound when attempting to interpret results obtained from the above behavioral assays. Thus, we transitioned to a sighted variant of these mice possessing the wild-type *Pde6b* gene (FVB/NJ $Pde6b^{WT}$) for all subsequent studies. Nevertheless, these preliminary results collectively indicated that DCP-LA appears to normalize anxiety-like behavior and social deficits in FVB/NJ *Fmr1* KO mice, at least in the assays we performed.

3.2. PKCε is under-expressed in the *Fmr1* KO ventral hippocampal CA1 region during postnatal hippocampal maturation.

Inspired by these preliminary findings, we hypothesized that PKCε-mediated signaling may potentially be compromised in *Fmr1* KO brain, and by extension, in FXS. This would provide a likely avenue by which the selective PKCε activator DCP-LA appears to restore anxiety levels and social behavior in FVB/NJ *Fmr1* KO mice, as we have observed. Further

bolstering this hypothesis, Darnell and colleagues have found that FMRP binds to PKCε mRNA.[43] Thus, expression of PKCε itself may be subject to translational regulation by FMRP, and therefore is likely dysregulated in FXS. However, given that FMRP seems to bimodally influence translation depending on the mRNA target, the precise consequences of absence of the FMRP on PKCε expression remained unknown. If PKCε expression is indeed altered in FXS, is the kinase over-expressed or under-expressed? Given our findings that DCP-LA seems to normalize anxiety and social behavior in *Fmr1* KO mice, we predicted that the drug may potentially compensate for a reduction in expression of PKCε. To address this hypothesis, we next investigated the integrity of PKCε expression in the *Fmr1* KO brain.

PKCε expression may be governed by different regulatory mechanisms depending on spatiotemporal factors such as age and brain region. Since PKCε is predominantly expressed in the hippocampal formation relative to most other brain regions,[306] we therefore honed in on this structure in our next set of studies. Particularly, we questioned whether PKCε expression was altered in the ventral portion of the hippocampus, which, as discussed, is a hub which influences behavior and emotion through its dense connections with other brain regions such as the cortex, amygdala, and hypothalamus. Therefore, it is plausible that altered PKCε expression in the ventral hippocampus may contribute to FXS-associated symptoms in these areas.

3.2.1. Immunohistochemical Analysis

Given our preliminary findings that neonatal administration of the PKCε activator DCP-LA appears to restore anxiety and social disturbances in the *Fmr1* mouse model, we next investigated whether ventral hippocampal PKCε expression was altered during the early postnatal period. We first examined PKCε expression levels at P6, at which point expression of PKCε within the hippocampus begins to steadily increase until P14-P15.[248] This was then

followed up a second, near-identical study in which we evaluated PKCε expression at P18, coinciding with the period shortly after eye-opening, and a subsequent dramatic rise in synaptogenesis.[305]

To resolve spatial expression patterns of PKCε within the ventral hippocampus and within cells, immunohistochemical labeling of the protein was performed. We observed that PKCε was highly expressed within the ventral dentate gyrus, (DG), CA3, and CA1 regions at both P6 and P18. High magnification imaging (63x with added zoom) revealed that the kinase was primarily localized the soma and dendrites (Figure 23).

Although we did not detect a meaningful difference in PKCε levels in the ventral hippocampus at P6 (Figure 24) between WT and KO groups, we did observe a significant reduction in ventral CA1 PKCε expression in the KO group at P18 ($p=1x10^{-4}$) (Figure 25), as measured by immunofluorescence intensity. This corresponded to a 25% decrease in expression. Importantly, the total number of neurons in this region remained unchanged (Figure 26), suggesting that the decrease in PKCε expression was not due to a reduced number of neurons. Intriguingly, this phenomenon was not observed in the CA3 region, although we did discover a possible trend towards significance in the dentate gyrus (DG) ($p=0.089$). We speculate this discrepancy may be attributed to functional differences between these regions that are only beginning to be understood. However, given that CA1 is the major output region of the hippocampus, our findings may be particularly relevant to FXS as it is likely that any disruption of CA1 activity may product effects on behavior.

Importantly, the fact that we were unable to detect a difference in PKCε expression at P6, combined with our observation that PKCε expression does not appear to be fully ablated in the *Fmr1* KO pups at P18, suggests that translational regulation by FMRP may be one of many

pathways governing PKCε expression. (Such redundancy in signaling pathways regulating gene expression may serve a protective function: in the event that one signaling pathway becomes compromised, another may take its place.) Different signaling pathways regulating the expression of a single protein, such as PKCε, may be active at different developmental time points, depending on the protein's function at that time point. Indeed, PKCε-mediated signaling appears to play a role in a variety of cellular processes.[243, 306] Here, we report that PKCε expression is normal in *Fmr1* KO mice at P6, which marks the beginning of postnatal hippocampal development, but is impaired by P18. Importantly, the immediate days following eye-opening at P15 are marked by a dramatic surge in hippocampal synaptogenesis. Considering that PKCε expression significantly rises in the hippocampus from P6 to P15 (Figure 11), it is likely that the protein plays a role in mediating these changes. Interestingly, P6 corresponds to the third term in humans, whereas P18 corresponds to 3 years of age, the average age of Fragile X diagnosis in boys. Given the link between the ventral hippocampus and emotion, we questioned whether reduced PKCε expression in the hippocampus during this time frame could potentially contribute to emotional symptoms in our *Fmr1* KO mouse model.

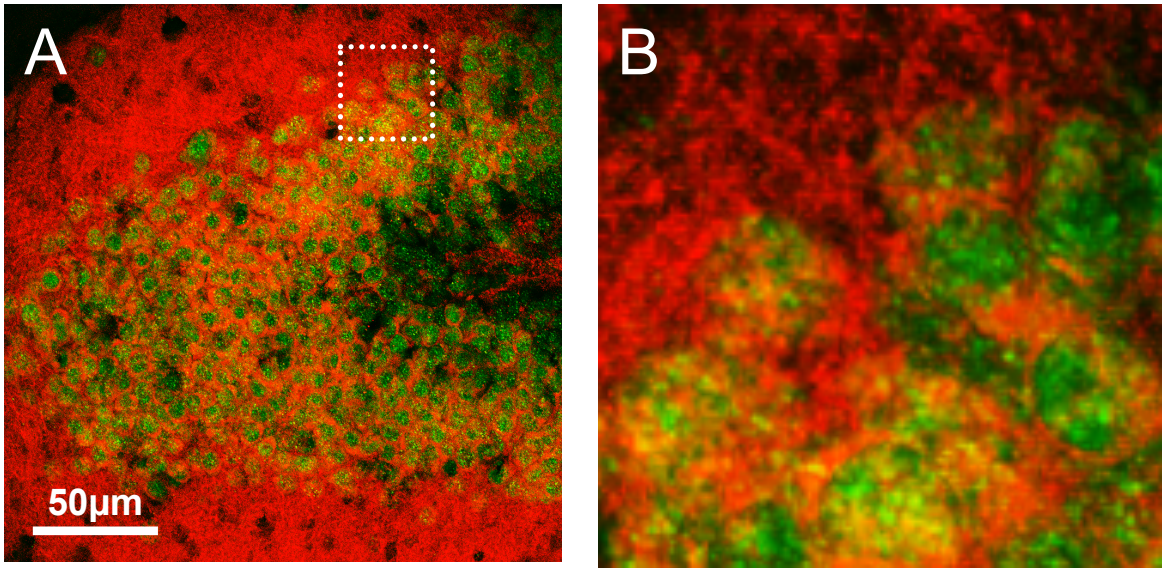


Figure 23. Hippocampal PKCε is localized to the soma and dendrites of neurons. Green indicates NeuN labeling of neuronal nuclei; red indicates PKCε labeling. Images acquired at 63x magnification. Images obtained from P6 WT pups.

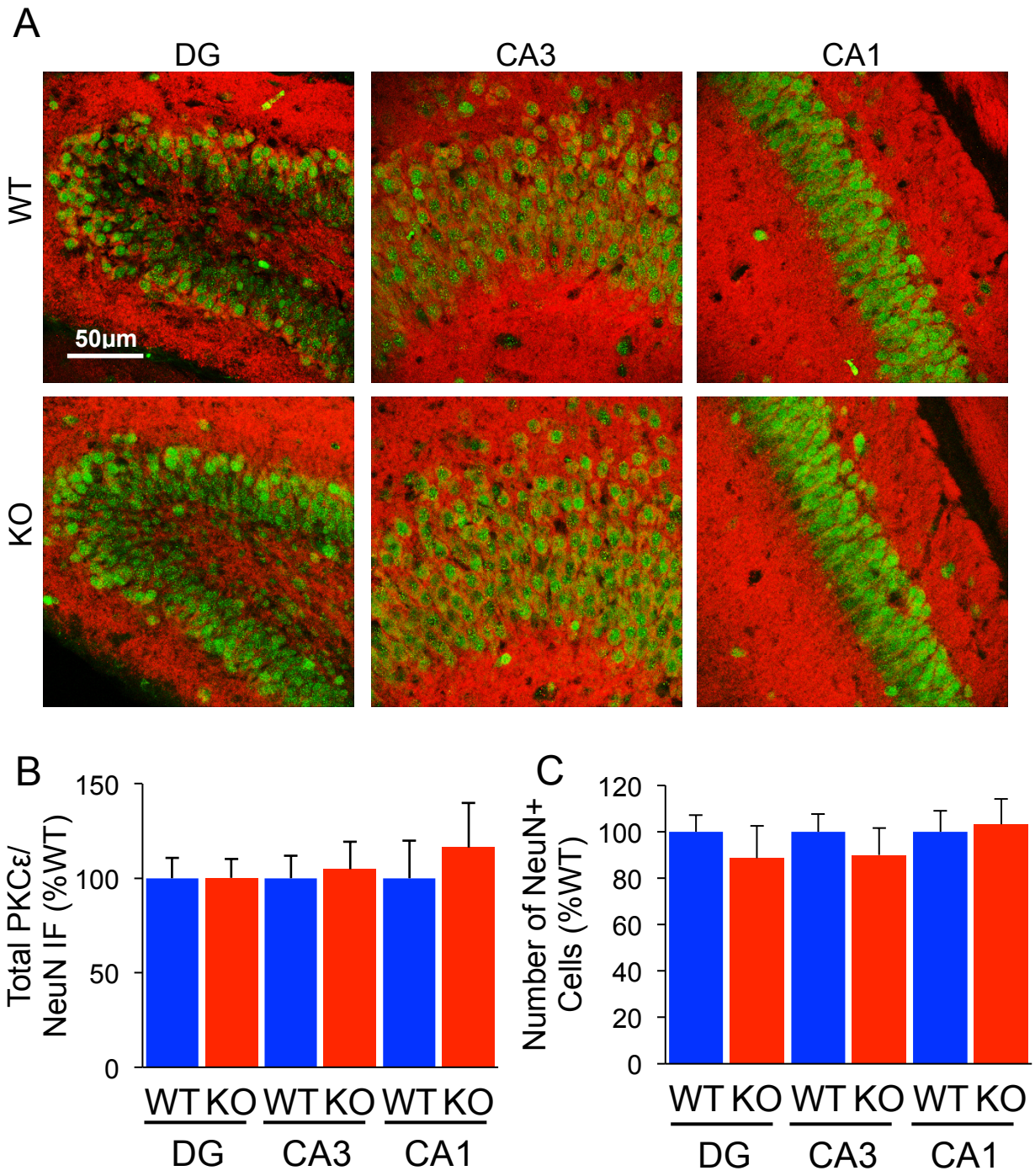


Figure 24. (A, B) Ventral hippocampal PKCε expression does not differ between WT and *Fmr1* KO mice at P6, as measured by PKCε immunofluorescence (IF) intensity normalized to NeuN IF. Red: PKCε, Green: NeuN. Images acquired at 63x magnification. (A,C) The number of NeuN+ neurons is also unchanged between the two groups at P6. Group n's: 5-6 per group.

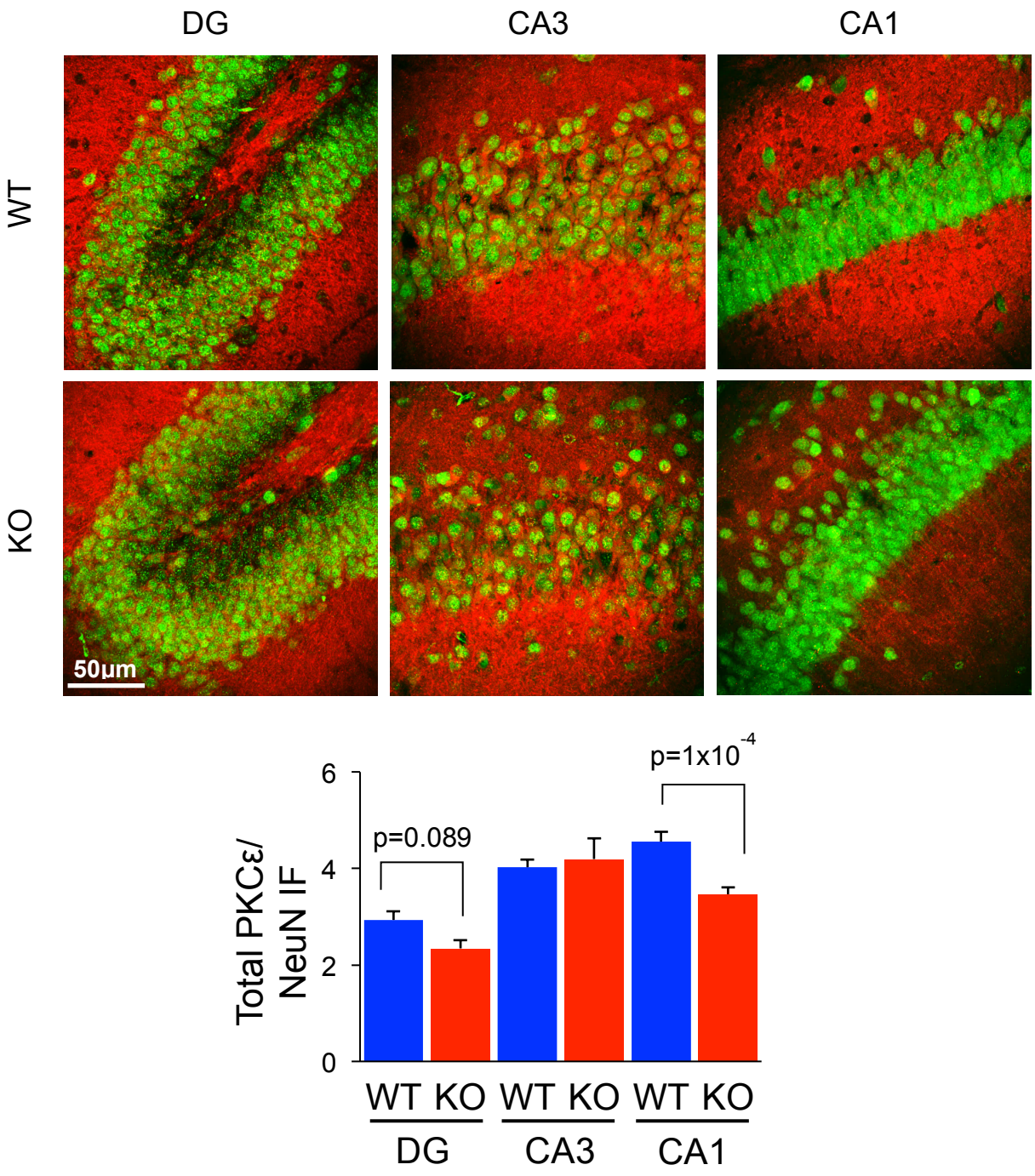


Figure 25. PKCε expression is suppressed in the ventral CA1 region of *Fmr1* KO mice at P18 compared to WT controls, as measured by PKCε immunofluorescence (IF) intensity normalized to NeuN IF. Red: PKCε, Green: NeuN. Green: NeuN; red: PKCε. Images acquired at 63x magnification. Group n's: 5-6 per group.

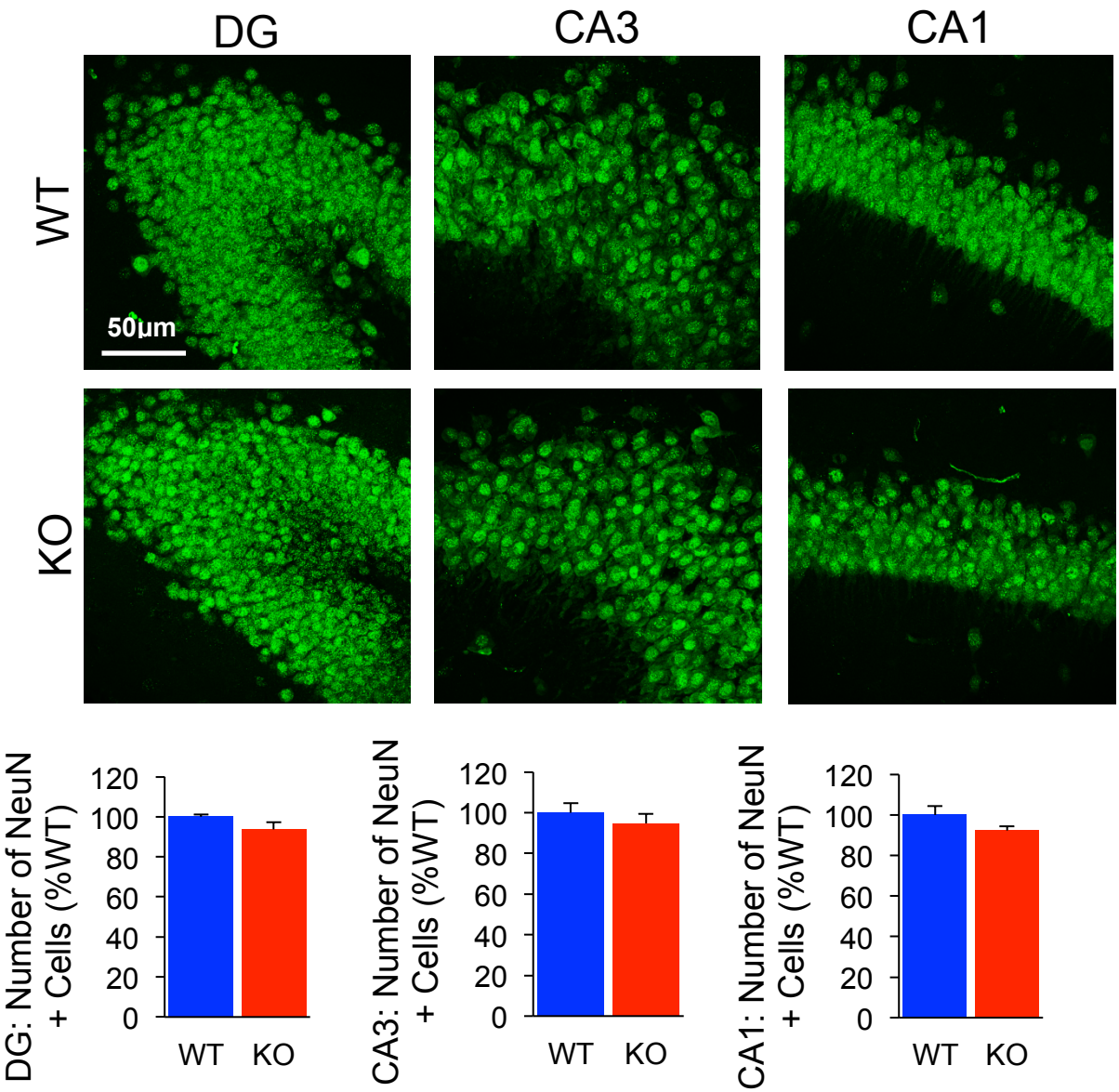


Figure 26. The number of NeuN+ ventral hippocampal neurons is unchanged in *Fmr1* KO mice compared to WT mice at P18. Green: NeuN. Images acquired at 63x magnification. Group n's: 5-6 per group.

3.2.2. Western Blot Analysis

To confirm our immunohistochemical findings, we next performed western blot analysis on ventral CA1 lysate obtained from P18 pups. Similar to our observations from the immunohistochemical experiments, we observed a roughly 33% decrease in ventral CA1 PKCε expression in the P18 KO pups, compared to WT controls (Figure 27). Taken together, these findings provide novel evidence that PKCε is altered during the later stages of postnatal hippocampal maturation, coinciding with the surge in synaptogenesis following eye-opening, in *Fmr1* KO pups.

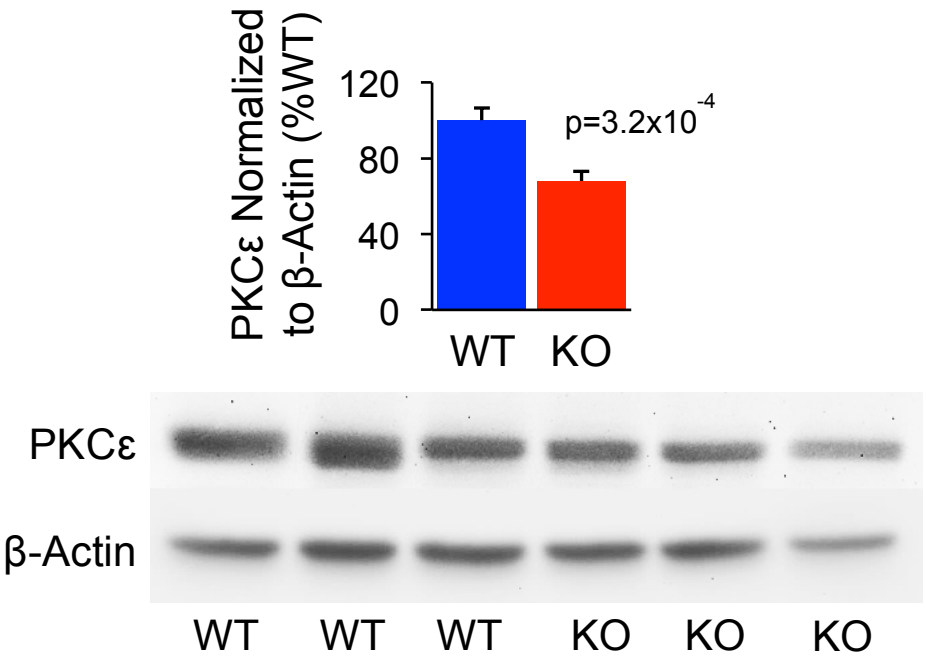


Figure 27. Ventral hippocampal CA1 PKCε expression is significantly reduced in *Fmr1* KO mice at P18, when normalized to beta actin (β-actin). Group n's: 6 per group.

3.3. Hippocampal neurogenesis is not altered in *Fmr1* KO mice at P6.

In our earlier studies, we have found evidence that that early postnatal PKCε-mediated signaling, downstream of the serotonin 1a receptor (5-HT_{1A}-R), may be required for the establishment of normal anxiety levels (Figure 13). Moreover, we have obtained evidence that anxiety-like behavior during adulthood appears to be linked to neonatal hippocampal neuroblast proliferation, where disruption of this process at P6 is associated with heightened anxiety-like behavior during adulthood in female 5-HT_{1A}-R KO mice (Figures 13 and 14).

Although we did not observe altered PKCε expression at P6 in our *Fmr1* KO mice, we nevertheless deemed it important to evaluate whether hippocampal neurogenesis was altered in these mice at this age, given that this process is linked to anxiety. In our earlier studies, stimulation of PKCε only partially rescued neuroblast proliferation in the female 5-HT_{1A}-R KO pups, and male 5-HT_{1A}-R KO pups did not exhibit reductions in neuroblast proliferation despite loss of activation of the 5-HT_{1A}-R → PKCε signaling pathway. Thus, while PKCε-mediated signaling does seem to influence neuroblast proliferation, it is most likely not the only signaling pathway that does so. Other signaling pathways regulated by FMRP may contribute to early postnatal hippocampal proliferation, yet remain unknown. Some reports have provided evidence that adult hippocampal neurogenesis may be altered in FXS, though the findings have been mixed and require further study.[315-317] To our knowledge, however, the integrity of early postnatal neurogenesis in FXS has not been reported. Therefore, in our next set of studies, we investigated hippocampal neuroblast proliferation at P6, the time-point at which this process begins to peak postnatally, in our *Fmr1* KO mice.

In brief, WT and *Fmr1* KO pups were injected with BrdU (100 mg/Kg, i.p.) at P6, and

then sacrificed 24 hours later to allow incorporation into dividing cells. Since males are more severely affected by the FXS mutation, only male pups were used. Immunohistochemical experiments were then performed to label BrdU and doublecortin (DCX) double-positive cells (newborn neurons) within the subgranular zone of the dentate gyrus. Lastly, the double-positive cells were then quantified using stereological methods (Section 2.3.6). Interestingly, we did not observe any differences in the number of proliferating neuroblasts under these conditions (Figure 28). However, this is consistent with our observation that PKCε expression did not appear to be altered in our *Fmr1* KO pups at this age.

Nevertheless, we do observe a substantial decrease in ventral CA1 PKCε expression in our *Fmr1* KO pups by P18. Importantly, at this time, neuroproliferation begins to wane as the dominant process driving hippocampal maturation; while synaptogenesis (in response to new visual inputs following eye opening) comes into play instead. Therefore, we explored possible mechanisms by which PKCε -mediated signaling may contribute to early postnatal hippocampal synaptogenesis, and how this process may potentially be disrupted in FXS. This may occur by several possible mechanisms. For instance, we have found that PKCε signaling influences gene expression via activation of transcription factors such as ERK1/2 (Figure 12). Thus, it is conceivable that the expression of genes related to synaptic function may be altered in FXS, not as a direct consequence of FMRP deficiency itself, but as a result of impairment in secondary pathways that would normally mediate the expression of such genes, such as PKCε signaling. Alternatively, PKCε might influence synaptic function in a more direct manner, for instance, by modulating receptor dynamics *via* phosphorylation. Therefore, our next aim was to explore such possibilities.

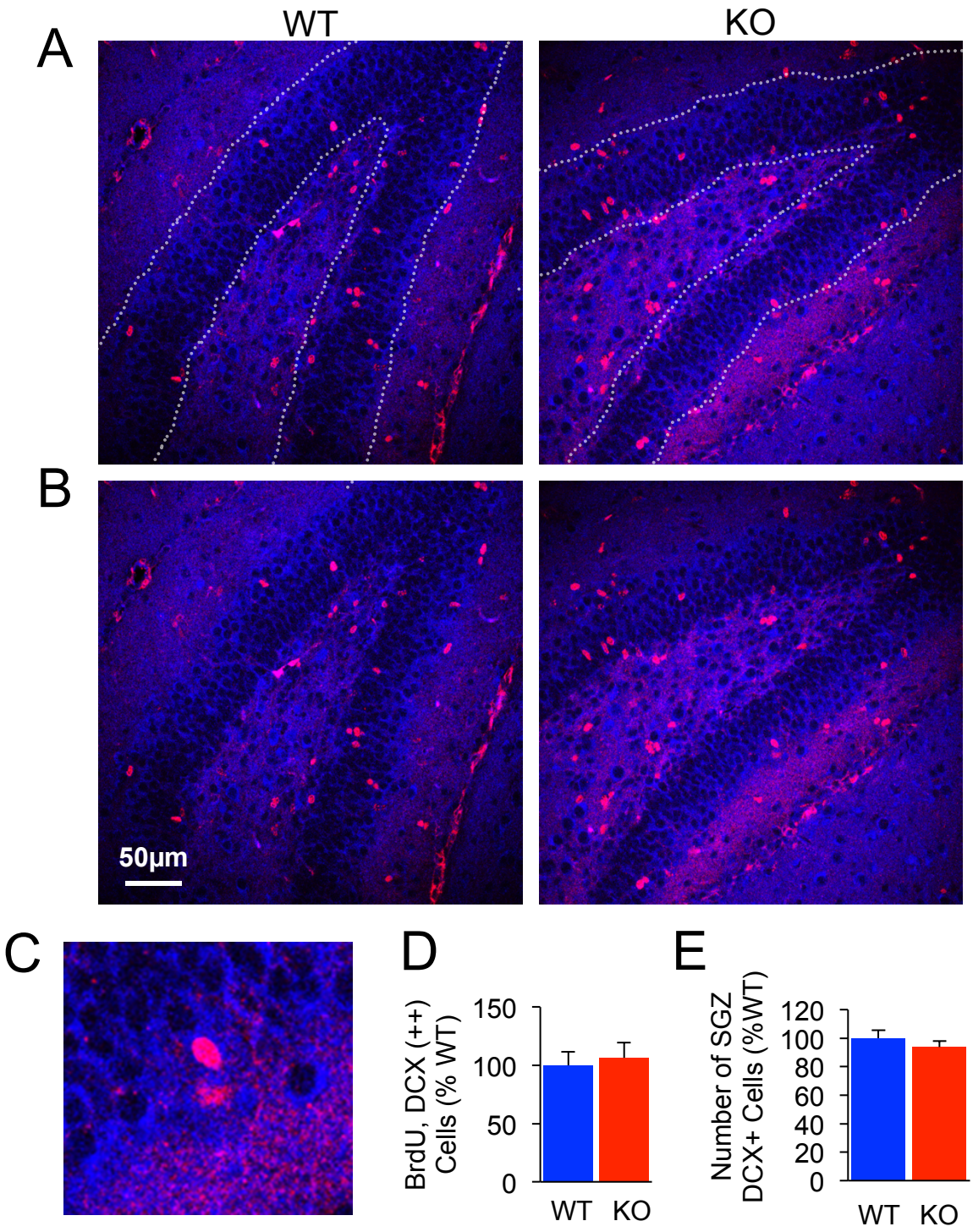


Figure 28. Hippocampal neuroblast proliferation is not altered in *Fmr1* KO mice at P6. (A) Representative images of the dentate gyrus obtained from WT and *Fmr1* KO pups at P6. Blue: doublecortin (DCX) labeling of immature neurons, red: BrdU labeling of proliferating and newly divided cells. Dashed lines demarcate the subgranular zone (SGZ). (B) Same images without SGZ demarcation. (C) Zoomed image indicating specificity of staining. (D) Stereological analysis detected no differences in the number of newborn neurons (DCX, BrdU double-positive cells) between WT and *Fmr1* KO pups in the SGZ at P6. Group n's:

3.4. Surface levels of GluR2-containing AMPA receptors are elevated in the *Fmr1* KO ventral hippocampal CA1 region at P18.

To ascertain potential consequences of reduced PKCε expression in the *Fmr1* KO hippocampus, we first honed in on excitatory synapses, which make up the vast majority of neuronal connections within this region. Ionotropic glutamate receptors - namely, the AMPA, NMDA, and kainate receptors - are requisite for excitatory post synaptic currents (EPSCs), in conjunction with metabotropic glutamate receptors. Most excitatory transmission in the brain is mediated by AMPA receptors,[252] and dynamic alterations in the number of synapse-localized AMPA receptors is an important factor contributing to synaptic plasticity.[255] AMPA receptors are tetramers consisting of combinations of four individual subunits, named GluR1-4.[253] During development, the majority of hippocampal AMPA receptors contain the GluR2 subunit, the expression of which is gradually up-regulated in the hippocampus from birth until adulthood.[255] Moreover, the GluR2 subunit is not only a suitable marker for hippocampal AMPA receptors, but may play a particularly important role in hippocampal synaptogenesis since it has been found to be required for both LTP and LTD.[259, 260] Since both of these processes are disrupted in FXS,[74] we therefore investigated the expression of GluR2-containing AMPA receptors in the ventral hippocampus.

3.4.1. Total expression of GluR2

Since expression of PKCε was most significantly reduced in the ventral CA1 region of our *Fmr1* KO mice (Figure 25), we honed in on this same region to examine GluR2 expression. Interestingly, western blot analysis did not detect any differences between the two groups in total GluR2 expression between our WT and *Fmr1* KO pups at P18 (Figure 29). This demonstrates that GluR2 expression may not rely on PKCε signaling, which is consistent with the finding that GluR2 mRNA does not appear to be regulated by FMRP.[43] Other studies have reported similar findings from hippocampal lysates obtained from WT and *Fmr1* KO pups.[318]

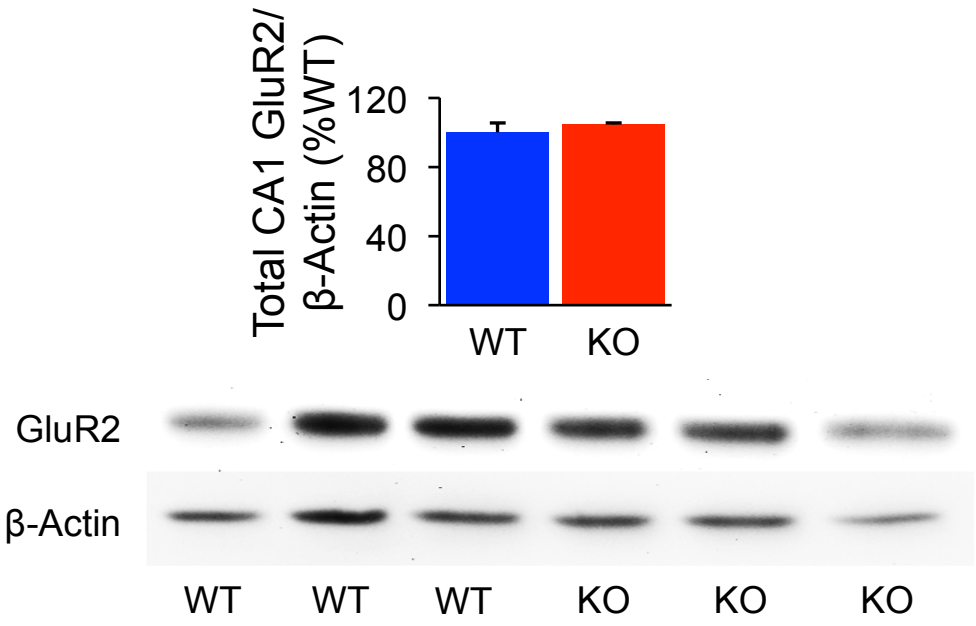


Figure 29. Total levels of CA1 GluR2 do not differ between WT and *Fmr1* KO mice at P18, when normalized to beta actin (β-actin). Group n's: 6 per group.

3.4.2. Surface GluR2 expression

Although total GluR2 levels were unchanged between our WT and *Fmr1* KO mice at P18, total levels of GluR2 may not necessarily reflect levels incorporated into synapses. Subunit phosphorylation is a common mechanism by which receptors are tagged for insertion to the synaptic membrane from the total internal receptor pool. Similarly, receptors may also be tagged for removal from the synapse (surface) via subunit phosphorylation. It has been shown that AMPA receptors are internalized via GluR2 phosphorylation at serine 880 in a PKC-dependent process.[319] Therefore, our next question was whether PKCε was the PKC isoform that mediated this process in the ventral hippocampus.

In order to most precisely study synaptic levels of GluR2, we performed immunohistochemical analysis of ventral hippocampal brain slices, obtained from WT and *Fmr1* KO pups at P18. Our preliminary study revealed, as expected, high GluR2 expression in hippocampal excitatory synapses, as indicated by its high degree of co-localization with the excitatory synaptic marker PSD-95 (Figure 30).

However, since PSD-95 is an internal structure, its immunohistochemical labeling requires the use of detergents that puncture the cell membrane, allowing antibodies to freely enter the cell. This presents a confound since this method could potentially result in labeling of internal GluR2 subunits adjacent to the synapse. In our next set of experiments, therefore, immunohistochemical analysis was performed under non-permeabilizing conditions to selectively and accurately label surface levels of GluR2 only (Figure 31). Most intriguingly, we discovered a 34% increase in surface GluR2 levels in the ventral CA1 region of *Fmr1* KO mice, compared to their WT counterparts, which was significant. A similar trend was also observed in the CA3 region. Interestingly, we did not observe an increase in surface GluR2 in the dentate

gyrus, possibly reflecting differences in function between these regions. The total number of cells in the CA1 region was also unchanged in the *Fmr1* KO group (Figure 31).

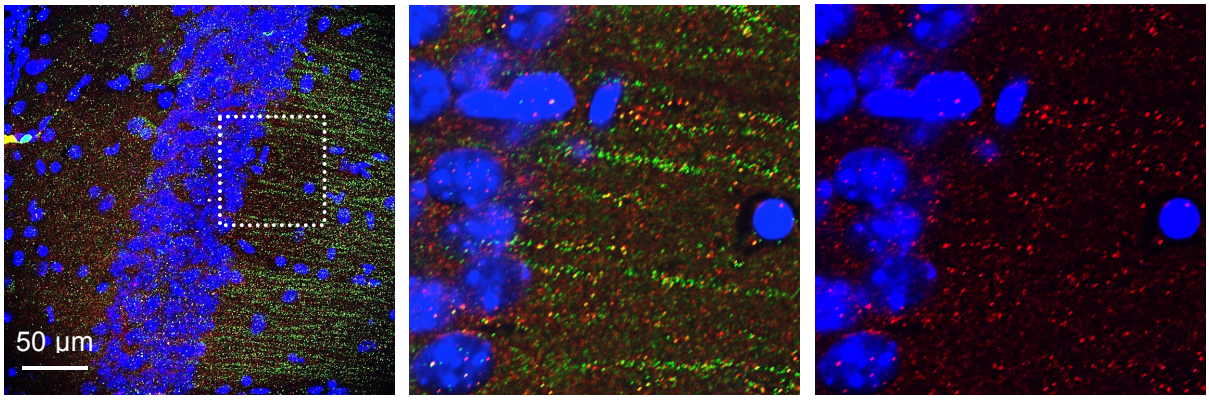


Figure 30. GluR2 co-localizes with PSD-95 in the CA1 region of the hippocampus. Representative images of GluR2 labeling in the WT hippocampus at P18 to demonstrate specificity of staining. Blue: Hoechst, green: PSD-95, red: GluR2. Yellow punctae indicate areas of PSD-95 and GluR2 co-localization. Images acquired at 63x plus zoom. Images obtained from P18 WT mice.

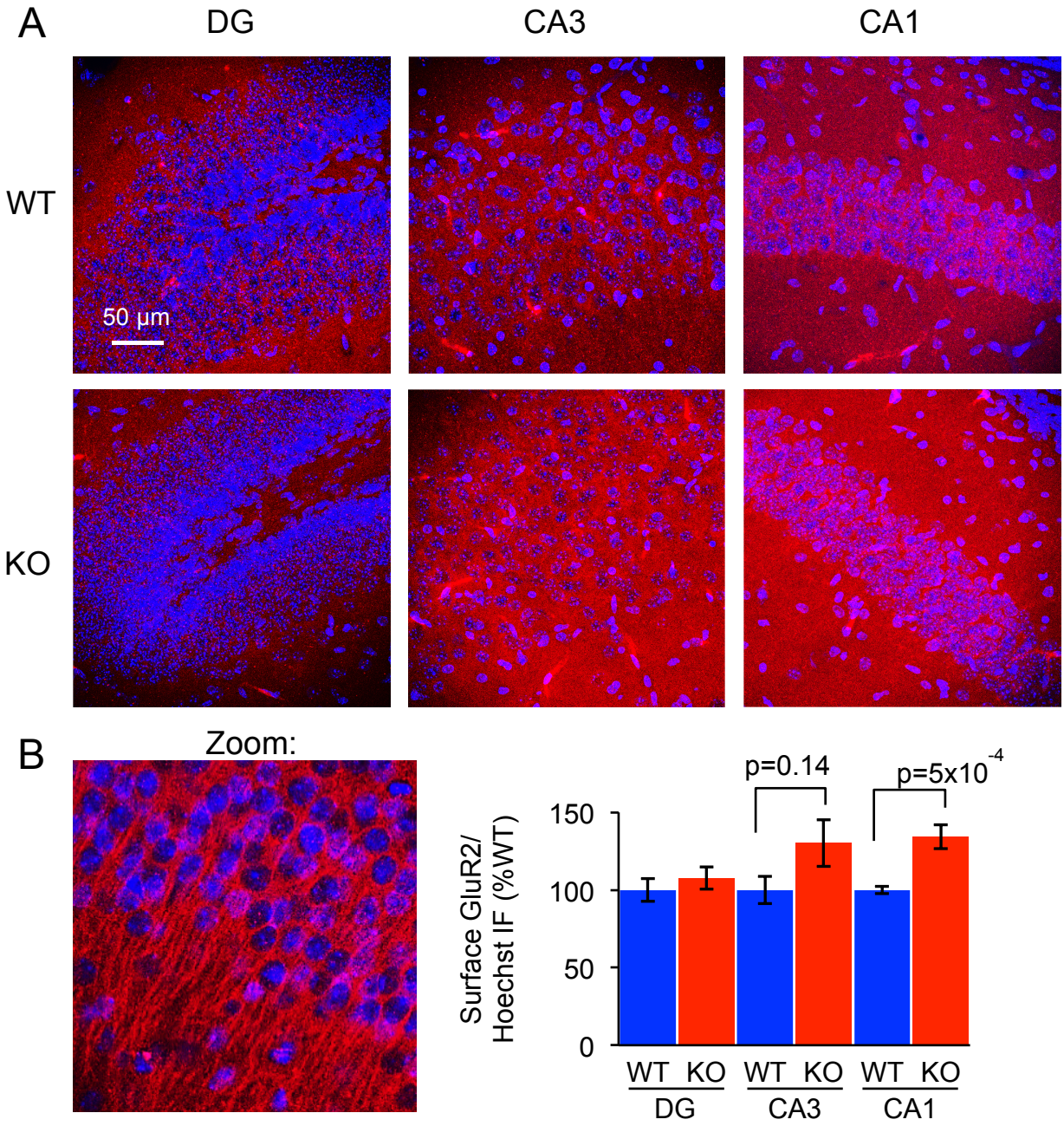


Figure 31. Surface GluR2 is increased in the hippocampal CA1 region in *Fmr1* KO mice at P18. (A) Under non-cell permeabilizing conditions, a significantly higher level of GluR2 fluorescence intensity is detected in the CA1 region of the hippocampus in P18 *Fmr1* KO mice compared to WT mice. Images represent Z-projections of 30µm thickness acquired at 63x magnification. Blue: Hoechst, red: GluR2 (extracellular N-19). (B) High-resolution image acquired at 63x plus zoom at a single z-position, to demonstrate specificity of staining. Group n's: 5-6 per group.

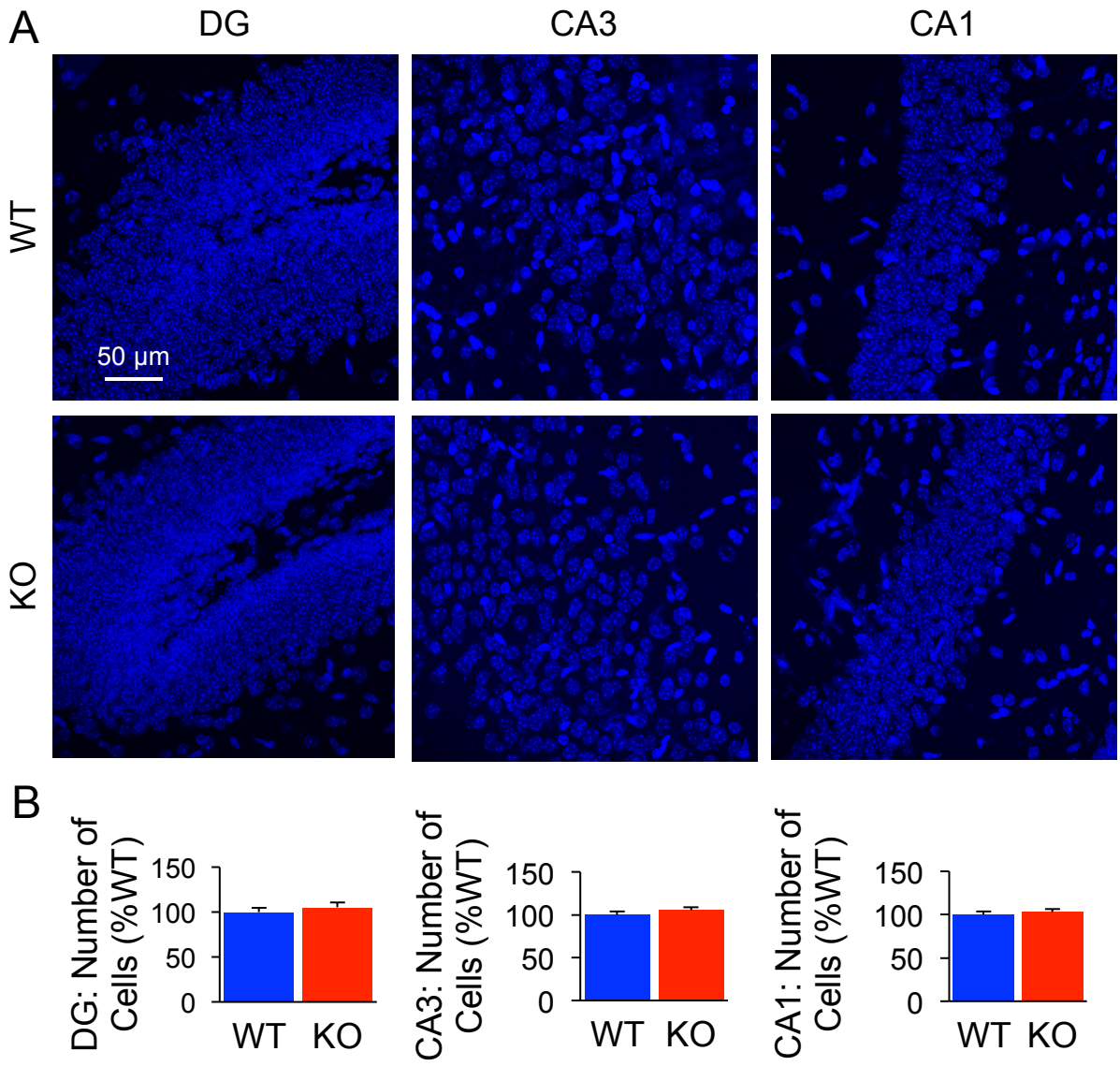


Figure 32. (A, B) The total number of Hoechst+ cells is not changed in the ventral hippocampus of *Fmr1* KO mice at P18, compared to WT mice. Blue: Hoechst labeling. Images acquired at 63x magnification. Group n's: 5-6 per group.

Taken together, these findings may be interpreted as evidence that at this age, the overall pool of GluR2-containing AMPA receptors is not increased in the *Fmr1* KO ventral hippocampus, but mechanisms governing trafficking of the receptor to and/or from the synapse may instead be altered, at least in the ventral hippocampus. This would presumably modulate synaptic responsiveness to incoming excitatory signals, thereby potentially leading to alterations in long-term synaptic modification during postnatal brain wiring. Intriguingly, when we performed the same analysis on adult mice (P90), a difference in CA1 surface GluR2 levels was no longer apparent (Figure 33). This suggests our observations at P18 may be a transient difference specific to early postnatal development, and that different regulatory pathways may oversee ventral hippocampal PKCε-mediated signaling and/or AMPA receptor dynamics during development versus adulthood.

What may be the consequences of our observation of heightened basal surface levels of GluR2-containing AMPA receptors in the juvenile *Fmr1* KO hippocampus? It is possible this may lead to an excess of glutamatergic signaling in the hippocampus at this age. If this were the case, then these findings may be compatible with evidence that the FXS hippocampus is hyper-excitable,[13, 320] contributing to heightened seizure susceptibility that is common in young FXS patients, but often remisses by adulthood. Importantly, excess hippocampal activity at this young age may lead to inappropriate signaling to downstream structures, such as the cortex, amygdala, and/or hypothalamus, resulting in altered brain wiring that may later manifest as behavioral symptoms.

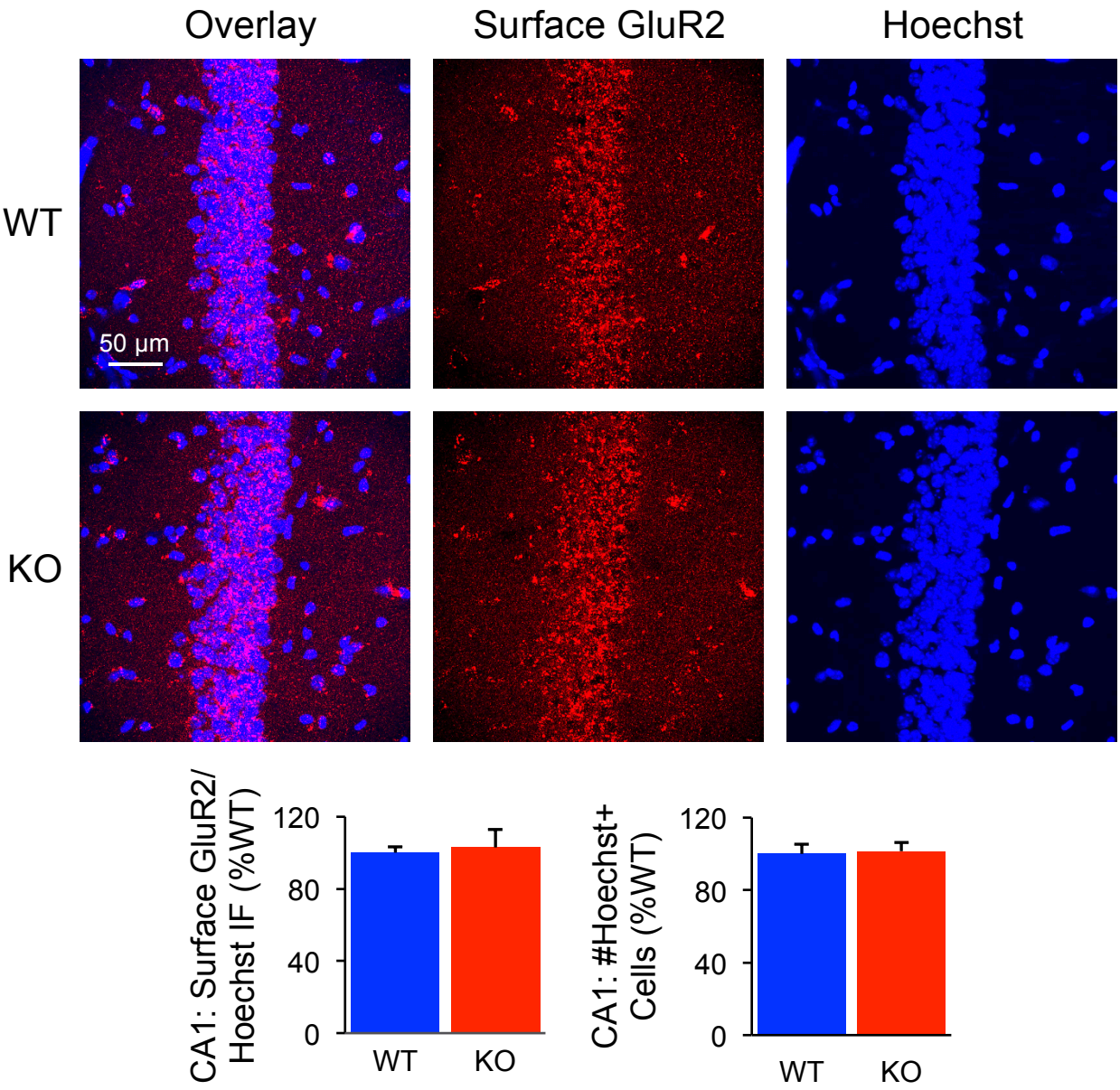


Figure 33. Surface GluR2 no longer differ between WT and *Fmr1* KO mice in the hippocampal CA1 region by adulthood (P90). Red: Surface GluR2 (N-terminus), blue: Hoechst. Images acquired at 63x magnification. Group n's: 5-6 per group.

3.5. Phosphorylated serine 880 GluR2 levels are decreased in the *Fmr1* KO ventral hippocampal CA1 region at P18.

Given our observations that reduced PKCε expression in the juvenile *Fmr1* KO ventral hippocampus coincides with heightened synaptic levels of GluR2, it is possible that PKCε signaling may be involved in AMPA receptor trafficking. It has been found that synaptic AMPA receptors may be internalized *via* phosphorylation of the GluR2 subunit at serine 880 in a PKC-dependent manner.[262] However, the PKC activator used in this study, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), is largely non-isoform specific. Therefore, we next questioned whether this process was potentially mediated specifically by the epsilon isoform of PKC.

To address this question, we first examined whether serine 880 phosphorylation of GluR2 was altered in *Fmr1* KO mice during the juvenile stage. True to our hypothesis, western blot analysis revealed a 52% reduction in levels of serine 880 phosphorylated GluR2 subunits in the KO mice at P18, when normalized to beta actin (Figure 34). Considering that earlier we observed that total levels of the GluR2 subunit were unchanged in the KO group, this finding is highly indicative of a specific reduction in phosphorylation of the AMPA receptor at serine 880.

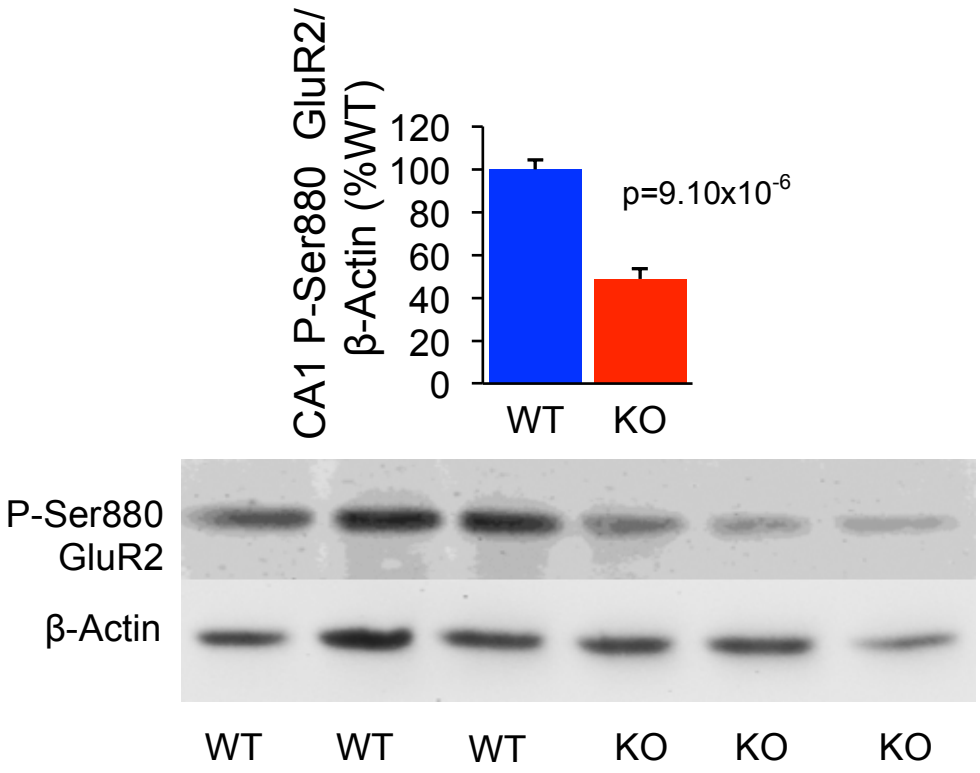


Figure 34. Serine 880 phosphorylated GluR2 subunits (P-Ser880 GluR2) are significantly reduced in the *Fmr1* KO ventral hippocampal CA1 region at P18 compared to WT controls, after normalization to beta actin (β-actin). Group n's: 6 per group.

3.6. PKCε interacts with the GluR2 subunit of the AMPA receptor.

If PKCε directly phosphorylates GluR2 at the serine 880 site, then a protein-protein interaction between the two proteins should be detectable. To investigate whether this was indeed the case, we performed co-immunoprecipitation studies on hippocampal lysate obtained from WT pups at age P18. Lysates were incubated with antibodies targeting PKCε, and then PKCε and bound proteins were immunoprecipitated with protein A/G agarose beads. The pulled down protein complexes were then resolved *via* SDS-Page and analyzed *via* western blotting. As expected, GluR2 bands were detectable following this procedure, indicating that GluR2 indeed co-immunoprecipitated with PKCε (Figure 35A, B). Similarly, when the experiment was performed in the reverse manner, PKCε was pulled down with GluR2 (Figure 35C). To our knowledge, this is the first reported evidence of an interaction between the two proteins. Given this interaction, we speculated that is likely that PKCε plays a hand in phosphorylation of the AMPA receptor at the GluR2 subunit. The GluR2 subunit has two known phosphorylation sites sensitive to PKC,[321] but as of yet, only phosphorylation of serine 880 has been linked to AMPA receptor endocytosis, which we report appears to be disrupted in the *Fmr1* KO hippocampus. FMRP regulates protein translation in response to synaptic activity, and this presumably includes translation of PKCε. Since reduction of PKCε expression in the *Fmr1 KO* ventral CA1 region coincides with increased surface levels of GluR2, it is plausible that PKCε may normally serve to phosphorylate the Ser880 site to modify activity-dependent synaptic plasticity, downstream of FMRP.

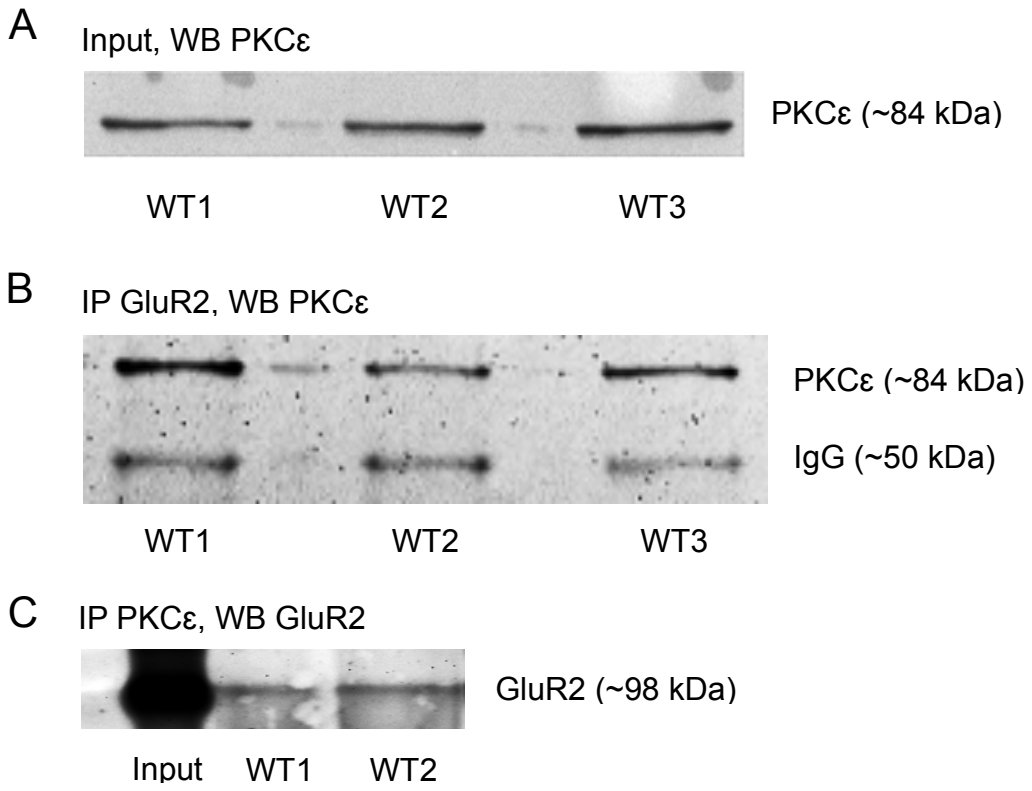


Figure 35. PKCε co-immunoprecipitates with GluR2 from P18 hippocampal lysate. (A) Input (non-immunoprecipitated samples) reveal that PKCε is normally expressed in hippocampus at P18. (B) When hippocampal GluR2 is isolated by immunoprecipitation and the isolate is subjected to Western blotting, PKCε bands (~84 kDa) are detected. Bands corresponding to the antibody used during the IP procedure are also present on the blot (IgG, ~50 kDa). WT=wild-type male. (C) Similarly, when hippocampal PKCε is isolated by immunoprecipitation and the isolate is subjected to Western blotting, GluR2 bands (~98 kDa) are detected.

3.7. Pharmacological PKCε stimulation by selective activator DCP-LA normalizes surface GluR2 levels in the *Fmr1* KO ventral hippocampal CA1 region.

If PKCε normally mediates AMPA receptor endocytosis at synapses, then a reduction in PKCε levels would lead to an increase in the number of AMPA receptors at the cell surface, which we have observed in our *Fmr1* KO mouse model (Figure 31). Similarly, if PKCε signaling were to be restored in *Fmr1* KO mice, then surface levels of the GluR2 should be returned to WT levels. To evaluate this possibility, we next examined surface GluR2 expression in KO mice that were treated systemically with the selective PKCε activator DCP-LA on P6, P10, and P14. As expected, we observed that our neonatal treatment regimen was sufficient to normalize surface levels of GluR2 completely in the ventral CA1 region (Figure 36), as determined by immunohistochemical analysis when non-cell permeabilizing conditions were used to label GluR2. Importantly, the number of cells in the CA1 region of the DCP-LA treated group did not differ significantly from the WT and vehicle-treated KO group, as determined by Hoechst labeling (Figure 37). Interestingly, surface GluR2 levels were also normalized in area CA3 in our DCP-LA treated KO mice, although we had been unable to detect a difference in PKCε in the CA3 region at this age. However, there still remains the possibility that PKCε levels may indeed be reduced in the CA3 region in *Fmr1* KO mice at P18, but the difference may be so subtle that it would not be easily detectable.

Based on this finding, it is possible that (1) PKCε-mediated signaling, downstream of FMRP, may be involved in AMPA receptor endocytosis in the juvenile ventral hippocampus to modify activity-dependent synaptic plasticity; and (2) that this process may go astray in FXS, presumably as a result of decreased PKCε expression.

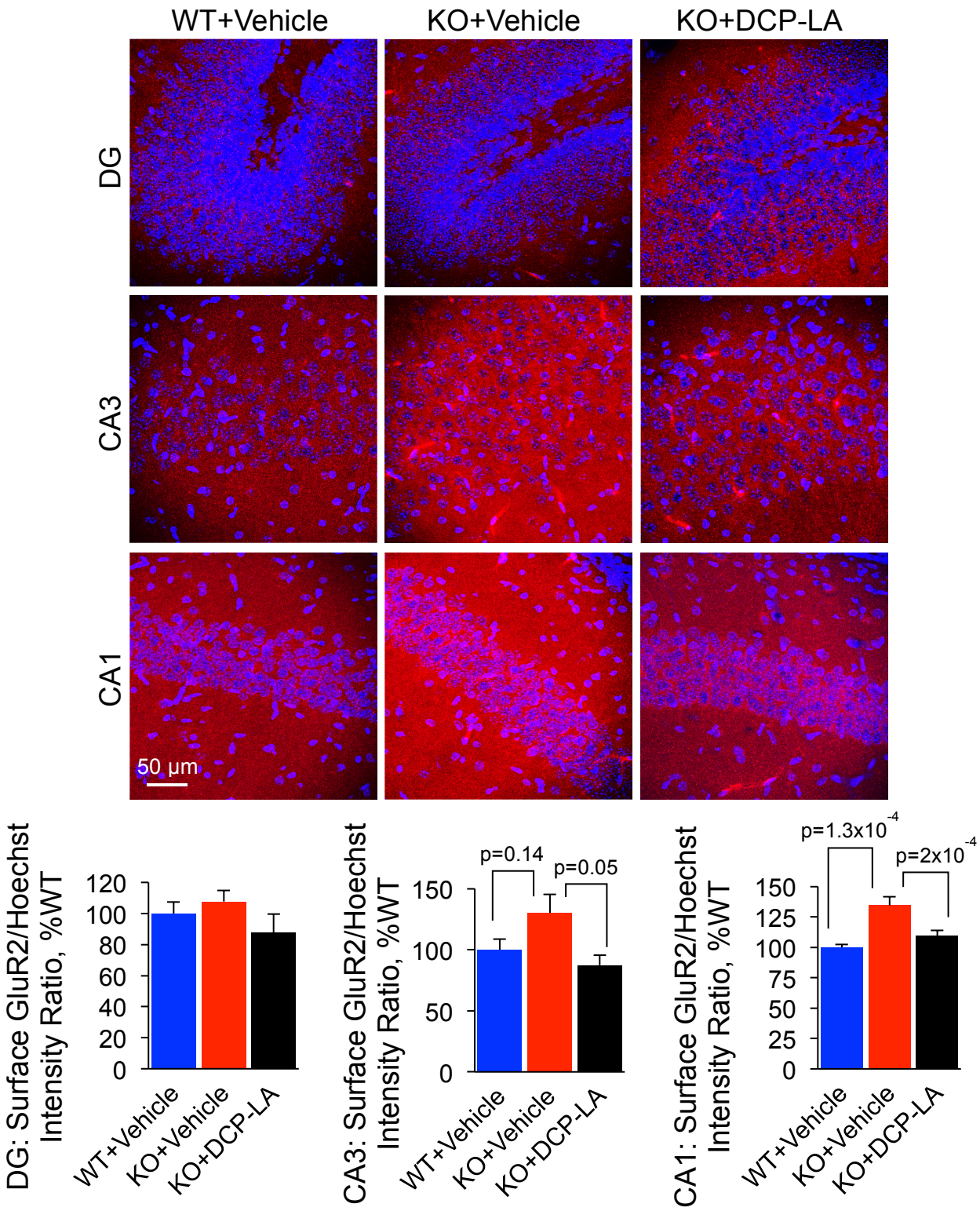


Figure 36. Surface GluR2 levels are increased in the hippocampal CA1 region of *Fmr1* KO mice at P18, but are restored to WT levels by intra-peritoneal injection of 3 mg/kg DCP-LA at ages P6, P10, and P14. Labeling conducted under non-cell permeabilizing conditions. Images acquired at 63x magnification. Blue: Hoechst, red: GluR2 (extracellular N-19). Group n's: 6.

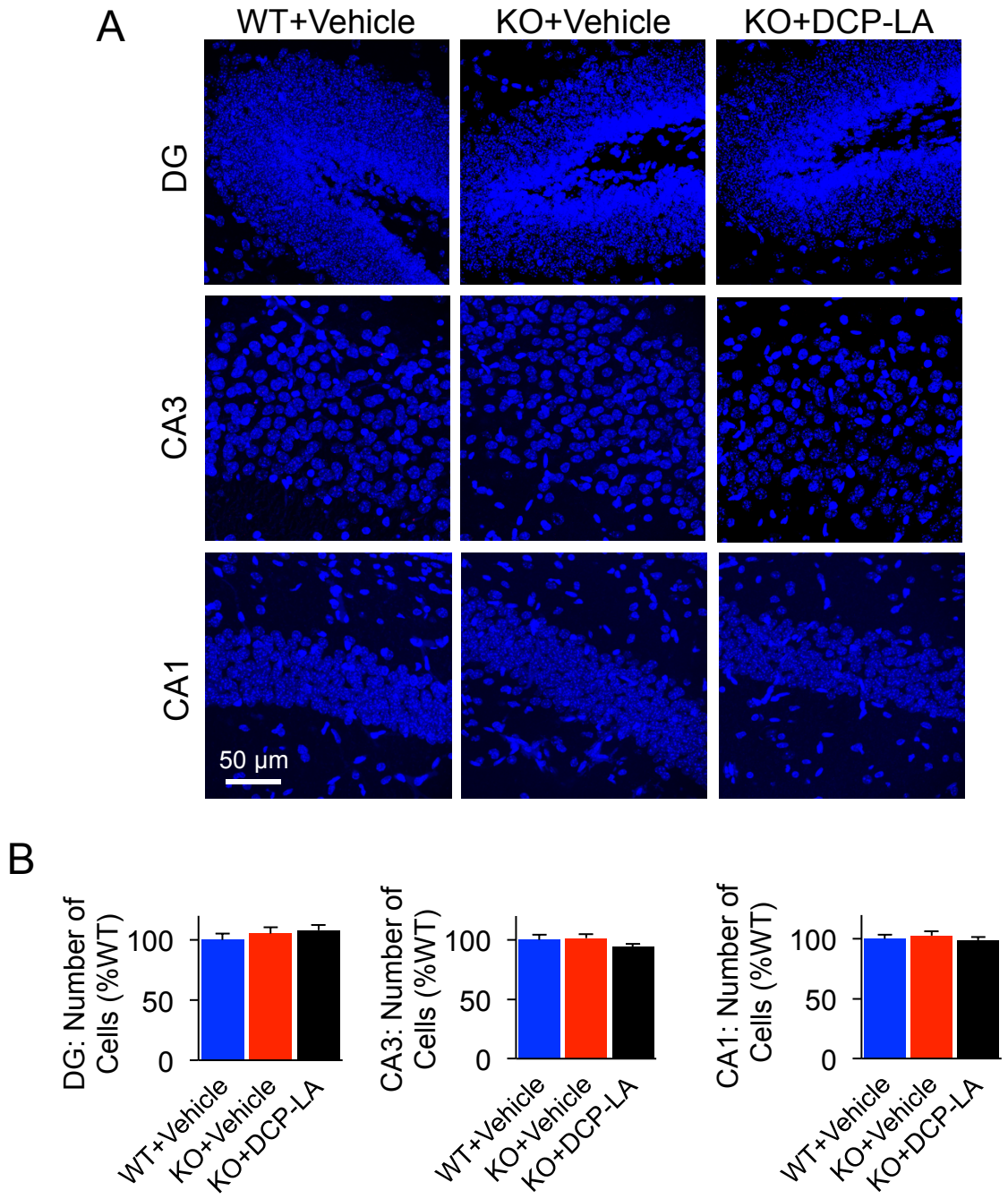


Figure 37. Neonatal DCP-LA treatment does not alter the number of Hoechst+ cells in the *Fmr1* KO hippocampus at P18. Blue: Hoechst labeling. Images acquired at 63x. Group n's: 6.

3.8. Oxytocin-positive cells are reduced in the *Fmr1* KO hypothalamic PVN at P20.

Given the many neurobehavioral symptoms associated with FXS, we next questioned the contributions of the synaptic aberrations we have observed within the hippocampus during the juvenile stage. The neuropeptide oxytocin is widely known to promote social behavior and temper anxiety levels (Section 1.5.2). Considering that many behavioral symptoms of FXS, including social deficits, can stem from anxiety, it is likely that oxytocinergic signaling may be compromised in FXS. To date, however, there has been a surprising dearth of studies investigating this possibility. One potential avenue by which oxytocinergic signaling may be altered in the FXS brain is as a consequence of upstream hippocampal aberrances. For instance, the hippocampus is known to inhibit activity of the paraventricular nucleus (PVN) of the hypothalamus, the main supplier of oxytocin in the brain.

A subset of parvocellular neurons within the PVN have been shown to produce centrally-distributed oxytocin that has been linked to behavioral effects.[174] Moreover, the ventral hippocampus tonically inhibits activity of the parvocellular PVN.[168, 222, 322] Does this inhibition include oxytocin-producing neurons? While the answer to this question remains unclear, it has been shown that activation of pyramidal neurons in the CA1 region of the ventral hippocampus produces anxiety-like behavior in rodents, possibly by suppressing central oxytocin signaling at its root, in the PVN.[155, 156] Supporting this hypothesis, studies have suggested that oxytocin production in this region may be especially sensitive to early life manipulation.[190]

To address this possibility, we first investigated whether oxytocin production was altered in the *Fmr1* KO brain relative to WT controls. We first studied oxytocin signaling at P20 (weaning age), at which point the hypothalamic oxytocinergic system is fully developed.

Immunohistochemical analysis was used to quantify oxytocin positive cells within the PVN. We found that the number of the oxytocin-positive cells was sharply suppressed in the *Fmr1* KO PVN compared to the WT group (Figure 38). To determine whether this deficit was due to a loss of neurons or loss of oxytocin synthesis within the PVN, we next quantified the total number of cells within the PVN. However, no differences between the two groups in PVN cell number (Figure 38D) were detectable, indicating that the reduction in oxtytocin-positive cells most likely reflected a depression in oxytocin synthesis. Thus, for the first time, we provide evidence that a deficit in oxytocinergic signaling appears to be associated with FXS, and moreover, this deficit appears to stem from a loss of oxytocin synthesis.

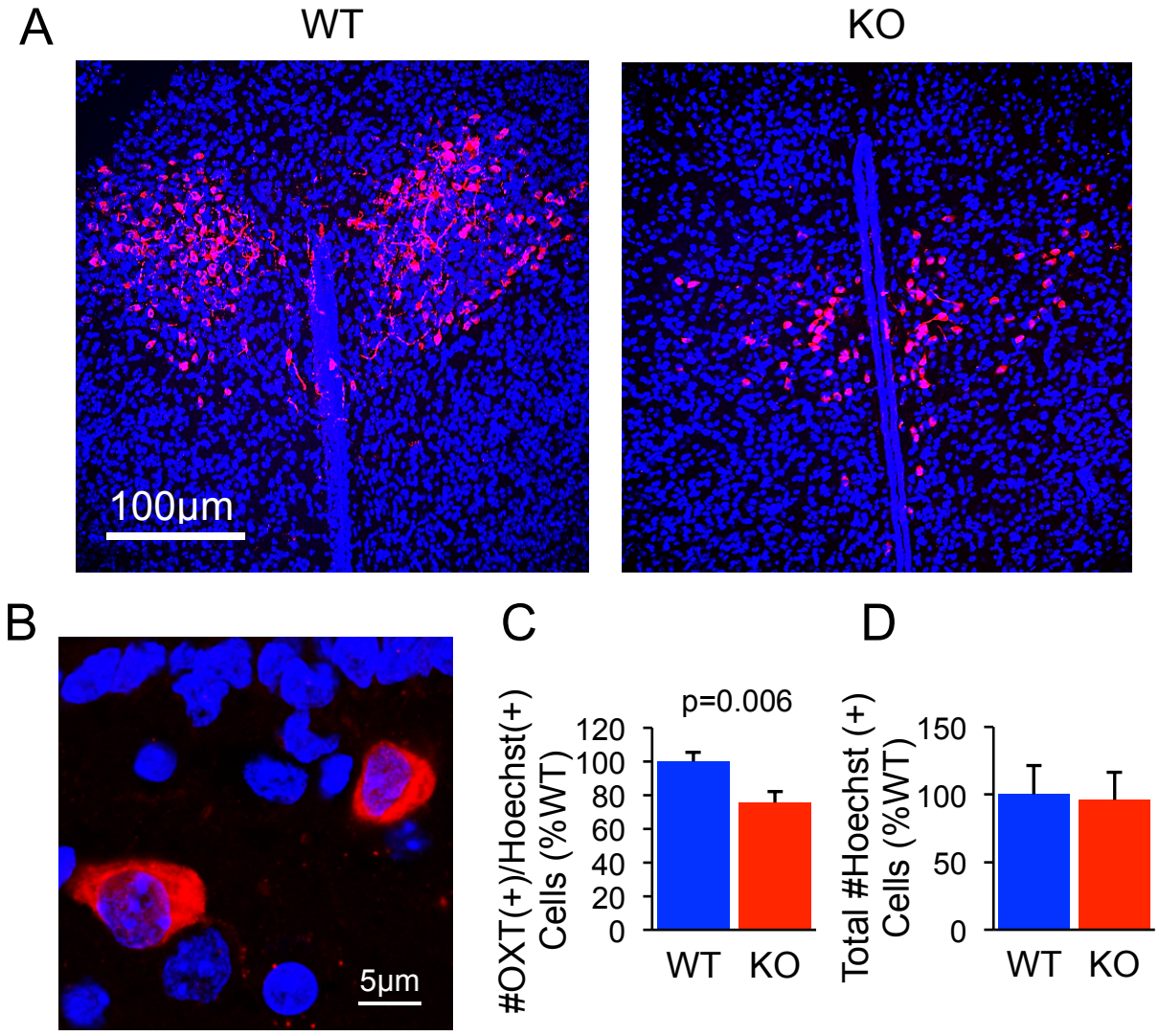


Figure 38. (A, C), The number of oxytocin positive cells (OXT+) in the paraventricular nucleus (PVN) is significantly reduced in male *Fmr1* KO mice at P20, compared to WT controls. (B) Zoomed image demonstrating specificity of staining. (D) The total number of PVN cells does not differ between WT and *Fmr1* KO mice. Images acquired at 20x magnification. Blue: Hoechst, red: oxytocin.

3.9. PKCε is under-expressed in the *Fmr1* KO hypothalamic PVN at P20.

Here, we report a significant reduction of oxytocin synthesis in the PVN in *Fmr1* KO mice at age P20. Concurrently, we observe increased levels of AMPA receptor surface expression in the CA1 region of the ventral hippocampus, which is indicative of increased excitation of the hippocampus, which in turn has been linked to anxiety.[323] The CA1 region is the major output site of the hippocampus, and the ventral hippocampus is known to exert an inhibitory effect on PVN neurons.[168, 222, 322] Thus, our observations of reduced oxytocin synthesis in the PVN could be a result of excessive inhibitory influence from the hippocampus. Alternatively, impaired oxytocin synthesis may instead be a direct effect of loss of PKCε expression in the PVN. For instance, we have previously found that in the hippocampus, PKCε is capable of activating ERK1/2 to influence gene expression (Figure 12). Could PKCε influence oxytocin gene expression in the PVN in a similar manner? To address this possibility, we first investigated whether PKCε expression was reduced in the hypothalamus of *Fmr1* KO mice, as it was in the hippocampus.

Western blot analysis was performed on PVN lysate obtained from P20 pups. Intriguingly, our densitometry analysis revealed a ~20% reduction in PKCε expression in the PVN at this time-point (Figure 39), which was significant ($p=0.03$). This raises the possibility that PKCε might normally influence oxytocin expression directly in PVN neurons, but this process is disrupted in FXS. We have previously found that PKCε is capable of mediating gene expression *via* ERK1/2, but as of yet no links between PKCε nor ERK1/2 and oxytocin expression have been identified. To examine this possibility, we next performed western blot analysis to measure activated (Thr202/Tyr204 phosphorylated) ERK1/2 within the same lysate.

If PKCε is involved in ERK1/2 activation in the hypothalamus, then the loss of PKCε in *Fmr1* KO mice would be reflected by a reduction in activated ERK1/2. However, we did not detect a significant difference in activated ERK1/2 relative to basal ERK (Figure 40) between the WT and KO groups. Therefore, a hypothetical pathway in which PKCε directly stimulates oxytocin expression through ERK1/2 may not be the mechanism at play here. This does not exclude the possibility that PKCε may influence oxytocin expression in the hypothalamus directly; however, no links between PKCε and pathways known to regulate oxytocin expression (the primary pathway being activation of estrogen-receptor beta[324]) have been discovered. On the other hand, considering that the hippocampus is a powerful inhibitor of PVN activity (Section 1.4.3), the suppression of oxytocin expression that we observe in the *Fmr1* KO brain may instead be due to heightened inhibitory drive exerted by the hippocampus, resulting from elevated levels of synaptic GluR2 in the CA1 region.

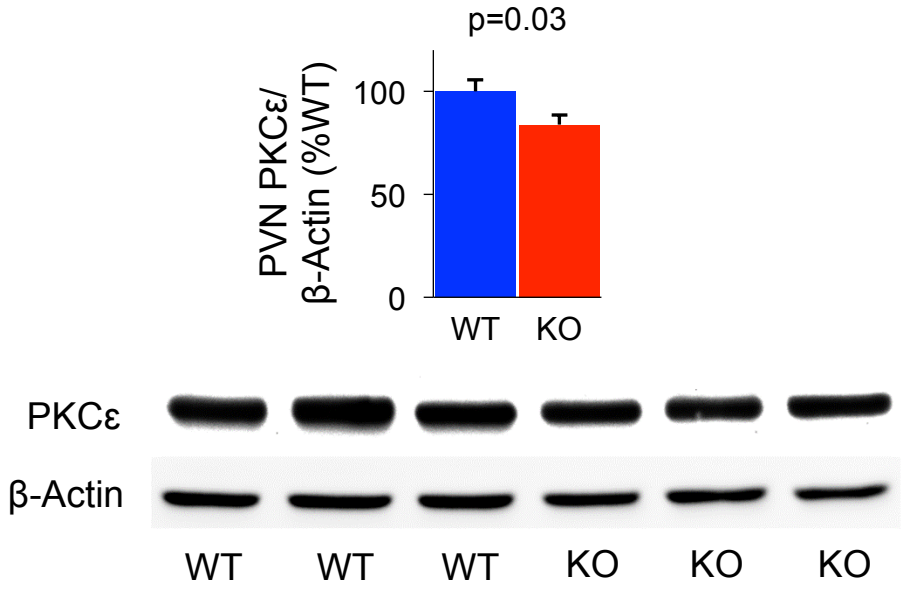


Figure 39. PKCε expression is significantly reduced in the *Fmr1* KO hypothalamic PVN at P20, compared to WT controls, after normalization to beta actin (β-actin). Group n's: 6 per group.

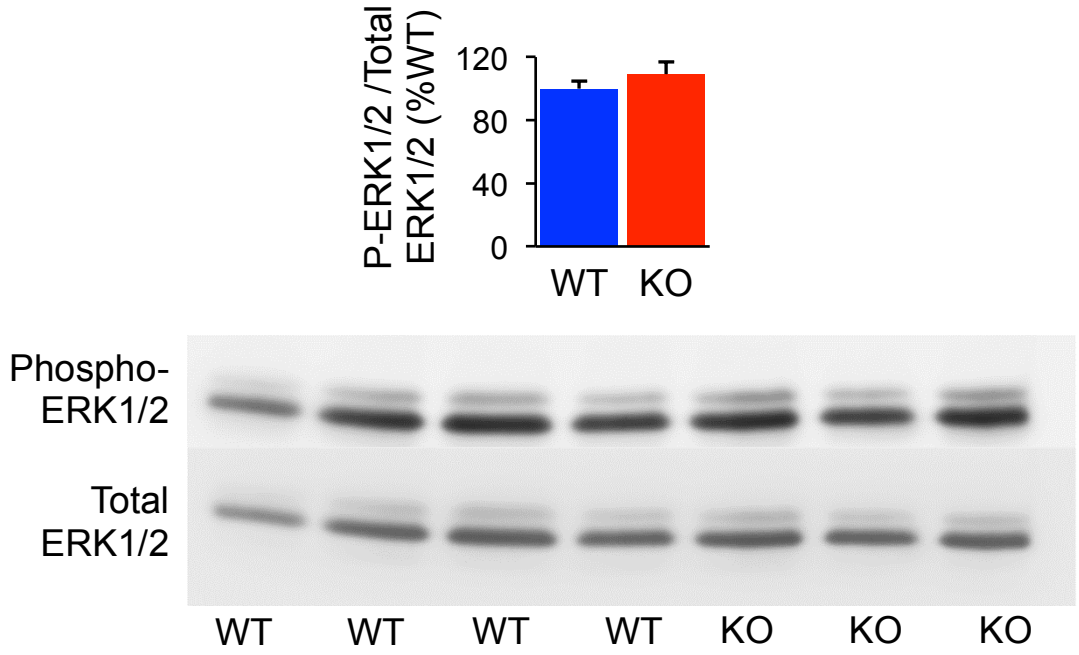


Figure 40. Phospho (activated) ERK 1/2 (Thr202, Tyr 204 phosphorylated ERK1/2) levels are not changed in the hypothalamus of *Fmr1* KO mice at P20, after normalization to total ERK 1/2 levels. Group n's: 6 per group.

Since we have shown that stimulation of PKCε appears capable of altering surface AMPA levels within the ventral hippocampus at P18 (Figure 36), we must also address the possibility that PKCε may influence PVN oxytocin synthesis by performing a similar action within the PVN itself. We have demonstrated here that PKCε co-immunoprecipitates with the GluR2 subunit of the AMPA receptor (Figure 35), which has been shown to signal for AMPA receptor internalization when phosphorylated by PKC at serine 880.[319] Moreover, we show that PKCε deficiency in the *Fmr1* KO ventral hippocampus is linked to reduced serine 880 phosphorylation of GluR2 (Figure 34), and consequently, surface GluR2 over-expression (Figure 31), presumably leading to heightened neuronal excitability in this region. On the other hand, neonatal pharmacological stimulation of PKCε in these mice appears to rescue surface GluR2 expression to normal levels (Figure 36).

Could this same mechanism be at play in PVN centrally-projecting, oxytocin-synthesizing cells? Heightened excitation of oxytocin-synthesizing cells in *Fmr1* KO mice would hypothetically lead to an increase in oxytocin synthesis, the opposite of our observations (Figure 38). On the other hand, heightened excitation of GABAergic interneurons within the PVN, which contact oxytocin-producing cells, could instead lead to a suppression of oxytocin synthesis. However, we predict that PKCε-mediated changes to GluR2 phosphorylation within the PVN is unlikely to explain oxytocin suppression in our *Fmr1* KO mice, due to inherent differences in AMPA receptor composition in the PVN: while the GluR2 subunit is found within the majority of AMPA receptors in the hippocampus, AMPA receptor composition within the PVN is much more heterogeneous. Here, GluR2 is predominantly found in hypophysiotrophic, peripherally-releasing cells, whereas GluR1 is the major AMPA receptor subunit found in centrally-releasing, pre-autonomic cells.[325]

Importantly, it has been found that AMPA receptor channel conductance can be enhanced *via* phosphorylation of the GluR1 subunit at serine 831 (Ser831).[326] Moreover, PKC is capable of catalyzing this phosphorylation.[327] Could decreased PKCε expression in the *Fmr1* KO PVN lead to reduced GluR1 serine 831 phosphorylation, and therefore reduced excitability of neurons within the PVN? To answer this question, we studied examined both total and phosphorylated Ser831 GluR1 levels in the WT versus *Fmr1* KO PVN at P20. However, our western blot analysis detected no significant differences between the two mouse groups in either measure (Figure 41). Although here we were unable to distinguish between oxytocin-synthesizing versus inhibitory neurons, these results suggest that PKCε signaling may not be as influential to AMPA receptor dynamics within the PVN, as it appears to be within the hippocampus. On the other hand, PKCε may serve different functions within the PVN that are, as of yet, unknown. Based on this finding and the supporting literature, we speculate that heightened inhibitory drive exerted by the ventral hippocampus is a more likely explanation for the suppressed oxytocin synthesis we observe in the neonatal *Fmr1* KO PVN, as opposed to altered AMPA receptor dynamics within the PVN itself, potentially resulting from PKCε deficiency.

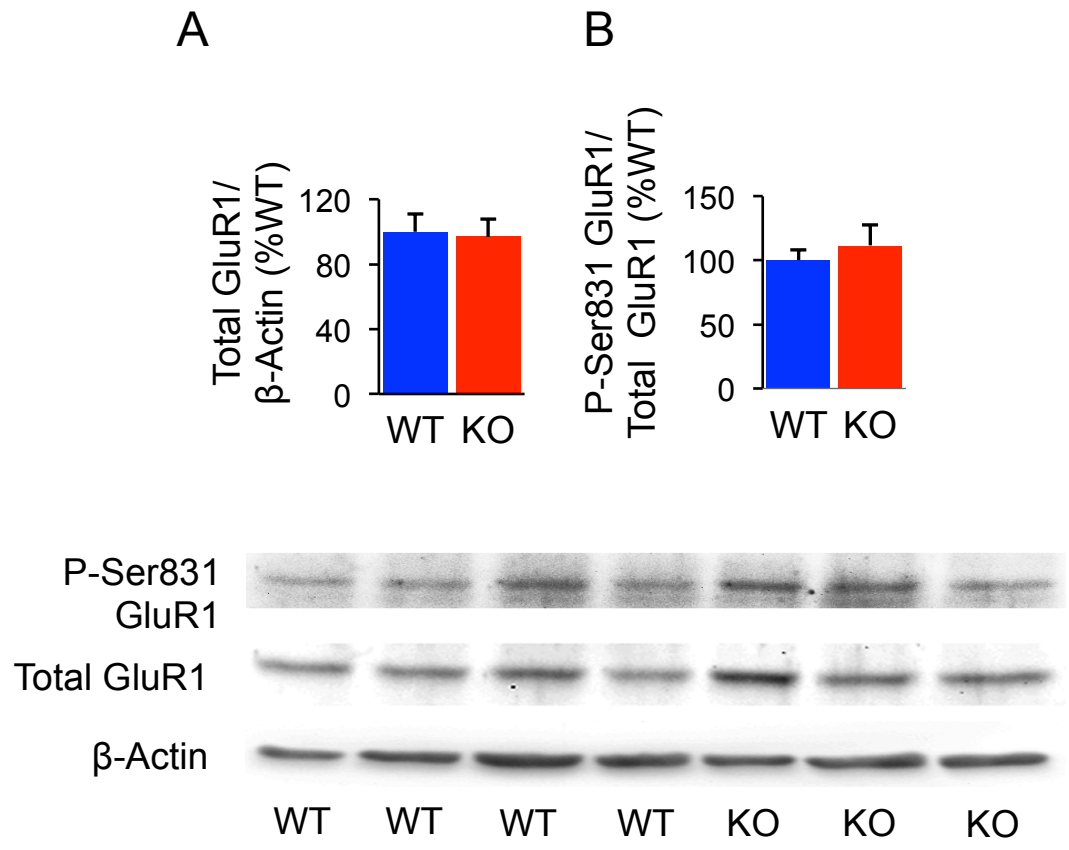


Figure 41. Levels of total (A) and serine 831-phosphorylated (ser-831) (B) GluR1 are not significantly different in the WT and *Fmr1* KO PVN at P20. Group n's: 6 per group.

3.10. Pharmacological stimulation of PKCε during the neonatal stage rescues oxytocin levels in the juvenile *Fmr1* KO hypothalamus in a PKCε-dependent manner.

We have obtained data indicative of reduced PVN oxytocin expression in the juvenile (P20) *Fmr1* KO mouse brain. We hypothesize that this reduction may be due to increased inhibitory drive exerted on the PVN by the *Fmr1* KO hippocampus. This in turn may be traced back to reduced PKCε expression in the CA1 area of the ventral hippocampus, which we have found may be involved in AMPA receptor trafficking during the juvenile developmental period (P18). To confirm whether oxytocin expression is PKCε dependent, we systemically treated *Fmr1* KO pups at P6, P10, and P14 with either the selective PKCε inhibitor, DCP-LA, or (2) DCP-LA plus the selective PKCε inhibitor, Myr-V1/2. Brain slices were then obtained from the pups at P20 and immunolabeled for oxytocin.

As expected, we found that DCP-LA treatment alone was sufficient to fully normalize the number of oxytocin-positive cells in the *Fmr1* KO PVN to wild-type levels (Figure 42). On the other hand, when DCP-LA was administered simultaneously alongside the PKCε inhibitor, Myr-V1/2, oxytocin expression was further suppressed compared to vehicle-treated KO controls. Both these changes occurred without any changes in the total number of PVN neurons, indicating that the process of oxytocin synthesis itself was altered. Based on this collective evidence, oxytocin synthesis within the PVN at this developmental stage appears to be dependent, in part, on PKCε signaling.

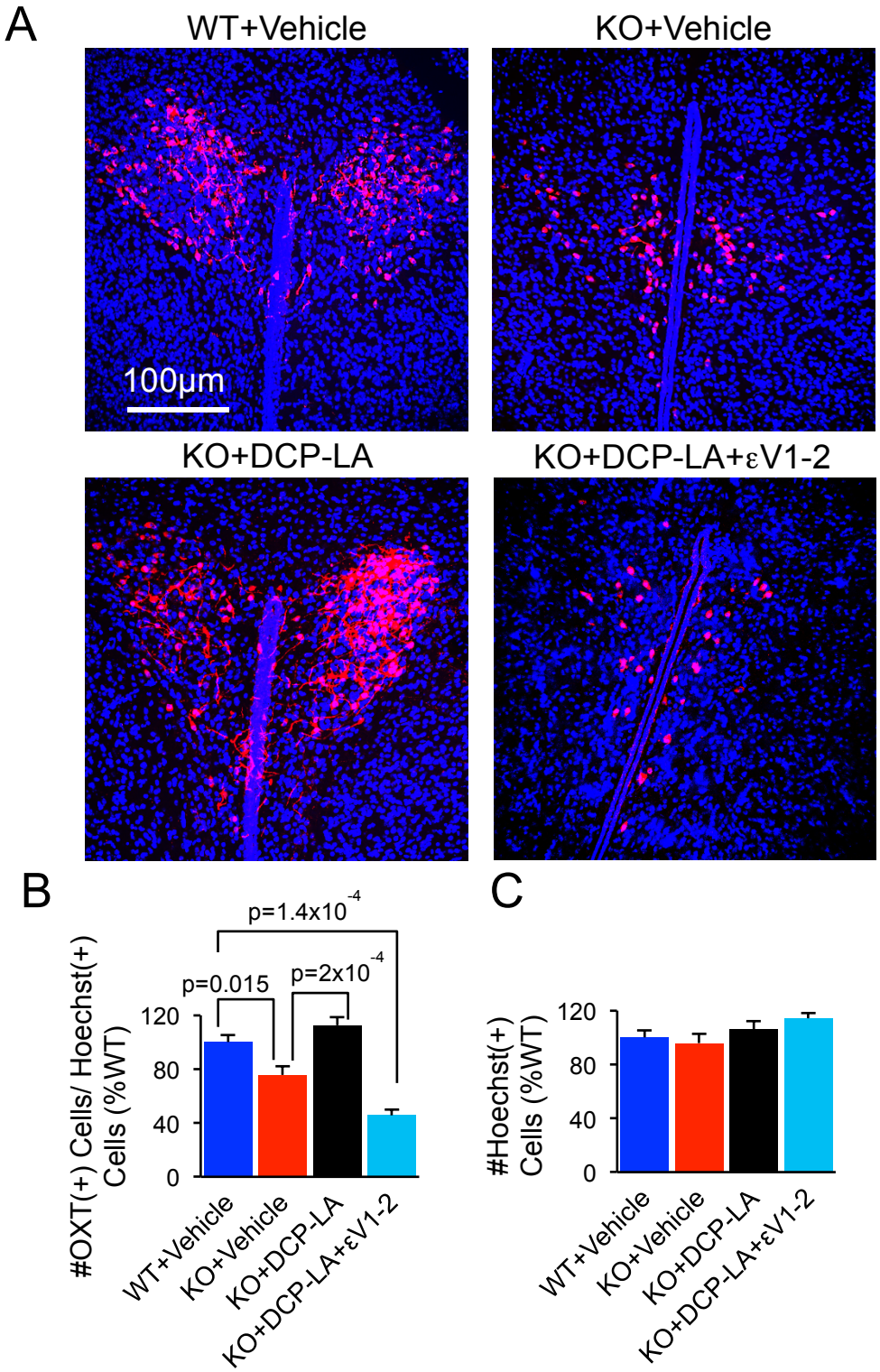


Figure 42. (A, B) Neonatal DCP-LA treatment fully rescues the number of PVN oxytocin positive (OXT+) cells in the *Fmr1* KO hypothalamic PVN at P20. On the other hand, co-administration of the selective PKCε inhibitor εV1-2 abolishes this rescue. Images acquired at 20x magnification. Red: oxytocin; blue: Hoechst. (C) The total number of PVN cells is comparable across groups. Group n's: 5-6 per group.

3.11. Pharmacological stimulation of PKCε during the neonatal stage rescues hyper-anxiety and social deficits in adult male *Fmr1* KO mice.

We have found evidence that PKCε is involved in AMPA receptor internalization in the CA1 region of the ventral hippocampus during the neonatal stage. This is significant because neuronal responses to excitatory stimuli are greatly dependent on AMPA receptor localization. During the early postnatal stage of brain wiring, synapse formation is largely activity dependent. However, this process appears to be impaired in the *Fmr1* KO brain, due to a reduction in PKCε expression, and as a result, surface levels AMPA receptors become abnormally elevated. Subsequently, altered responses to excitatory signals in *Fmr1* KO CA1 neurons may lead to aberrant synapse formation between the hippocampus and efferent targets, such as the PVN, the major source of brain oxytocin. The effect of the hippocampus on the PVN is predominantly inhibitory.[119, 120] Accordingly, we hypothesize that in the neonatal *Fmr1* KO brain, ventral CA1 neurons are hyper-responsive to incoming excitatory signals, and in turn exert increased inhibitory drive upon the PVN, potentially suppressing oxytocin production. These changes begin early in development, and may lead to permanent consequences on downstream oxytocin-dependent circuits involved in behavior, particularly anxiety and social behavior. If this is the case, then behavioral aberrances in adult *Fmr1* KO mice could theoretically be rescued by neonatal PKCε stimulation. To test this hypothesis, we treated *Fmr1* KO pups with either vehicle or 3 mg/Kg DCP-LA at P6, P10, and P14 and then assessed anxiety and social behavior upon adulthood (2 months of age). Unlike the studies previously outlined in this thesis, for these behavioral experiments we treated both male and female pups. This was done to determine whether DCP-LA would exert a behavioral effect on the generally asymptomatic KO females.

3.11.1. Anxiety-like Behavior

We first assessed anxiety-like behavior in the elevated plus maze (EPM), the gold standard for evaluation of anxiety in rodents. Consistent with reports of heightened anxiety disorders in human FXS patients, we observed that vehicle-treated *Fmr1* KO males made significantly less entries to the open arms (Figure 43A), and demonstrated a trend of spending less time in the open arms, compared to vehicle-treated WT males (Figure 43B). On the other hand, this phenomenon was not observed in our homozygous *Fmr1* KO females, which behaved comparably to WT females. In line with our hypothesis, KO males that were treated systemically with DCP-LA from P6-P14 were indistinguishable from WT males, and displayed significantly higher entries to the open arms, and a trend of higher time spent in the open arms, than vehicle-treated KO males. DCP-LA treated KO females were also statistically indistinguishable from WT and KO females, indicating that DCP-LA had no effect on EPM performance in the KO females.

While these data are indicative of reduced anxiety in DCP-LA treated KO males, we sought to confirm these findings using a second assay. For this purpose, the light dark test was selected based on its high degree of validity. Here, we observed that vehicle-treated *Fmr1* KO males spent significantly less time in the lit chamber than WT control males, once again indicative of heightened anxiety (Figure 44). Consistent with our observations in the EPM, DCP-LA-treated KO males behaved identically to the WT males, indicating a complete rescue of the anxiety phenotype observed in the vehicle-treated KO males in this test. Interestingly, we observed that in this more naturalistic test, KO females spent slightly less time in the lit chamber than WT females, but this difference was ultimately non-significant ($p=0.27$). On the other hand, DCP-LA treated KO females were indistinguishable from the WT females ($p=0.99$).

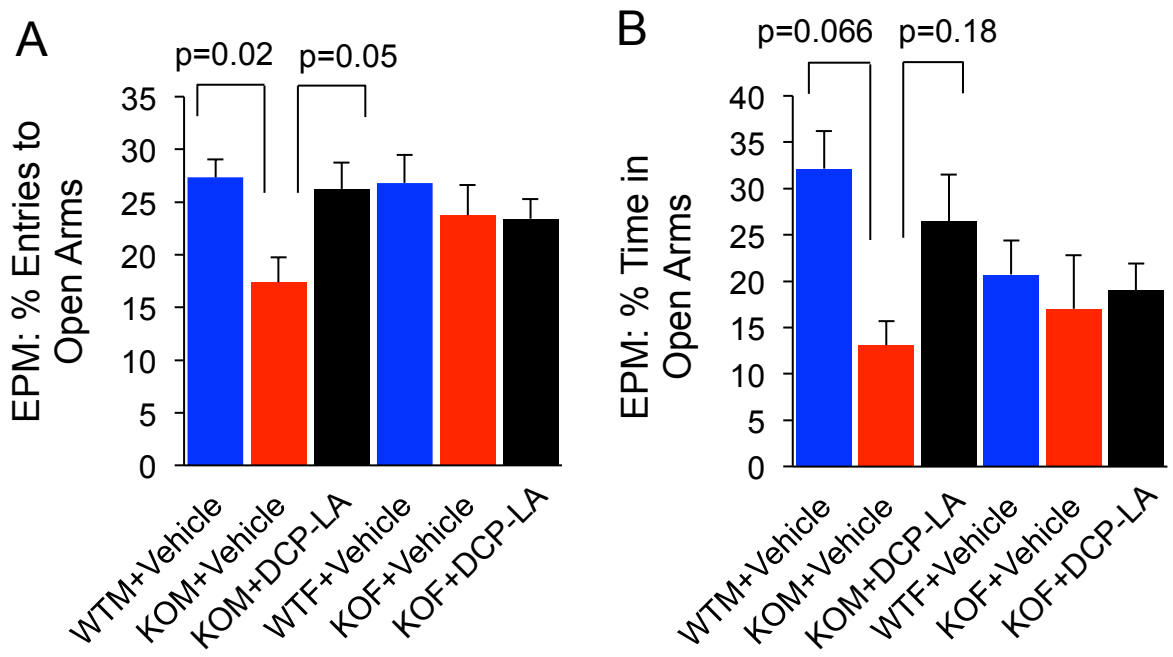


Figure 43. Neonatal DCP-LA treatment rescues male-specific anxiety behavior in visually intact, adult *Pde6b*^{WT} *Fmr1* KO mice in the elevated plus maze (EPM). Male *Fmr1* KO mice (KOM) make significantly less entries to and spend less time in the open arms compared to wild-type males (WTM), but this is corrected with administration of 3 mg/kg DCP-LA at P6, P10, and P14. On the other hand, KO females (KOF) do not differ significantly from WT females (WTF), and neonatal DCP-LA treatment has no effect on these behaviors in KO females. Group n': 12-14 per group.

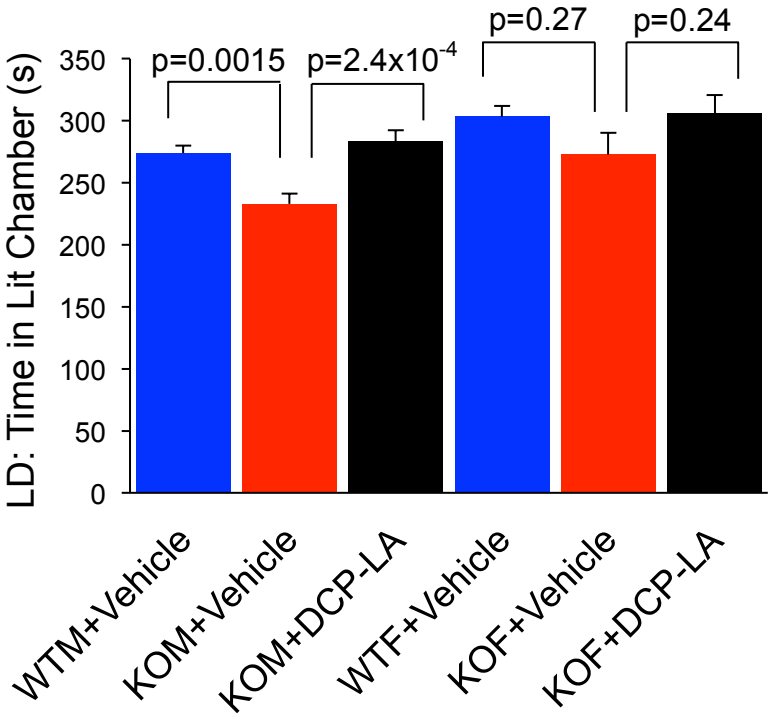


Figure 44. Neonatal DCP-LA treatment rescues hyper-anxiety behavior in *Fmr1* KO mice in the light-dark test (LD). Male *Pde6b*^{WT} *Fmr1* KO mice (KOM) spend less time in the lit chamber of the apparatus compared to wild-type males (WTM), but this is corrected with administration of 3 mg/kg DCP-LA at P6, P10, and P14. In contrast, KO females (KOF) do not differ significantly from WT females (WTF), and neonatal DCP-LA treated KO females are indistinguishable from WT females. Group n's: 12-14 per group.

3.11.2. Social Interaction

Taken together, our results from the EPM and light-dark tests demonstrate that neonatal DCP-LA administration is capable of rescuing anxiety-like behaviors in adult male *Fmr1* KO mice in these tests. Moreover, this treatment did not seem to exert any negative effects when administered to our asymptomatic *Fmr1* KO females. Thus, we next questioned whether the same neonatal regimen was capable of rescuing social deficits in our *Fmr1* KO mice. To answer this question, we performed the three-chambered social interaction test, which is considered the gold standard for measuring sociability in mice.

Consistent with human FXS patients, we observed noticeable social deficits in our vehicle-treated *Fmr1* KO male mice. Whereas WT control males spent a significantly greater amount of time investigating the novel mouse than the novel object, the vehicle-treated KO males investigated the novel mouse and novel object roughly equally (Figure 45). However, this deficit was fully corrected in DCP-LA-treated KO males, which, similarly to the WT males, spent significantly more time investigating the novel mouse than the novel object. Thus, the social deficits in our *Fmr1* KO mice, as measured by this assay, appeared to be reversed by neonatal DCP-LA treatment.

Using the same assay, we next examined social behavior in female *Fmr1* KO mice. Interestingly, while the WT control females spent a significantly greater amount of time investigating the novel mouse than the novel object, the vehicle-treated KO females displayed a trend towards spending more time with the novel mouse that was approaching significance (Figure 45). Thus, any social deficits in the vehicle-treated KO females were noticeably milder than the vehicle-treated KO males. Curiously, however, we found that neonatal DCP-LA treatment caused the *Fmr1* KO females to spend equivalent amounts of time investigating the

novel mouse and novel object, in a similar manner to our vehicle-treated *Fmr1* KO males. One explanation for this unexpected discrepancy could possibly be attributable to down-regulation of oxytocin receptors or oxytocin-synthesizing neurons. Since KO females did not demonstrate an appreciable deficit in social interaction in the first place, the oxytocinergic system in these animals was likely, for the most part, intact. As a result, DCP-LA treatment may have potentially lead to excess oxytocin synthesis in KO females during the juvenile stage. Interestingly, a recent study has shown that oxytocin receptors in the BNST appear to stimulate social avoidance in female, but not male, mice in response to social stress, possibly explaining our observations in our DCP-LA treated KO females. [328] Supporting this hypothesis, we observed no effect of neonatal DCP-LA treatment on social behavior in our wild-type males (Figure 46), indicating DCP-LA may have a female-specific detrimental effect on social behavior. However, data from DCP-LA treated WT females is still needed at this time. Nevertheless, since our vehicle-treated KO females appear to be, for the most part, asymptomatic, there would be no need for DCP-LA treatment in the first place, and so the observed effect would not be an issue. On the other hand, these results demonstrate that DCP-LA treatment appears sufficient and capable of fully reversing hyper-anxiety and social deficits in symptomatic *Fmr1* KO males.

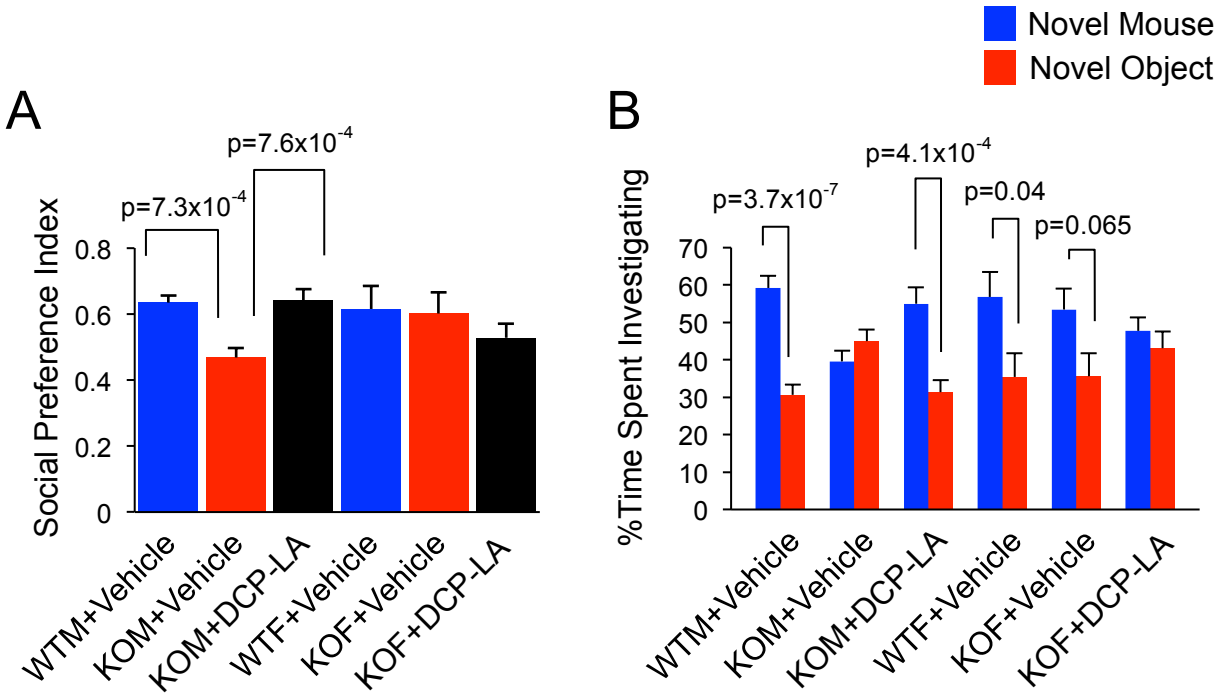


Figure 45. Adult *Pde6b*^{WT} *Fmr1* KO males (KOM), but not females (KOF), displayed lack of a preference for the novel mouse over a novel object in the three-chambered social interaction test (SI). Social preference index = time spent with novel mouse / (time spent with novel mouse + time spent with novel object). This deficit was eliminated in KOM neonatally treated with 3 mg/kg DCP-LA. WTM: Wild-type males, WTF: wild-type females. Group n's: 12-14 per group.

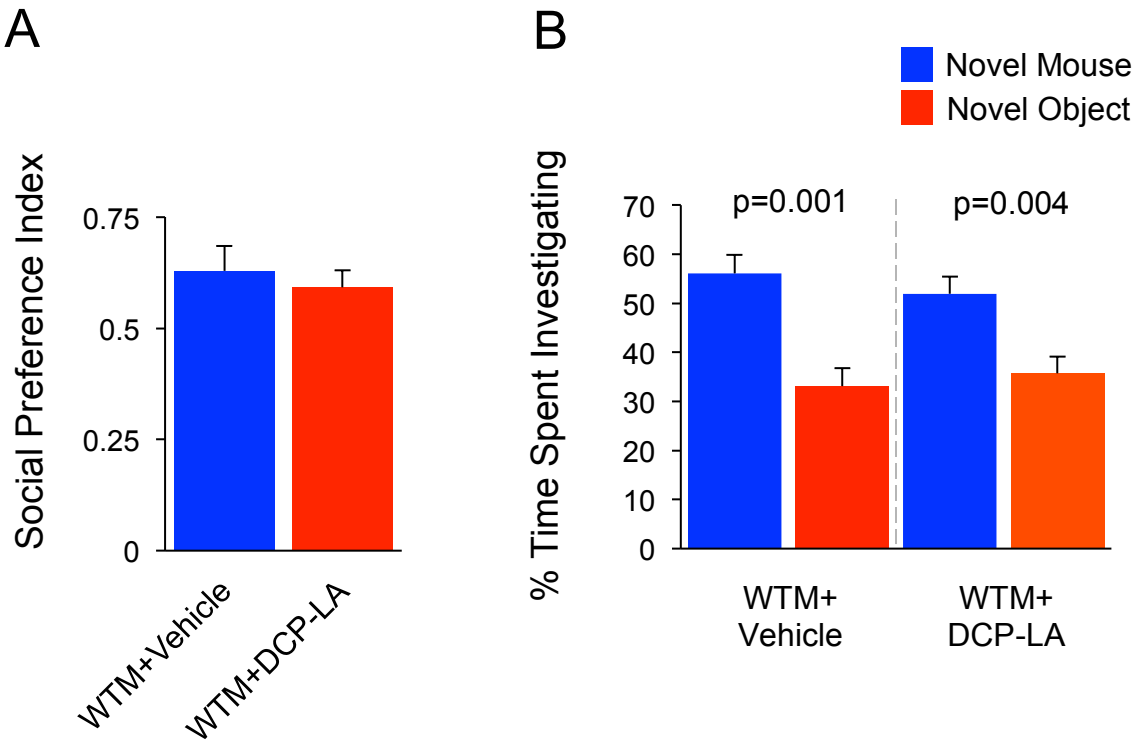


Figure 46. Administration of 3 mg/kg DCP-LA from P6-P14 does not seem to alter later-life social behavior in male WT mice. Group n's: 7-8 per group.

3.11.3. Locomotor Behavior

To ensure that our results from the EPM, light-dark, and social interaction tests were not influenced by differences in locomotor activity, we lastly performed the open field test on these mice. However, we were unable to detect statistically significant differences in distance traveled or time spent mobile between any of the groups tested (Figure 47). Since the open field did not reveal any discernable differences in locomotor activity between groups, we have reasonable confidence that our reported effects of neonatal DCP-LA treatment on EPM, light-dark, and social interaction test performance were not due to altered locomotor activity, and thus can be attributed to anxiolytic effects. Therefore, for the first time, we have demonstrated that a novel, early-intervention strategy is capable of rescuing the abnormal anxiety and social behavior phenotype of *Fmr1* KO mice, by rescuing aberrant PKCε signaling.

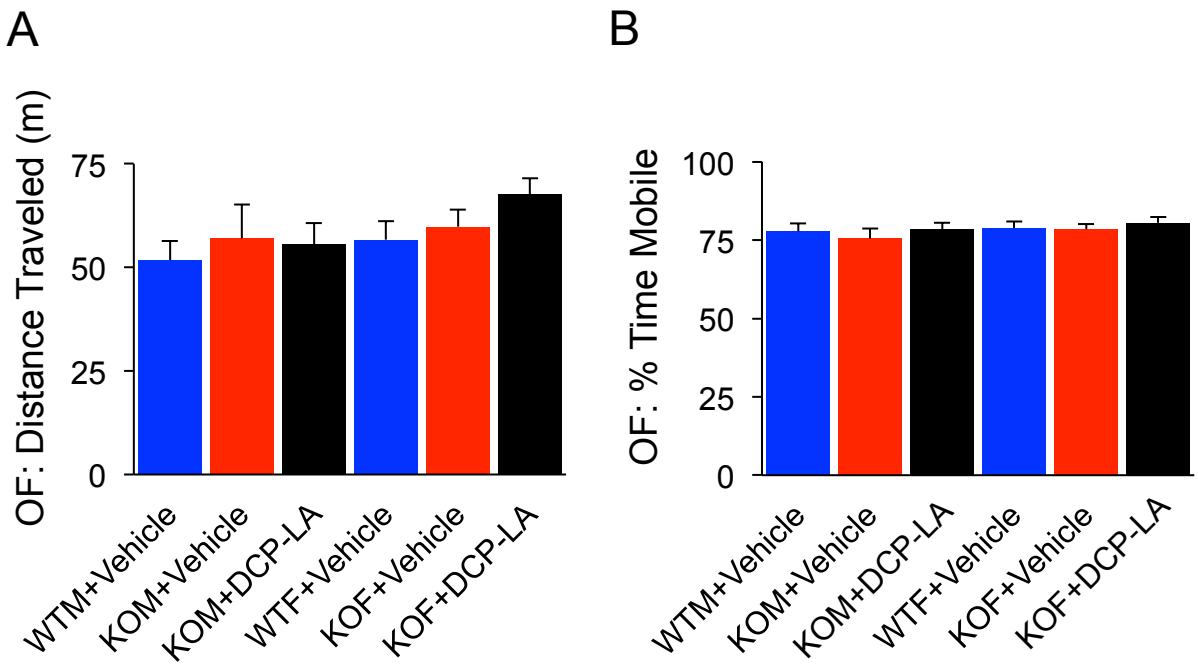


Figure 47. Adult *Pde6b*^{WT} *Fmr1* KO mice show normal locomotor behavior when compared to WT mice in the open field. Neonatal DCP-LA treatment does not alter these behaviors. Group n's: 12-14 per group.

IV. Discussion

4.1. Discussion

Fragile X Syndrome (FXS) is a heritable genetic disorder that is one of the leading causes of monogenic autism. However, unlike classical autism, many of the symptoms of FXS are attributed to hyper-anxiety, including social interaction deficits. Because FXS is a lifelong disorder that significantly affects the quality of life of the patient, the need for effective treatments for FXS remains imperative. Despite this, no direct cure for FXS exists, and current treatment strategies have significant disadvantages.

In this thesis, we have proposed an innovative, early-intervention strategy for the treatment of FXS. It has been widely accepted that developmental disorders like autism and FXS involve aberrant brain development during early development, which is highly difficult or almost impossible to correct in adulthood. However, the majority of translational studies have attempted to ameliorate the symptoms of FXS in adult subjects, after most of the neural circuitry has been permanently established. On the other hand, we propose that early intervention can redirect brain development onto a normal path, mitigating these symptoms before they fully develop.

Using the well-established *Fmr1* KO mouse model of FXS, we have discovered a novel, PKCε-dependent pathway by which ventral hippocampal communication with downstream brain regions may be altered during the early postnatal brain wiring. In our previous studies, we have discovered that PKCε is abundantly expressed during early postnatal hippocampal maturation, peaking after eye opening.[248] Although it has been reported that PKCε mRNA is a binding partner of FMRP, it was unclear how PKCε expression was affected in individuals with FXS.

Here, we report that in the juvenile *Fmr1* KO ventral hippocampus, PKCε is significantly under-expressed (Figures 25 and 27). In our attempts to elucidate the consequences of this under-expression in these mice, we discovered that shortly after eye-opening (P18), PKCε appears to interact with the GluR2 subunit of the AMPA receptor (Figure 35), and that PKCε deficiency coincides with increased surface incorporation of GluR2-containing AMPA receptors in the ventral hippocampus (Figure 31). This is significant because hippocampal responses to sensory stimuli are greatly dependent on AMPA receptor localization, and any changes to this process can upset the critical balance of excitation and inhibition in the hippocampal circuitry. However, we find that this process of AMPA receptor localization appears to be disturbed in the *Fmr1* KO brain, possibly as a consequence of reduced PKCε expression. As a result, AMPA receptors appear to be overexpressed in ventral CA1 pyramidal neuron synapses during the basal state, potentially leading to hyper-excitability of these neurons. Interestingly, these findings are consistent with an independent study that has reported heightened hippocampal excitability in *Fmr1* KO mice at this age.[85] Taken together, these observations may help explain, at least partially, heightened network excitability in FXS,[329] and the fact that many children with FXS are more susceptible to seizures.[108] Moreover, the fact that we no longer observe a difference in surface GluR2 levels in our *Fmr1* KO mice by adulthood (Figure 33) is consistent with the fact that for a significant proportion of these children, seizures remiss by adulthood. [108] On the other hand, the changes we observe in surface AMPA receptor levels appear limited to early postnatal hippocampal maturation. Importantly to note, the time point at which we observe this difference, P18, corresponds to the average age of FXS diagnosis in humans (roughly 3 years old).

At this point, it is necessary to address the fact that an independent study by Nakamoto and colleagues has found evidence of heightened AMPA receptor internalization in FXS,

presumably contributing towards the heightened LTD that has been observed in these mice.[330] While at first glance, our observations reported here may appear contradictory to these earlier findings, this is not necessarily the case. The results from Nakamoto's study are postulated to result from excess expression and activation of group 1 metabotropic glutamate receptors (mGluRs); however, we instead report that basal surface levels of the receptor appear to be increased. Moreover, the aforementioned study was conducted on cultured hippocampal neurons originating from embryonic rats transfected with siRNA against *Fmr1*. Given the great differences in model systems and developmental stages studied, these results may not necessarily contradict our *in vivo* findings. It should also be mentioned that in these independent studies, relatively high concentrations of the mGluR agonist DHPG (dihydroxyphenylglycine) were used (50uM), which may not reflect physiological conditions. On the other hand, we report intrinsic, baseline differences between *Fmr1* KO and WT mice in the juvenile stage.

In a separate study by Huber examining the phenomenon of heighted group I mGluR-mediated LTD in the *Fmr1* KO model, hippocampal slices were taken from P21-P30 mice from the C57 strain.[331] However, this corresponds to a later time-frame than the one studied in this thesis - a time-frame closer to early puberty. On the other hand, we report differences in surface AMPA receptor levels earlier on (P18), during the peak of early postnatal hippocampal synaptogenesis. Although further study is required, it is not unreasonable to assume that mechanisms governing AMPA receptor dynamics may be largely different between these two distinct stages of development. Aside from the age difference between the mice used in this study and ours, one must also pay attention to the strains used. Importantly, it has been shown that *Fmr1* KO mice on the C57 background, as used in Huber's study, have been reported to display normal levels of sociability and conditioned fear.[295] In this thesis, we have honed in on

the social and emotional aspect of FXS, and therefore used *Fmr1* KO mice on the FVB strain, which do express altered social and emotional behavior.[295] Because of this, we have specifically targeted the ventral hippocampus, whereas the origin of the hippocampal slices in the aforementioned study (dorsal versus ventral) was not specified. Lastly, group I-mGluR inhibitors have so far failed to produce meaningful improvements in human FXS patients, compared to placebos.[87]

Nevertheless, based on our findings, we next questioned whether pharmacological stimulation of PKCε could compensate for its reduced expression in the hippocampus, reversing our observed aberrances in surface AMPA receptor levels. To answer this question, we enlisted the selective PKCε activator, dicyclopropyl-linoleic acid (DCP-LA), which binds to PKCε's phosphatidylserine binding site. We have previously reported that PKCε expression in the mouse hippocampus rises between the ages of P6 and P15.[248] We therefore treated *Fmr1* KO pups with 3 mg/Kg DCP-LA at three points during this time-frame: P6, P10, and P14. Being a hydrophobic linoleic acid derivative, DCP-LA readily crosses the blood-brain barrier,[281] and hence, the treatments were administered *via* intra-peritoneal injection. Confirming our hypothesis, DCP-LA treatment fully reversed GluR2-overexpression in the ventral CA1 region at P18 (Figure 36).

Internalization of the AMPA receptor has been linked to PKC-mediated phosphorylation of the GluR2 subunit at serine 880 (Ser880).[319] Consistent with our findings that surface GluR2 is over-expressed in the *Fmr1* KO CA1 region, we observe a reduction of phosphorylated Ser880 GluR2 in these mice (Figure 34). Given our observation that that PKCε co-immunoprecipitates with GluR2 (Figure 35), this can be interpreted as evidence that PKCε may phosphorylate GluR2 at the Ser880 subunit to regulate synaptic AMPA expression. However, it is also possible that PKCε may not directly catalyze the Ser880 phosphorylation of GluR2. The precise nature of

PKCε regulation of GluR2 Ser880 phosphorylation could be determined *via* an *in vitro* kinase assay. In such an experiment, purified PKCε and GluR2 protein could be incubated together with ATP as a phosphate donor. Phosphorylation of GluR2 at the Ser880 residue could be assessed *via* Western blotting under basal conditions, as well as following treatment with PKCε activators and inhibitors.

What are the potential global consequences of GluR2 over-expression and net hyper-excitability in the FXS hippocampus? The hippocampus is a hub which receives sensory stimuli, and in response, influences the activity of several downstream brain regions relevant to behavior, such as the cortex, amygdala, and hypothalamus. Consequently, altered responses to excitatory signals in *Fmr1* KO CA1 neurons may lead to aberrant synapse formation between the hippocampus and its efferent targets during brain wiring.

Because a high proportion of patients with FXS suffer from anxiety disorders, especially social anxiety, we studied the integrity of the central oxytocinergic system in FXS, as a downstream target of ventral hippocampal regulation. The major source of oxytocin in the brain is the paraventricular nucleus of the hypothalamus (PVN). Ventral hippocampal neurons are known to project to and activate GABA-ergic BNST neurons, which in turn inhibit the PVN.[119, 120] We speculate that in the neonatal *Fmr1* KO brain, CA1 neurons may be hyper-responsive to incoming excitatory signals, and in turn exert increased inhibitory drive upon the PVN, potentially suppressing oxytocin production. Such changes may begin early in postnatal development, and may lead to permanent consequences on the development of downstream oxytocin-dependent circuits involved in behavior, particularly anxiety and social behavior (Figure 48). Supporting our hypothesis, we discovered that at P20, *Fmr1* KO mice displayed a reduced number of oxytocin-positive cells in the PVN compared to wild-type controls (Figure

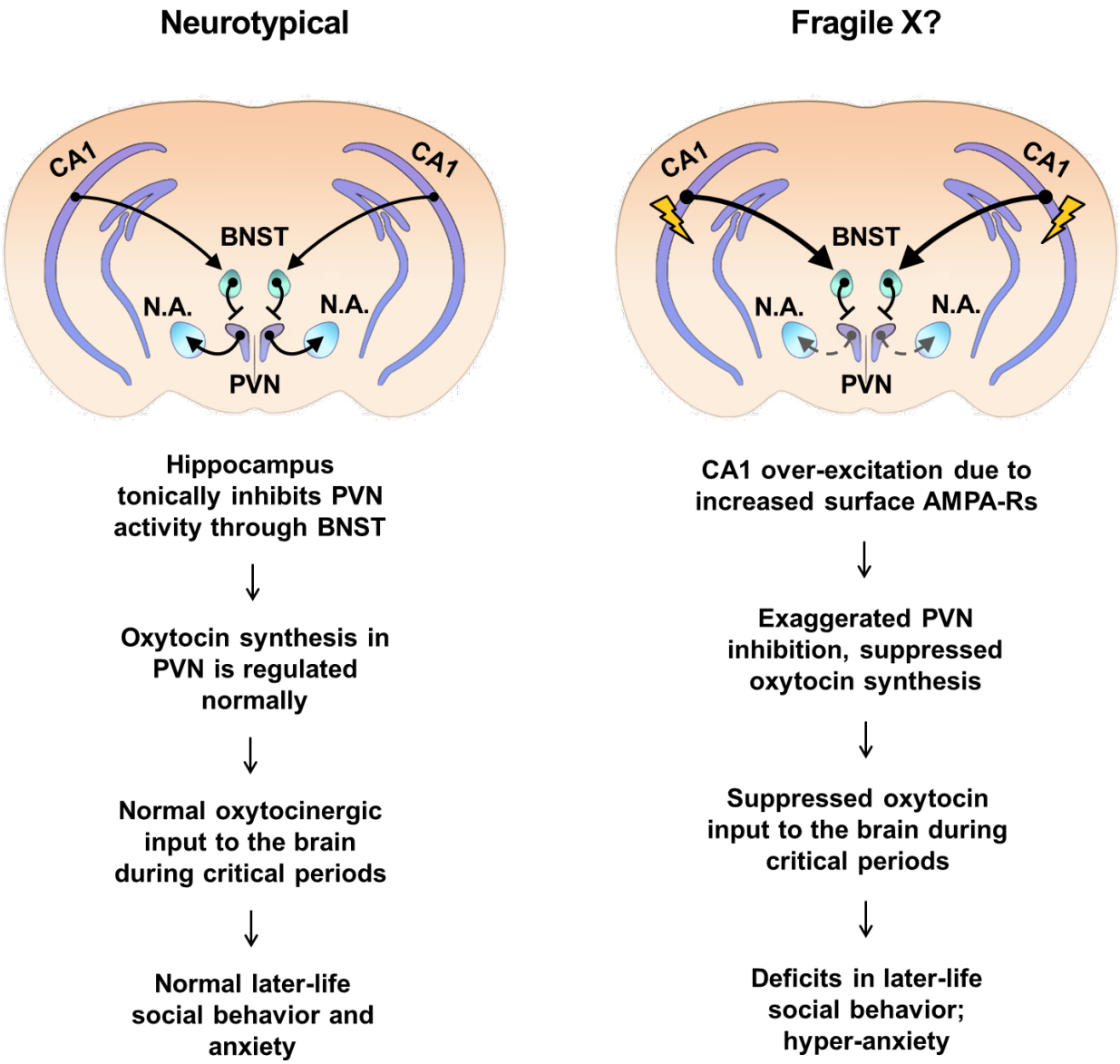


Figure 48. Overall hypothesis of the study. The findings reported in this thesis point to heightened basal levels of surface AMPA receptors in the *Fmr1* KO ventral hippocampal CA1 region during the juvenile period (P18). This would presumably lead to heightened excitability of the CA1 neurons, and in turn, heightened inhibitory drive upon the PVN through recruitment of the BNST. As a result, oxytocin synthesis is suppressed, leading to reduced oxytocin input to the brain during critical periods in which circuits relevant to emotion and social behavior are being established. In consequence, *Fmr1* KO mice exhibit deficits in later life social behavior, as well as exaggerated anxiety levels. BNST: bed nucleus of the stria terminalis; PVN: Paraventricular nucleus of the hypothalamus; NA: Nucleus accumbens.

38). Although we do not distinguish between centrally-projecting and peripherally-releasing cells in this study, given the deficits in social behavior that are characteristic of *Fmr1* KO mice,[287] it is very likely that centrally-projecting cells are affected.

If our hypothesis that the *Fmr1* KO hippocampus exerts increased inhibitory drive on the PVN - thus suppressing oxytocin synthesis - is correct, then reversal of synaptic GluR2 over-expression in the *Fmr1* KO hippocampus would relax this inhibitory drive and consequently boost oxytocin synthesis. To address this possibility, we examined PVN oxytocin synthesis in *Fmr1* KO pups treated with 3 mg/Kg DCP-LA at P6, P10, and P14. Supporting our prediction, we observed that the DCP-LA treated KOs displayed normal levels of oxytocin-positive cells in the PVN, when compared to WT controls (Figure 42). Thus, DCP-LA treatment during the neonatal stage seems capable of not only correcting synaptic GluR2 expression in the *Fmr1* KO ventral hippocampus, but also rescuing oxytocin synthesis within the PVN. To confirm that this rescue was PKCε-dependent, we also treated a separate group of KO pups with DCP-LA and the selective PKCε inhibitor, Myr-εV1/2, simultaneously. In this group, we observed a further decline in the number of oxytocin-positive cells in the PVN (Figure 42) relative to the vehicle-treated KO group. Because the inhibitor negated the effects of DCP-LA, it is most likely that our observed pro-oxytocin effect of DCP-LA is mediated through PKCε signaling.

Although we observed a reduction of PKCε expression in the KO hypothalamus, the possibility of a role of PKCε in oxytocin synthesis within PVN neurons remains unclear. It is unknown whether PKCε influences oxytocin gene expression through the recruitment of transcriptional regulators. However, we plan to more comprehensively address this question in our upcoming experiments. The most practical way to accomplish this would most likely be through *in vitro* experiments. For instance, primary cultured neurons obtained from the PVN

could be treated with PKCε activators and inhibitors, and then oxytocin mRNA levels could be measured via real-time PCR. Oxytocin itself could be directly quantified via radioimmunoassay.[190] Additionally, oxytocin-positive cells could be quantified visually via immunocytochemistry, in a similar manner to the experiments outlined in this thesis. In the event that we observe that PKCε activators enhance oxytocin levels while inhibitors suppress them, similar follow-up experiments could be performed to identify the downstream signaling molecules in the pathway, since the role of PKCε in oxytocin synthesis could be indirect (through phosphorylation of other, intermediary kinases). However, to our knowledge, no link has been found between PKCε and pathways mediating oxytocin synthesis in the PVN (the main pathway being estrogen-receptor beta activation).[324]

There also exists the possibility that PKCε could be involved in the stimulation of PVN oxytocin-synthesizing neurons by altering AMPA receptor dynamics. We have shown that PKCε co-immunoprecipitates with the GluR2 subunit of the AMPA receptor (Figure 35), and propose that PKCε may serve to internalize surface AMPA receptor levels in the hippocampus through phosphorylation of GluR2 at the serine 880 residue. However, we predict this is unlikely to occur within centrally-projecting, oxytocin-synthesizing neurons due to the inherent AMPA receptor subunit composition in the PVN. Although most hippocampal AMPA receptors contain GluR2 subunits,[252] AMPA receptor composition in the PVN is much more heterogeneous. Here, GluR2 is more predominantly found in hypophysiotrophic cells, whereas GluR1 is more highly expressed in centrally-projecting, pre-autonomic cells.[325] Interestingly, it has been found that the catalytic unit of PKC is capable of increasing AMPA receptor conductance by phosphorylating the serine 831 (Ser831) residue of GluR1.[327] However, our western blot analysis determined that phosphorylated Ser831 GluR1 levels were comparable in WT versus *Fmr1* KO mice at P20

(Figure 41). This suggests that PKCε may have a more influential role in AMPA receptor dynamics in the hippocampus, as opposed to within the PVN. Thus, we predict it is more likely that oxytocin suppression in the *Fmr1* KO mouse is due to enhanced hippocampal inhibition of the PVN, rather than an autonomous effect within the PVN. While this scenario is strongly backed by the existing literature, in order to truly establish whether the hippocampus is the source of the oxytocin suppression in our *Fmr1* KO mice, future studies must be performed in which PKCε activators and inhibitors are delivered directly to the hippocampus (for example, *via* stereotactic injections). Inherent effects of PKCε within the BNST must also be examined.

Correcting anomalies in the FXS brain is of little use to human patients, unless these corrections meaningfully improve the patients' symptoms. We hypothesize that by correcting hippocampal communication with targets such as the PVN during brain wiring, neonatal DCP-LA treatment may aid in re-directing the development of behaviorally-relevant circuits back onto a normal course, before FXS symptoms become permanent. To examine whether this was indeed possible, we treated KO pups with either vehicle or 3 mg/Kg DCP-LA at P6, P10, and P14, and then tested them behaviorally when they reached adulthood. As expected, we found that unlike our vehicle-treated group, our DCP-LA-treated mice were statistically and visually indistinguishable from WT controls in the elevated plus maze, light-dark test, and social interaction test (Figures 43-45). These data provide compelling evidence that neonatal DCP-LA treatment is capable and sufficient of permanently rescuing the exaggerated anxiety and social impairments in *Fmr1* KO mice.

Intriguingly, the aforementioned behavioral anomalies were only observed in male KO mice. At first glance, this finding seems compatible with the observation that females typically display milder symptoms than male patients. This is attributed to the fact that all human females

with the full mutation are heterozygous, because the full mutation can only be transmitted maternally. Consequently, due to lionization, all full mutation females are mosaics for activity of the affected X chromosome. Based on this, it is presumed that tissue-specific skewed X inactivation may underlie the variability of FXS in women. However, the female KO mice used in this study were homozygous for the *Fmr1* KO mutation, and therefore, the sex-specific effect of *Fmr1* knockout on anxiety and social interaction profiles remains to be explained.

This study introduces early postnatal PKCε-mediated signaling as an important regulator of brain wiring, in regards to social and anxiety behavior. For the first time, we have provided evidence that PKCε expression is impaired in the *Fmr1* KO hippocampus and hypothalamus. We hypothesized that during the early postnatal period, this loss of normal PKCε expression may produce long-lasting changes in the formation of circuits involved these behaviors. Supporting this idea, we report that early-life pharmacological stimulation of PKCε is sufficient to fully rescue male-specific hyper-anxiety and social deficits in our *Fmr1* KO mice, in three well-validated behavioral assays: the elevated plus maze, light-dark box, and social interaction test.

While these results are an encouraging start towards a viable early-intervention treatment for FXS, they also raise several important questions. To begin, further work needs to be done to elucidate the finer mechanisms by which DCP-LA exerts its therapeutic effect, as such studies would provide valuable insights into potential new avenues of treatment for FXS. First, the manner in which PKCε expression in the *Fmr1* KO brain affects the basal excitability of CA1 neurons at this young age still remains to be fully confirmed. This can be most directly addressed through electrophysiological studies. For instance, measurement of field excitatory synaptic potentials and population spike amplitudes in CA1 neurons, following paired-pulse stimulation of the Schaffer collaterals, could greatly illuminate the relationship between CA1 inputs and cell

outputs.[131] Moreover, the question of whether PKCε directly phosphorylates GluR2 must also be confirmed. This can be accomplished via a combination of *in vitro* kinase assays including recombinant PKCε and GluR2 proteins. In addition, cryo-electron microscopy could be used to further analyze the molecular architecture of the PKCε:GluR2 complex. Given our co-immunoprecipitation data (Figure 35), these studies would likely be fruitful in illuminating the nature of this novel interaction.

The safety of DCP-LA itself as a potential treatment for FXS is another significant concern, especially considering the treatment regimen outlined in this thesis takes place during early postnatal brain development. While we have not detected any adverse side effects of DCP-LA in our male KO mice, rigorous screening will be required to ensure it is suitable for human use. Beginning with the first treatment through adulthood, DCP-LA-treated mice should be closely monitored in the home cage for the presence of any external symptoms, such as aberrant behaviors, twitching, seizures, or abnormal eating or sleeping patterns. In order to assess abnormal effects on liver, blood collected from the 24-hour time point should be used to assess the levels of the enzymes alanine aminotransferase (ALT) and alkaline phosphatase (ALP), which rise sharply in the blood during liver damage. The fact that DCP-LA treatment appeared to modify social behavior in our originally asymptomatic female KO mice (Figure 45) should also be addressed. It is possible that DCP-LA treatment may prove effective in severe cases of FXS, but should be avoided in mild cases. Moreover, this effect may be sex-specific, since we did not observe any effect of DCP-LA on social behavior in our WT males (Figure 46).

If no other adverse side effects of DCP-LA are observed, then the best regimen for neonatal treatment will also need to be determined. Besides the dose used in this thesis (3 mg/Kg), both lower and higher doses should be tested to identify the safest effective dose. Such

preclinical testing will be necessary to predict human responses to DCP-LA as a drug candidate and to screen for unwanted CNS and peripheral side effects. Moreover, the most effective time-course of DCP-LA treatment must still be clarified. Although in this thesis, we have treated our *Fmr1* KO mice at P6, P10, and P14, a single treatment at one of these time-points may be sufficient to produce the therapeutic effects reported here. Since P6 is considered pre-term in humans, our future studies will evaluate whether a single treatment at P10 or P14 will be sufficient. The ideal regimen would be a single treatment at P14, which corresponds to ~2 years of age, at which point symptoms are beginning to emerge in the patients. Given the fact that we observe a negative effect of DCP-LA on social behavior in asymptomatic females, we would caution against preventative DCP-LA administration in humans, until more studies are performed to determine which populations of patients would respond best to the treatment.

Lastly, the extent to which DCP-LA exerts its therapeutic effect must be more thoroughly evaluated. While in this thesis we have only examined anxiety and social behavior, DCP-LA may prove beneficial in treating other symptoms of FXS, such as learning delays and hyper-sensitivity to sensory stimuli. Rescue of the former could very well be likely, since it has already been discovered that DCP-LA improves age-related cognitive impairments in senescence-accelerated mice. In a similar vein, Alkon and colleagues have demonstrated that bryostatin, a non-specific PKCε inhibitor, is capable of rescuing spatial learning and memory in adult *Fmr1* KO mice, by correcting structural abnormalities in hippocampal dendritic spines. Thus, DCP-LA may be of use in combating cognitive impairments in FXS patients, by normalizing hippocampal synapses. The efficacy of DCP-LA in treating cognitive impairment can be tested in *Fmr1* KO mice using well-validated assays such as the Morris water maze or Barnes maze, which provides insight into spatial and reversal learning similarly to the Morris water maze, but without the

anxiety provoked by being forced to swim. Similarly, hippocampal-dependent declarative memory can be studied using a simple object recognition assay.

As mentioned, DCP-LA may also be effective in rescuing FXS-associated hyper-sensitivity to sensory stimuli. It is believed that in FXS, hippocampal neurons are hyper-excitable,[332-334] leading to over-activity of circuits involved in processing visual, auditory and tactile stimuli. In the patient, this may result in feelings of being overwhelmed by sensory stimuli in their environment, in turn causing difficulty focusing, distress, or even seizures. However, by normalizing basal levels of synaptic AMPA receptors in CA1 neurons, DCP-LA may counteract these defects, normalizing sensory processing in the FXS brain. The hyper-arousal state can be gauged in vehicle versus DCP-LA treated *Fmr1* KO mice in several ways. For instance, exaggerated responses of the auditory cortex in response to tones have been well-described in both FXS patients[335, 336] and mice,[337] and can be measured through *in vivo* electrophysiological recording. Additionally, serum cortisol can be measured in response to various environmental stressors, for instance, during exploration of novel environments.

4.2. Concluding Remarks

In our efforts toward a therapeutic strategy for FXS, we have adopted a unique approach of focusing on the early stages of postnatal brain development. In the process, we have discovered an FXS-associated defect in an FMRP target (PKCε) that is likely to be linked to multiple behavioral deficits. We demonstrate that early intervention, using a highly selective stimulator of PKCε, is able to correct later-life social behavior deficits and hyper anxiety in the leading FXS model; and attempt to identify possible mechanisms by which this rescue takes place. The work described in this thesis introduces the possibility that early intervention using PKCε activators may serve as a promising new avenue of treatment for FXS during a time where

existing treatments are still desperately lacking. Consequently, it is my hope that this work and our described future experiments will, in some way, ultimately contribute to our understanding of FXS and pave the way for better treatments for human patients.

V. References

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