Understanding the Molecular Basis of Memory Defects in Fragile X Syndrome

by

Daniel Brent Chambers

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Centre for Neuroscience, Faculty of Medicine and Dentistry
University of Alberta

Abstract

Fragile X Syndrome (FXS) is the most common genetic cause of Intellectual Disability, affecting 1 in 4000 boys and 1 in 6000 girls. Work in our lab using *Drosophila melanogaster* and since replicated in the mouse model has shown that excess protein synthesis is a major mechanism connecting the observed learning and memory problems and the mutation in the fragile x gene. My masters thesis project aims at understanding further the molecular mechanisms leading to dysregulation in protein synthesis in Fragile X. Previous research has found a major protein synthesis regulation pathway, the AKT-mTOR pathway to be disrupted in Fragile X mice (Sharma et al., 2010). I initially carried out an analysis of potential target proteins within the AKT-mTOR axis of the Insulin Receptor pathway in a *Drosophila melanogaster* (fruit fly) model of Fragile X syndrome. This identified multiple targets that are dysregulated in Fragile X. We show that decreasing Insulin signaling in neurons throughout the brain results in impairments of both learning and protein synthesis dependent memory specifically. The final phase of this project was to identify treatment methods for Fragile X syndrome. We selected drugs targeting key candidate proteins misregulated in Fragile X mutant flies. Acute administration of Metformin, a hypoglycemiant, and Rolipram, an inhibitor of phosphodiesterase, led to significant rescuing of the learning and memory defects seen in Fragile X. Taken together the data may offer new insights into the molecular mechanisms underlying the learning and memory defects seen in Fragile X syndrome and identify potential treatment methods to improve learning and memory defects.

Quote

"Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure. It is our light, not our darkness that most frightens us."

- Marianne Williamson

Dedication

I would like to dedicate this work to my family, my father Dan, mother Sandy, my little sister Chelsea and our late beloved dog Jake. Without the support you have provided I never would have been able to do this. Even though Jake did not live to see me finish my graduate work, he saw me through the hardest times with the constant wag of his stubby tail.

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List of Abbreviations

FXS	Fragile X Syndrome
FX	Fragile X
FMRP	Fragile X Mental Retardation Protein
LTM	Long Term Memory
LTM^d	Protein synthesis dependent long-term memory
LTM^i	Protein synthesis independent long-term memory
ID	Intellectual Disability
NDD	Neurodevelopmental disorder
IQ	Intellectual Quotient
CNS	Central Nervous System
mGluR	Metabotropic Glutamate Receptor
mTOR	Mammalian Target Of Rapamycin
PI3K	Phosphoinositide 3-Kinase
TSC	Tuberous Sclerosis Complex
p70S6K	p70 ribosomal protein S6 kinase
WT	Wild Type
cAMP	Cyclic Adenosine Monophosphate
PDE	Phosphodiesterase
PKA	Protein Kinase A
MAPK	Mitogen-Activated Protein Kinase
AMPK	AMP-activated protein kinase
Inr	Insulin Receptor

PBS	Phosphate Buffered Saline
SDS	Sodium Dodecyl Sulfate
PVDF	Polyvinylidene Difluoride
WTR	Wild Type Rescue
Dilps	Drosophila insulin-like peptides
IRS	Insulin Receptor Substrate

Chapter 1. GENERAL INTRODUCTION

1.1 Definition of ID

Intellectual disability (ID), also known as mental retardation, represents a group of disorders in which intelligence development is affected. Attempts at studying a genetic basis to intellectual disability date back to the beginning of the 19th century, but only recently has an enormous amount of clinical data emerged that has taught us about the molecular basis of intellectual disabilities (Raymond and Tarpey, 2006). ID affects 3% of the population (Ropers and Hamel 2005, Yeargin-Allsopp and Boyle 2002) and costs $1.3 billion each year for schooling and specialized medical services for those affected in Canada (CDC, 2004). There are numerous other conditions known to have high co-morbidity with intellectual disability such as: cerebral palsy, epilepsy, severe hearing impairment or deafness, severe vision impairment or blindness, hydrocephalus, autism and psychiatric disorders (Berry-Kravis et al., 2010).

Intellectual disability consists of a significant sub-average general intellectual functioning that is accompanied by limitations in adaptive functioning in at least two of the following areas: communication, self care, home living, social/interpersonal skills, use of community resources, self-direction, functional academic skills, work, leisure, health and safety (Srivastava and Schwartz, 2014). Intellectual disability is described as an intellectual quotient (IQ) less than 70; the IQ is relative performance of the individual compared to the general population for the same age. Intellectually disabled can be divided into separate categories of severity; mild (50-70), moderate

(35-50) or severe (less than 35) based on the IQ number. Mild intellectual disability is seen to be the most common form, and is more common in males.

1.2 Classification systems of ID

There are various classification methods that have been used in intellectual disability (Moog, 2005; Shalock et al., 2007). One such method relates to the chromosomal localization: autosomal versus X-linked. X-linked is further divided between syndromic, neuromuscular and non-specific. Intellectual disability has additionally been divided between syndromic and non-syndromic (Shalock et al., 2007). Syndromic intellectual disability is associated with dysmorphic or other neurological features. Examples of syndromic intellectual disability include Down syndrome, Rett syndrome, Rubinstein-Taybi syndrome and Fragile X syndrome (Moog, 2005; Shalock et al., 2007). Non-syndromic intellectual disability has been defined by the presence of intellectual disability as the sole clinical feature. However, it has been a challenge to rule out the presence of more subtle neurological anomalies and psychiatric disorders in these patients, as they may be less apparent, or difficult to diagnose due to the cognitive impairment. Many causes of ID for both syndromic and non-syndromic have been linked to genetic mutations, which has allowed the use of animal models to bring to light the mechanisms by which IDs affect cognition.

1.3 Fragile X is the most common cause of ID

1.3.1 Clinical description of Fragile X syndrome

Fragile X Syndrome (FXS) is the most common genetic cause of ID, affecting 1 in 4000 boys and 1 in 6000 girls. Clinically, features such as a narrow face, large head, large ears, flexible joints, flat feet, and a prominent forehead can identify Fragile X patients (Aziz et al., 2003; Berry-Kravis et al., 2007; Visootsak et al., 2014). These physical features become more obvious with age. At the root of the issues in ID and in particular FX are abnormalities within the nervous system. FXS is caused by a lack of Fragile X mental retardation protein (FMRP), most often due to increased CGG repeats in the 5' UTR region of the *fragile X mental retardation 1* (*fmr1*) gene, leading to the methylation of that region which in turn leads to the transcriptional silencing of the gene (Honeycutt et al., 2003; Bolduc et al., 2008; Sharma et al., 2010; Callan et al., 2012; Visootsak et al., 2014).

1.3.2 Molecular function of Fragile X Protein

The nervous system begins as a flat sheet of cells within the embryo, which then folds, twists and grows into a mature organ containing hundreds of billions of cells that are organized into functional units to receive, process and respond to information (Okray and Hassan 2013). While this process takes place, complex genetic and molecular interactions ensure that all cells follow a specified developmental program that leads to their appropriate role in the system. Defects in

the development and maintenance of a healthy nervous system can compromise cognitive abilities, with most of these defects leading to diagnoses of neurodevelopmental disorders (NDD) characterized by intellectual disability (Bolduc and Tully, 2009). How mutations affect the development of the brain by disrupting molecular and cellular networks, ultimately contributing to neurodevelopmental disorder phenotypes are notable outstanding questions that require further research research to attempt to understand.

FMRP is ubiquitous in the CNS, where it associates with ~4% of mammalian neuronal mRNAs (Bolduc et al., 2008). FMRP is an RNA-binding protein for which numerous mRNA targets have been identified (Edbauer et al., 2010). Numerous studies have demonstrated that FMRP interacts with both coding and non-coding RNAs and represses protein synthesis at dendritic and synaptic locations (Zalfa et al., 2003). In the absence of FMRP, the basal protein translation is enhanced and not responsive to neuronal stimulation. FMRP negatively regulates translation (Verkerk et al., 1991; Zalfa et al., 2003), and it has been observed that *Fmr1* knockout mice show enhanced global translation (Laggerbauer et al., 2001). In the absence of FMRP, synaptic local translation occurs independently of synaptic activity, leading to abnormally shaped and functioning synapses (Reeve et al., 2005; Reeve et al., 2008; Connor et al., 2011). Within neurons FMRP is found in the granules containing mRNA, RNA-binding proteins and ribosomes. Local translation of these granules at activated synapses allows encoding of memory at the cellular level. The dendritic spines represent the site of synapse between neurons and have been found to be

malformed in FXS humans, mice and even flies (Bagni and Greenough 2005; Irwin et al., 2001).

FMRP loss can result in elevation of proteins whose translation is negatively regulated. Loss of this ubiquitous translational regulator likely impacts multiple neuronal circuits, mediating deficits in distinct behavioral areas in both patients and animal models (Kanellopoulos et al., 2012). FMRP, as an RNA-binding protein has shown to repress translation when present at the base of synapses, therefore seems to be an excellent candidate for the proper regulation of translation in the synapses. The loss of FMRP is responsible for a constellation of symptoms including seizures, sleep disorders, anxiety, autism and mild to severe cognitive impairment (Jacquemont et al., 2007).

1.4 Drosophila and neurobiology

The fruit fly *Drosophila melanogaster* is one of the premier genetic model organisms used in biomedical research today owing to the extraordinary power of the genetic tools that have been developed (Rubin and Spradling 1982). It serves as an effective tool for studying brain development and other related disorders since the principles of nervous system development and function are conserved across lineages (Bolduc and Tully, 2009). In addition, its experimental manipulability allows extensive analysis of the mechanisms of the brain along with discovering new avenues of brain function.

Many of the fundamental principles of the nervous system appear to have remained unchanged throughout evolution (Bolduc and Tully, 2009). The use of *Drosophila melanogaster* as an animal model to investigate and understand the genetics and molecular mechanisms of ID hinges upon this fact. In the context of human diseases and disorders, up to two thirds of human disease genes reported in the Online Mendelian Inheritance in Man (OMIM) database have a homolog in the fruit fly (Reiter et al., 2001), and the conservation of ID genes is particularly high: up to 87% have *D. melanogaster* homologs (Inlow and Restifo, 2004). The genomes of mammalian models are more complex than Drosophila due to the fact that animals such as the mouse have a larger gene family (Bolduc and Tully, 2009). Members of a gene family can be functionally redundant, or a common function can be partitioned among paralogs. This buffers against genetic mutations and allows development of new functions from genes (Ohno, 1970), however this makes mammals much more difficult to understand from a genomic function perspective. For example, many genes linked to NDD's have several paralogs in humans but in the fly, are found as only a single homolog (Okray and Hassan, 2013). For instance, *fxr-1* and *fxr-2* are two paralogs of the Fragile-X mental retardation (*fmr-1*) gene in humans and mice, whereas Drosophila has a single fmr-like gene, *dfmr1* (Wan et al., 2000). Along with a more complex genome, the interconnections between neurons of the human brain are orders of magnitude more complex than that of the fly brain. Despite the overall simplistic organization of the fly brain, the cellular and molecular components of the

mammalian and fly nervous systems are structurally and developmentally comparable (Sanchez-Soriano et al., 2005).

At the base of ID characterization are defects in cognition and behavior, and presumably a functioning human brain produces more complex and sophisticated cognitive behaviors than that of a fruit fly. The fly is capable of its own repertoire of complex behaviors, and the most basic behaviors of humans and flies are often shared- think of circadian rhythm for instance (Greenspan and van Swinderen, 2004). The high level of plasticity of their nervous systems allows flies to habituate to sensory stimuli, and learn and form memories of associations through numerous training modules (Pitman et al., 2009). Many genetic and molecular foundations of behavior are shared between humans and flies (Greenspan and Dierick, 2004). The genes driving human neurodevelopmental disorders like Fragile X Syndrome, Neurofibromatosis type 1, and Angelman Syndrome have homologs in the fly that lead to learning and memory defects similar to those seen in humans (McBride et al., 2005; Wu et al., 2008). A large fly genetic toolbox has been created for reverse genetic approaches to allow precise cell-type and developmental-stage specific control of gene expression (Venken et al., 2011). By altering a specific gene's expression in specific cell-types we are able to study phenotypes that the system's inherent mechanisms would otherwise obscure. The most commonly used tools from a fly genetic toolbox are microbial-derived binary expression systems (Okray and Hassan, 2013). These expression systems consist of two genetic elements: an exogenous transcription factor and a synthetic promoter containing its binding

sequence. A very specific endogenous fly gene promoter drives expression of the transcription factor, after which this transcription factor binds to a specific synthetic promoter to drive the expression of the selected gene. The main binary system used in the fly involves the yeast-derived transcription factor Gal4, which in turn binds to the Upstream Activator Sequence (UAS) to drive gene expression (Duffy, 2002). Brand and Perrimon (1993) developed the procedure by restricting the expression of a gene only to cells of particular interest during a select developmental stage one is capable of looking at the impacts of mutations during the course of development and the different impacts that they can have on cognitive processes such as memory.

1.5 Drosophila learning and memory

Drosophila has been used in various ways to study learning and memory. Learning and memory can be measured in the context of appetitive or aversive conditions as well as in courtship. We used a method based on olfactory classical conditioning as developed by Tully and Quinn (1985). This well established assay allows us to study various stages of memory from the initial learning to the formation of long-term memory (LTM). Interestingly, using various training protocols allows us to dissect protein synthesis dependent and independent phases of memory. This proved very useful in the study of Fragile X syndrome. Indeed, Bolduc et al. (2008) showed that the Drosophila homologue for Fragile X, d*fmr1*, was required for learning and protein synthesis dependent memory. In addition, they showed for the first time that d*fmr1*'s role in memory could be divided between a development

function and an acute role. They also showed that d*fmr1* interacted with the RNA binding protein staufen and with the RNA interference protein Ago1. Bolduc also identified that excess in protein synthesis may be responsible for the memory problem when they were able to rescue the acute memory function using protein synthesis inhibitors such as cycloheximide and puromycin. Interestingly, the effect was specific to protein synthesis dependent long-term memory. It remains unclear though which pathway controlled protein synthesis and was disrupted in the absence of Fragile X protein, dFMRP.

Memory is stored via the precisely regulated interaction of neuronal networks of the nervous system. It has been previously demonstrated that there are different forms of Long-Term Memory (LTM). There is an LTM training called Space Training that is considered protein synthesis dependent memory (LTM^{d}), due to the fact it requires protein synthesis to take place in order for a stronger memory to be formed (Goodwin et al., 1997, Yin et al., 1995). In addition there is a form of LTM training called Mass Training that does not require protein synthesis and is therefore known as protein synthesis independent memory (LTM^{i}) (Bolduc et al., 2008). Assays have been developed to test these different types of memory, Space training and Mass training. Space training, as the name implies exposes the flies to 10 training sessions but allows for a rest period of 15 minutes between each session; during this time protein synthesis takes place. Mass training exposes flies to the same 10 training sessions however there is no rest period between sessions, not allowing protein synthesis to take place (For more information please see Chapter 2: Materials and Methods). Earlier work done on Drosophila has demonstrated key pathways that

are involved in the formation of memories (Goodwin et al., 1997). One of them is the cyclic AMP pathway that will be discussed further in the chapter on therapy. In brief, adenylate cyclase leads to the production of cAMP whereas phosphodiesterase leads to its destruction. cAMP activates the kinase PKA that, in turn, phosphorylates the transcription factor CREB. The CREB pathway was also validated in mouse and human.

1.5.1 The Drosophila model of FX: role of protein synthesis

The *Drosophila* homologue of *fmr1* is d*fmr1*. There is a high degree of functional conservation between the two genes, especially in the RNA-binding domains (Gao, 2002). There are many advantages to using *Drosophila* for the study of FXS. It is inexpensive to raise and has a short generation time of two weeks, allowing for large numbers of flies to be economically experimented on in a short period of time. More importantly, several strains of fly's mutant for *fmr1*s are readily available.

There exists a *Drosophila fmr1* null mutant, d$fmr1^{B55}$ that demonstrates impairments in both learning and long-term memory (Bolduc et al., 2008). Moreover, it was seen that d$fmr1^{B55}$ flies were defective in protein synthesis dependent memory tasks. The memory defects observed in d*fmr1* mutants are the result of the additive contribution of developmental brain malformation and post-developmental effects. The developmental effect is in part due to the malformation of the Mushroom Body (MB) (Bolduc et al., 2008), a large structure present in each side of the brain, which is

thought to be important for the formation of LTM. In the fly model of FXS, there is an abnormal fusion between the MB from each hemisphere (Bolduc et al., 2008).

Bolduc also showed that the memory defects seen in FX models such as the fly are due to excess protein synthesis taking place (Bolduc et al., 2008). Bolduc et al. (2008) applied potent protein synthesis inhibitors, Cyclohexamide and Puromycin and observed that when flies were given either one of the protein synthesis inhibitors it lead to a rescue of the learning and memory defects that were previously seen. This provided strong evidence that protein synthesis plays a critical role in memory formation (Bolduc et al., 2008).

1.5.2 Fragile X Courtship Memory Defect

Another line of investigation of memory in Fragile X has been with courtship. In order for Drosophila to reproduce they must undergo the ritual of courtship, which involved a series of behaviors (Hall, 1994). Research has demonstrated that d*fmr*1 mutants have a defect in courtship behavior (McBride et al., 2005; McBride et al., 2012). The mutants initiate normal numbers of courtship attempts, however are not able to maintain a courtship interest (Dockendorff et al., 2002).

In *Drosophila*, cognitive ability can be assessed utilizing the conditioned courtship associative memory paradigm. A male fly will display a semi-stereotyped set of courtship behaviors when paired with a female. These behaviors can be scored

and the percentage of time spent engaged in these courtship behaviors during a testing period is referred to as a courtship index (CI) (Siegel and Hall, 1979). If a male is paired with a previously mated female over the course of one hour, his courtship will decrease during the training period due to the female's aversive cues and rejection of his advances. This decrease in courtship during the training period is known as learning during training (LDT) (Joiner Ml and Griffith, 1997; Kane et al., 1997). Additionally, the male will continue to have lower courtship activity when subsequently paired with a virgin female, compared to males that are not paired with a previously mated female. This lower courtship activity is indicative of a memory of the training. An alternative version of this paradigm pairs the trained male with a novel previously mated female target after training (Siegel and Hall, 1979; Kamyshev et al., 1999; McBride et al., 2005). The comparison is then between the courtship index (CI) during the initial 10 minute period of training and the CI during the testing period (Kamyshev et al., 1999; McBride et al., 2005). Again a reduction in CI during the testing period is indicative of memory. Males can be tested immediately after training to assess immediate recall memory or 60 minutes after training to assess short-term memory. FX flies have impairments in immediate recall, short-term memory and long-term memory in the conditioned courtship paradigm (McBride et al., 2005; Banerjee et al., 2010; Choi et al., 2010).

1.6 Mechanisms of protein synthesis control

The AKT-mTOR pathway (Fig.3-1).

The synapse is the essential cellular unit of memory and is a site of communication between neurons; these connections are known to be "plastic"(Hoeffer and Klann, 2010). Synaptic plasticity can be defined over time, some alterations lasting only seconds whereas some last over the lifetime of the organism. The most durable forms of synaptic plasticity come about through biochemical processes via the expression of new proteins. The crucial role that protein synthesis plays in synaptic plasticity has been demonstrated numerous times through both pharmacological and genetic approaches (Huber et al, 2002; Tang and Schuman, 2002; Sutton and Schuman, 2006). Protein synthesis or translation is a highly regulated process that can be subdivided into three different steps: initiation, elongation and termination. Where the majority of regulation takes place is at the level of translation initiation.

Key to the regulation of translation initiation is the activity of a ubiquitously expressed kinase, mammalian target of rapamycin (mTOR). mTOR is a serine/threonine kinase that plays important roles in regulating functions of cellular proliferation and growth and is activated by a variety of different receptors (Hoeffer and Klann, 2010; Hoeffer et al., 2012). mTOR exists in two biochemically and functionally distinct multi-component complexes known as mTORC1 and mTORC2 (Kim et al., 2012). mTORC1 couples nutrient availability with hormonal and growth factor signals to regulate metabolism, cell growth and proliferation (Hoeffer and Klann, 2010). mTORC2 has been shown to function as an important regulator of the cytoskeleton through its stimulation of F-actin stress fibers (Hoeffer and Klann,

2010). mTORC1 is the complex that will be referred to from now on. In order for mTOR to function, upstream receptors such as mGluR or Insulin Receptor must first be activated. Activation of the receptors triggers the recruitment of phosphoinositide 3-kinases (PI3K) and activates the downstream target molecules including the serine/threonine kinase AKT (Sharma et al., 2010; Hoeffer and Klann, 2010). Active AKT phosphorylates and inactivates the tuberous sclerosis complex protein 2 (TSC2), leading to a loss of suppression of mTOR by the TSC1-TSC2 complex. This process in turn leads to an activated mTOR complex. Active mTOR regulates protein translation by activating the p70 ribosomal protein S6 kinase (p70S6K) and inhibiting the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Many different groups have looked at various targets of the AKT-mTOR pathway in Fragile X models however there has been variability in results obtained thus far (Table 1).

The RNAi pathway

microRNA (miRNA) and short interfering RNA (siRNA) have been shown to regulate protein synthesis by inhibiting the function of their target mRNAs through complimentary binding (Bredy et al., 2011). Previous research has shown that the d*fmr1* interacted with a major miRNA protein argonaute 1 (AGO1) in long-term memory (Bolduc et al., 2008). AGO1 along with Dicer and FMRP have all been closely associated with the miRNA pathway. Bredy et al. (2011) propose three mechanisms by which miRNA contribute to plasticity and memory. They suggest a) miRNAs might influence cognitive capacity by regulating dendrite morphogenesis during early development, b) miRNAs might fine-tune gene function by regulating

translation locally within synapses of individual dendrites or c) miRNAs might serve to constrain or destabilize memory upon retrieval in order to allow new learning or memory updating to occur.

1.6.1 Mechanisms of increased protein synthesis in FX

The AKT pathway is upregulated in FX

There are many pathways that are known to be involved with protein synthesis and its constant regulation. A key pathway is the AKT-mTOR pathway; this pathway has been shown to contain many important regulators of protein synthesis (Crowder and Freeman, 1998; Shioi et al., 2002; Sharma et al., 2010; Callan et al., 2012). Work done previously on the AKT-mTOR pathway analyzed numerous targets in a Fragile X mouse model, thought to be involved and found that AKT levels across the brain showed no difference between FX mouse models and WT models (Sharma et al., 2010). They also found that p-AKT levels were increased in the FX model when compared to the WT control (Sharma et al. 2010). These results on p-AKT support to the theory that excess protein synthesis; which has been demonstrated as a cause for the learning and memory problems associated with FX (Bolduc et al., 2008; Liu et al., 2011), is caused in part by dysregulation of the AKT-mTOR pathway. Another group was able to genetically remove p70 S6 Kinase 1, a downstream target within the AKT-mTOR pathway, from a Fragile X mouse model which then lead to correction of the synaptic and behavioral phenotypes seen in a FX mouse model, adding further

evidence that p70S6K plays a critical role in the molecular mechanisms of fragile x syndrome (Bhattacharya et al., 2012). There are conflicting results from different research sources and it is hard to agree on a consensus regarding the levels of different targets within the AKT pathway (Table 3-1).

Numerous signaling pathways have been shown to have an effect on learning and memory (Bolduc and Tully, 2009). Several of these signaling pathways that act upstream of the AKT pathway could be affected in FX. We will focus our experiments and discussion on 1) the mGlu receptor signaling, 2) the cAMP signaling and 3) the insulin signaling pathway.

The mGluR signaling in FX.

One important potential mechanism of disease in FX was proposed by Bear et al. (2004) who suggested that elevated signaling via the metabotropic glutamate receptor (mGluR), alters synaptic properties and dynamics in multiple neuronal circuits leading to the FXS behavioral deficits.

FMRP has been shown to control translational efficiency of dendritic mRNAs in response to stimulation of metabotropic glutamate receptors (mGluRs) (Sharma at el., 2010). mGluRs are G-protein-coupled receptors enriched at excitatory synapses throughout the brain in which they act to regulate glutamatergic neurotransmission (Bear et al., 2004; Sharma et al., 2010). FMRP in translational regulation acts in

response to the action of the neurotransmitter glutamate and certain metabotropic glutamate receptors, mGluR1 and mGluR5. Together these two receptors are known as the group 1 mGluRs and are coupled with the family of G protein a subunits. mGluRs have been implicated in synaptogenesis, spine morphogenesis, and activity-dependent synaptic plasticity (Huber et al., 2000; Illiff et al., 2013). Support for this hypothesis has emerged (Shoenfeld et al., 2013) however experimental evidence is still lacking to provide evidence on the consequences of increased mGluR activity in different neuronal circuits on a larger scale.

The cAMP pathway regulates protein synthesis

The cAMP-PKA pathway has been found to be abnormal in platelets and cells derived from patients with FX (Berry-Kravis and Sklena, 1993; Berry-Kravis et al., 1995; Berry-Kravis and Ciurlionis, 1998). The second messenger cAMP (cyclic adenosine monophosphate) is produced in response to a broad range of extracellular stimuli that act upon G-protein couple receptors. cAMP is synthesized by the action of adenylate cyclase (AC) and its degradation is mediated by the action of cAMP phosphodiesterases (PDEs) (Berry-Kravis and Huttenlocher, 1992). The majority of effects of cAMP are dependent upon the activation of its downstream effectors protein kinase A (PKA) and exchange protein directly activated by cAMP. There is a large amount of evidence that suggests there is a good deal of cross talk between the cAMP and mTOR pathways (Hoeffer and Klann, 2010). For example, cAMP can either stimulate or inhibit mTOR depending upon the cell type. This is most likely

due to activation or suppression of signaling transduction cascades upstream of mTOR such as the PKB, the MAPK (mitogen-activated protein kinase) and the AMPK (AMP-activated protein kinase) pathways (Schmelzle et al., 2004). PKA activates AMPK that in turn inhibits mTOR activity. This is accomplished through its inhibition of tsc1/2 protein that activate rheb that in turn activates mTOR. In addition, PKA can also directly inhibit mTOR activity. Previous work has demonstrated that cAMP inhibits mTOR via PKA. Additionally, increased concentrations of cAMP activity caused dissociation of the mTOR complexes leading to inhibition of mTOR catalytic activity (Hoeffer and Klann, 2010). mTOR activity has been previously shown to be increased in FXS mouse models (Sharma et al., 2010) suggesting decreased cAMP-PKA input. Earlier work has shown that cAMP levels are suppressed after synaptic stimulation in Fragile X shown in both animal models and patient derived cell lines (Berry-Kravis and Sklena, 1993; Berry-Kravis et al., 1995; Berry-Kravis and Ciurlionis, 1998; Berry-Kravis et al., 2007).

The Insulin Pathway regulates AKT

One of the initiators of the AKT pathway is the binding of a ligand such as Insulin Growth factors (IGF) to the Insulin Receptor. The insulin pathway is highly conserved across species, and it aids in the translation of nutritional status into neural stem cell behavior, which moderates neural tissue growth (Callan et al., 2012). Studies in Drosophila have characterized Insulin receptor/Phosphoinositide 3-kinase (Inr/PI3K) signaling as a potent regulator of cell growth (Potter et al., 2001; Junger et al., 2003; Hwangbo et al., 2003). Inhibiting Inr/PI3K signaling phenocopies the

cellular and organismal effects of starvation, whereas activating this pathway bypasses the nutritional requirement for cell growth, causing starvation sensitivity at the organismal level. Drosophila insulin-like peptides (Dilps) promote tissue growth through the single InR (Gao and Pan, 2001; Hwangbo et al., 2003; Callan et al., 2012). Binding of Insulin Growth Factors to the Insulin Receptor leads to a promotion of tissue growth through the activation of PI3K/AKT pathway (Gao and Pan, 2001; Callan et al., 2012). Research has demonstrated that FMRP plays a key role in the modulation of the Insulin pathway in a Drosophila model and that lack of FMRP can lead to negative outcomes for the organism (Callan et al., 2012).

1.7 Potential Pharmacological Treatments

One drug that appears to have potential to treat some of the impairments seen in FX is Metformin. It is the first-line drug of choice for the treatment of type 2 diabetes, in particular, in overweight and obese people and those with normal kidney function. First synthesized and found to reduce blood sugar in the 1920s, metformin was neglected for the next two decades as research shifted to insulin and other hypoglycemiant drugs. Interest in metformin was rekindled in the late 1940s after several reports that it could reduce blood sugar levels in people, and in 1957, French physician Jean Sterne published the first clinical trial of metformin as a treatment for diabetes (Kender et al., 2014; Koh et al., 2014). Metformin is thought to act on AMPK, which is downstream of the cAMP-PKA pathway. It has been shown to

directly increase AMPK activity, increasing PTEN expression and ultimately leading to the inhibition of mTOR activity.

First, one of the main targets of Metformin is AMPK, which is downstream of the cAMP-PKA pathway. G protein coupled receptor activation (including mGluR) leads to activation of adenylate cyclase which then leads to phosphorylation of PKA. PKA activates AMPK, which in turn inhibits mTOR activity. AMPK is highly conserved in evolution. This is accomplished through its inhibition on Tsc1/2 that activate rheb, which activates mTOR (Inoki et al., 2006; Inoki et al., 2002; Inoki et al., 2003; Li et al., 2002; Choo et al., 2006). Additionally PKA can directly inhibit mTOR activity (Xie et al., 2011). mTOR activity has been shown to be increased in FXS mouse model (Sharma et al., 2010), suggesting decreased cAMP- PKA input. Indeed, cAMP levels are suppressed after synaptic stimulation in fragile X as has been demonstrated in the mouse model and patient derived cell lines (Berry Kravis et al, 1992; Berry-Kravis et al., 1993; Berry-Kravis et al., 1998; McBride et al., 2005; Kelley et al., 2007; Kelley et al., 2008). Metformin has been shown to directly increase AMPK activity (Hecquet et al., 2002; Ciullo et al., 2001; Valverde et al., 1995), increasing PTEN expression and ultimately leading to the inhibition of mTOR activity (Kim and Choi et al., 2012; Zang et al., 2004; Walker et al., 2005; Zou et al., 2004).

As previously discussed, cAMP is seen to be lower in Fragile X Syndrome patients. Previous work in CBP mutant mice, model for Rubinstein Taybi has shown

that PDE inhibitors could be used to enhance cAMP signaling and rescue memory. An additional drug that has generated some interest for treating both STM and LTM deficits seen in FX patients is called Rolipram. Rolipram is a selective phosphodiesterase 4 inhibitor. It inhibits phosphodiesterase 4 or PDE4, particularly the PDE4B subtype. This should in theory enhance the activity of cAMP due to the inhibition of the enzyme that degrades cAMP, namely PDE-4.

1.8 Objectives

This thesis identifies pathways that are disrupted in Fragile X mutant flies and further investigates the potential role of protein synthesis control in relation to memory defects in Fragile X mutant flies. There are three aims that were set forth at the beginning of my graduate work. **1)** determine the molecular levels of different proteins involved in the AKT axis of the insulin pathway (chapter 2); **2)** assaying for impaired learning and memory capabilities of mutant flies with different expression levels of proteins within the AKT axis of the insulin pathway (chapter 3) ; **3)** investigate a pharmacological approach to rescue the fragile x phenotype based on drugs targeting the AKT axis of the insulin receptor pathway (chapter 5).

In Chapter 3, I characterize the proteins levels of multiple targets within the AKT-mTOR pathway, which is downstream of the Insulin receptor pathway. Targets within this pathway have been investigated in previous work and found to be dysregulated in FX mice (Sharma et al., 2010; Liu et al., 2012; Callan et al., 2012) but

not in a Drosophila model and usually without taking into account activity dependent plasticity. Further to this, I wanted to investigate the levels of these different targets after Drosophila have undergone LTM training. It has been observed previously that FX Drosophila models have impairments in LTM (McBride et al., 2005; Bolduc et al., 2008) however; the protein levels of targets within the AKT-mTOR pathway have not been investigated after training has taken place. This work may give us further insight into the reasons why FX perform poorly on protein synthesis dependent memory tasks but perform normally on protein synthesis independent memory tasks.

In Chapter 4, I studied the role of insulin pathway gene in classical olfactory learning and memory. This chapter's main objective was to target different proteins using the UAS-Gal4 system and observe the resulting learning and memory phenotypes. Beginning with FMRP, the protein responsible for Fragile X syndrome we look at the learning and memory phenotype produced when it is missing. From there we look at the resulting learning and memory phenotype when insulin receptor or the insulin receptor substrate is knocked down. Insulin has been shown to be misregulated in FX during development but not previously in adult flies, which may lead to effects on the AKT-mTOR pathway (Callan et al., 2012). To try and pinpoint where the deficiency in Insulin signaling results in a memory defect I used a tissue specific driver to knock down the translation of Insulin receptor and Insulin receptor substrate in the Mushroom Body, a critical structure for the formation of memories, of Drosophila. This chapter provided evidence that Insulin signaling is involved in

the learning and memory phenotype observed in FX therefore I wanted to investigate pharmacological agents that could rescue the behavior phenotype.

In the final experimental chapter, we demonstrate that acute pharmacological interventions targeting the Insulin pathway and the cyclic AMP pathway are able to rescue the acute memory function in Fragile X mutant flies. This section will include a description of the mechanisms by which these drugs perform their action along with the rationale as to why these particular agents were chosen.

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Chapter 2: MATERIALS AND METHODS

2.1 Fly stocks

We used established *Drosophila* mutant rescue lines dfmr1 Wild type rescue (WTR); dfmr1^{3}/ TM3, Ser, Sb. These flies have a deletion of the d*fmr* gene but also have a full-length wild-type genomic fragment including flanking regions of the d*fmr1* gene. The null mutant line dfmr1^{3}/ TM3, Ser, Sb was extensively characterized and used previously to show olfactory memory defect previously in Bolduc et al. 2008.

2.2 Western blot analysis.

In brief, heads of WTR; fmr1^{3} and *fmr1^{3}* were separated from their respective bodies and protein was extracted with a protein extraction sample buffer. Enough heads were collected so that each lane on the gel contained sample from 10 heads. Only female heads were gathered to avoid sex related differences in neural structure acting as a confounding variable. Samples were placed in -80 degrees Celsius until needed. Western blot was started by adding 5x sample buffer containing 5% β-mercaptoethanol to the heads where they were then boiled at 95^{oC} for 5 minutes and then spun in a centrifuge at 15, 000 rpm for an additional 5 minutes. Aliquots of protein were subjected to SDS-PAGE (10%) at 200V over 45 minutes. Once that was complete the gel was transferred to a nitrocellulose membrane at 95V over 1 hour. When transfer was complete the membrane was put in blocking solution of PBS and

Licor (50:50) solution, for 2 hours. Then the membrane was processed for incubation with primary antibodies (1:1000) contained in a Licor:PBT solution (50:50) for 2 hours. Membrane was put through a series of washes, 4 rounds of 5 minutes in PBT. This was followed by fluorescent secondary antibodies (1:3000) contained in a Licor:PBT solution (50:50) for 45 minutes. Membranes were washed 4 times for duration of 5 minutes per wash and imaged using LiCor Odyssey scanner. Band intensities were measured using ImageJ. Statistical analysis was performed with Graphpad Prism software.

2.3 Antibodies

Primary antibodies: rabbit anti-p70S6K (1:1000) was obtained from Developmental Hybridoma, mouse anti-AKT (1:1000) (pan) and mouse anti-p-AKT Ser^{505} (1:1000) were obtained from Cell Signalling, rabbit anti-GAPDH (1:3000) was a gift from Dr. Luc Berthiaume (Department of Cell Biology, University of Alberta). Secondary antibodies were Alexa Fluor® 680 goat anti-rabbit (Cell Signaling) and Alexa Fluor® 750 goat anti-mouse (Cell Signaling).

2.4 UAS-GAL4 System.

In order to create mutants for genes we needed to either over-express genes or decrease their translation by "knocking them down". These can both be done using the UAS-GAL4 driver system. GAL-4 drivers can be cell specific (i.e. the Mushroom Body alone) or pan-neuronal. UAS system is placed at the site of the

target gene for either over-expression or knockdown. The GAL-4 driver then binds to the UAS. In over-expression the gene is targeted and more RNA for that gene is created leading to increased protein synthesis. In knockdown or RNAi systems the UAS is set to target a gene then when the GAL-4 driver binds it, resulting in dsRNA and the formation of hairpin loops which the cell takes as irregular and then targets them for breakdown. This leads to the decreased translation of this gene and in turn decreased protein synthesis.

2.5 Fly Stocks, Virgin Female Collection and Fly Sorting

Fly stocks were maintained at 22^{o}C on standard cornmeal agar medium. Fly stocks were flipped onto fresh medium on a weekly basis in order to maintain healthy stocks and ensure a standardized age range of the flies. When preparing for and performing a genetic cross for experimentation, virgin females of a particular genotype are collected and allowed to mate with males of a different genotype, thereby producing a F1 of interest. The selection of virgin females is crucial in order to avoid allelic combinations resulting from sexual interactions between unintended and undesired genotypes. This procedure is based on the observed behavior that female flies will generally not be sexually receptive to a male mate until 10-12 hours after the time they emerge from the pupa as a fully developed adult fly (Ashburner and Roote, 2007). Virgin females can be identified by their lack of male sexual characteristics, the stripe pattern on the backside of their abdomen, their overall larger

size, as well as by features such as unexpanded wings and lack of pigmentation (Ashburner and Roote, 2007).

For sorting, flies are first transferred to a hard plastic funnel pre-charged with carbon dioxide (CO_2). After approximately 10 seconds, the flies are transferred to a porous CO_2 pad and viewed under a dissecting light microscope. Manipulations of flies are performed using a round-tipped paintbrush, while the flies are kept lightly anesthetized by enough CO_2 to keep the flies from moving. Flies are sorted using phenotypic markers and subsequently transferred to small plastic vials containing the same fly media used for stock maintenance and sealed by a ball of cotton.

Fly stocks for Fmr RNAi (2-1) and (1-7) were generated previously by Bolduc et al (2008). Other RNAi lines were obtained from the Vienna Drosophila RNAi center (VDRC). The annotation corresponds to the accession number. Wild type, OK 107 Gal4 and Elav Gal4 were obtained from Dr. Tim Tully (Cold Spring Harbor Laboratory). *dfmr*1^{B55} was obtained from Dr. Kendal Broadie (Vanderbilt University). *Fmr*1^{3} and dmfr1 WTR;Fmr1^{3} were obtained from Dr. Tom Jongens (U Penn). Both *dfmr*1^{3} mutants are null and carried over the balancer TM3, Ser, Sb. Homozygous flies can survive but reproduce at very low rate. All stocks were brought to the same genetic background by outcrossing to wild type for 6 generations as previously described (Tully and Quinn, 1985).

2.6 Pavlovian olfactory learning and memory

Drosophila were raised at 22°C and placed at 25°C overnight prior to behavioral experiments. Adult *Drosophila* 1-3 days old were trained and tested with the classical conditioning procedure of Tully and Quinn. About 100 flies were trapped inside a training chamber, covered with an electrifiable copper grid. Flies were allowed 90 seconds to acclimate and then were exposed sequentially to two odors, 3-octanol (OCT) and 4- methylcyclohexanol (MCH), carried through the chamber in a current of air (750mL/min; relative concentrations of OCT and MCH were adjusted so that naïve flies distributed themselves 50:50 in the T-maze). Flies were first exposed for 60 seconds to the conditioned stimulus (CS+; either OCT or MCH), during which time they received the unconditioned stimulus (US; twelve 1.25 seconds pulses of 60V DC electric shock at 5 second interpulse intervals). After the CS+ presentation, the chamber was flushed with fresh air for 45 seconds. Then flies were exposed for 60 seconds to a second, control stimulus (CS-; either MCH or OCT depending on the odor the flies were shocked to in the first step), which was not paired with electric shock. After the CS- presentation, the chamber was again flushed with fresh air for 45 seconds. To test for conditioned odor avoidance after classical conditioning, flies were tapped gently from the training chamber into an elevator-like compartment that transports them to the choice point of the T-maze. Ninety seconds later, the flies were exposed to two converging current of air one carrying OCT, the other MCH, from opposite arms of the T-maze. Flies were allowed to choose between the CS+ and CS- for 120 seconds, at which time they were trapped inside their respective arms of the T-maze (by sliding the elevator out of register), anesthetized

and counted. In order to test the learning abilities of flies they are tested immediately after training whereas in LTM they are tested a day later. To form a stronger memory, repeated training is required. Previous groups have shown that LTM forms when flies are trained 10 times with 15-minute rest periods between each training (Bolduc et al., 2008). This style of training is called Space Training otherwise known as protein synthesis-dependent memory. There is another form of memory training where flies are trained 10 times without any rest periods between training sessions, this is called Mass training or protein synthesis independent memory.

Two groups of flies were trained and tested in one complete experiment. The CS+ was OCT and the CS- was MCH for one group; the CS+ was MCH and the CS- was OCT for the second group. The performance index (PI) was calculated as the average of the fraction of the population avoiding the shock-associated odor minus the fraction avoiding the control odor for each group of flies trained in one experiment. In other words, the PI enumerates the distribution of flies in the T-maze as a normalized "percent correctly avoiding the shock-paired odor" and ranges from 0 for a 50:50 distribution to 100 for a 100:0 distribution. The average PI for learning for WT flies should be around 80 whereas the average PI for Space training is 40 for WT flies and Mass training should give a score around 20 for WT flies.

2.7 Task-relevant sensori-motor responses

Briefly, odor avoidance at the concentrations used for the conditioning experiments was quantified in mutant and control flies. Naïve flies were placed in the T-maze and given a choice between an odor (OCT or MCH) and air. The odor is

naturally aversive, and flies usually avoided the T-maze arm containing the odor (OCT or MCH) by running into the opposite arm (air). After the flies distributed themselves for 2 minutes, they were trapped, anesthetized and counted. For shock reactivity, flies were given a choice between an electrified grid in one T-maze arm and an unconnected grid in the other. After the flies distributed themselves for 2 minutes, they were trapped, anesthetized and counted.

2.8 Statistical Analysis

Data from an experiment were subjected to a one-way ANOVA (JMP from SAS, Inc.), followed by planned pairwise comparisons as indicated in text and figure legend. An alpha = 0.05 was corrected for multiple comparisons using Bonferroni. Post-test analysis was performed with Tukey test. Comparisons with one, two or three asterisks indicate significances of $P < 0.05$, 0.01 and 0.001, respectively. All graphs depict mean +/- s.e.m.

2.9 Drug Administration

Flies are starved in empty plastic bottles for 4 hours on day 1. Drugs are prepared in solution and a 5% sucrose solution is used as the vehicle. Metformin (Sigma) is prepped to a 1mM concentration, while Rolipram (Sigma) is prepared at a 50μM concentration. After the 4-hour starvation period, flies are placed into vials, approximately 100 flies/vial. At the bottom of the vial there is a small filter paper with 250μL of either the drug solution or vehicle solution absorbed into the filter paper. Approximately 100 flies are then left overnight to consume either the drug or

vehicle solution. On day 2, flies are either trained using the Space or Mass training protocol or tested on their learning abilities as described earlier. In addition they are put through the sensory assays to test their ability to sense the odor and foot shock. Results are inputted into Jump to calculate the PI.

2.10 Genetic Crosses

For all experiments, we use the same paradigm where each genetically appropriate controls are raised in parallel in the same environment as the target genotype. All flies are therefore raised at the same temperature and humidity, for the same time and within the same room. The WT control line is known as *2U-Cold Spring Harbor Laboratory (CSHL)—w^{1118}* outcrossed 10 times with *Canton-S* (Kuo et al., 2012).

Pan-neuronal FMRP knockdown (using Bolduc FMRP RNAi 1-7)

2U-CSHL

2U-CSHL (males) crossed with Elav Gal4 (females)

2U-CSHL (females) crossed with FMR $RNAi^{1-7}$ (males)

Elav Gal4 (females) crossed with FMR $RNAi^{1-7}$ (males)

Pan-neuronal FMRP knockdown (using Bolduc FMRP RNAi 2-1)

2U-CSHL

2U-CSHL (males) crossed with Elav Gal4 (females)

2U-CSHL (females) crossed with FMR RNAi$^{2\text{-}1}$ (males)

Elav Gal4 (females) crossed with FMR RNAi$^{2\text{-}1}$ (males)

Pan-neuronal Insulin receptor substrate knockdown (using VDRC Insulin Receptor Substrate RNAi 7777)

2U-CSHL

2U-CSHL (males) crossed with Elav Gal4 (females)

2U-CSHL (females) crossed with Chico RNAi^{7777} (males)

Elav Gal4 (females) crossed with Chico RNAi^{7777} (males)

Pan-neuronal Insulin receptor substrate knockdown (using VDRC Insulin Receptor Substrate RNAi 7776)

2U-CSHL

2U-CSHL (males) crossed with Elav Gal4 (females)

2U-CSHL (females) crossed with Chico RNAi^{7776} (males)

Elav (females) crossed with Chico RNAi^{7776} (males)

Pan-neuronal Insulin receptor knockdown (using VDRC Insulin-Like Receptor RNAi 992)

2U-CSHL

2U-CSHL (males) crossed with Elav Gal4 (females)

2U-CSHL (females) crossed with Insulin-like receptor RNAi^{992}(males)

Elav (females) crossed with Insulin-like receptor RNAi^{992} (males)

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Chapter 3. KEY TARGETS WITHIN THE AKT PATHWAY ARE MISREGULATED IN FRAGILE X SYNDROME

3.1 Introduction

It has been demonstrated in previous work that excess protein synthesis is a causal factor leading to learning and memory impairments in Fragile X syndrome (McBride et al., 2005; Bolduc et al., 2008). As outlined in Chapter 1, the AKT pathway is a critical modulator of protein synthesis (Whiteman et al., 2002). This has led it to be a popular target of research in Fragile X Syndrome. Research has demonstrated that it is impaired in FX, leading to defects in Fragile X model organisms (Sharma et al., 2010; Bhattacharya et al., 2012). It is seen that targets within the AKT-mTOR pathway are mysregulated in FX but these targets have never been investigated after memory training has taken place. It is thought that this mysregulation may be a leading cause to the defects seen after memory training has taken place in a Drosophila FX model.

3.2 Results

Our first aim was to look at the level of expression of various members of the AKT pathway. The AKT pathway has been shown by previous work to be dysregulated in Fragile X mice and human cell lines (Hoeffer et al., 2012; Liu et al., 2012; Sharma et al., 2010; Callan et al., 2012). However, the degree of dysregulation

has been controversial with no consensus between different groups in the field (Table 2-1).

3.2.1 Verification of the FMRP level in dfmr1 mutants used in this study

FMRP is involved in multiple aspects of RNA metabolism, leading to tight regulation of protein synthesis (Till, 2010; Till et al., 2010). FMRP is predicted to specifically bind approximately 4% of the mRNA's found in the brain, many of which code for proteins that are involved in neuronal maturation and plasticity (Brown et al., 2001; Darnell et al., 2001). As previous research has demonstrated, in a FX Drosophila model there is an absence of FMRP (Bolduc et al., 2008). Through western blot analysis we were able to confirm that the models of FX are protein null for FMRP (Fig.3-2). This lack of FMRP very likely leads to the behavioral phenotypes that have been seen in Fragile X syndrome.

3.2.2 Increased AKT activity in Drosophila dmfr1 mutants

Activation of the AKT pathway is reflected by increased level of the phosphorylated form of AKT in comparison to the total level of AKT. This was used previously by Sharma et al. (2010) to show hyperactivity of the AKT pathway in the mouse model of Fragile X. One of the first targets was one of the phosphorylated forms of AKT, p-AKT^{Ser505}, which is homologous to an active form of AKT in mice and humans, p-AKT^{Ser473}. It can be seen that similar results to previous research have been obtained (Fig.2-3A).

I observe higher levels of p-AKT within the heads of *fmr1*^{3} flies compared to their genetic controls containing a genomic rescue fragment for dfmr1 WTR; dfmr1^{3} (Fig.3-3B, C). In contrast, there was no significant difference in the AKT levels between *fmr1*^{3} and their genetic controls containing a genomic rescue fragment for dfmr1 WTR; dfmr1^{3} (Fig.2-3D). This mirrors previous findings in mouse hippocampus and cortex (Sharma et al., 2010). I examined if the defect in memory observed in Drosophila mutant for dfmr1 was related to aberrant levels of p-AKT. Interestingly, we observed no significant difference between *dfmr1*^{3} mutants and their genetic controls in level of p-AKT after spaced or massed training (Fig.3-3B,C). This was unexpected due to the fact that excess protein synthesis has been implicated in the memory defects of FX (Bolduc et al., 2008). It was expected that there would be higher levels of p-AKT in FX mutants after spaced training had taken place. I extracted proteins from trained flies at 24 hours, which is the time where flies would be tested for 1-day memory. It is possible that there is an earlier difference between groups that was not tested for here. Another possibility is that the memory defect is due to the lack of change from untrained to trained. To follow up on this point I examined the levels of AKT after space or mass training have taken place. Interestingly there was no significant difference between *fmr1*^{3} and their genetic controls containing a genomic rescue fragment for dfmr1, WTR; dfmr1^{3} even after LTM training has taken place (Fig.3-3D).

3.2.3 Increased P70 S6K activity in Fragile X mutants.

In addition to the phosphorylated forms of AKT, p-AKT^{Ser505}, which is homologous to an active form of AKT in mice and humans, p-AKT^{Ser473}, we also investigated p70S6K. Earlier work examined this target within the AKT-mTOR pathway and found in most cases there to be no differences between FX and WT (Sharma et al., 2010; Osterweil et al., 2010; Hoeffer et al., 2012).

In contrast, our results show significantly higher levels of p70S6K within the heads of *fmr1*^{3} flies when compared to their genetic controls containing a genomic rescue fragment for dfmr1, WTR; dfmr1^{3} (Fig.3-4B). I investigated further if there were differences between controls and fragile X mutant flies after training. I observed a significantly increased level of p70S6K in *dfmr1*^{3} mutant brain after spaced training but no significant difference in flies trained with massed training (Fig.3-4B). Again, as in the AKT experiments, proteins were extracted at 24 hours after training, which is the time point where flies would usually be tested for their memory. These findings mirror what have been previously demonstrated by Bolduc et al., 2008 in olfactory classical conditioning. Indeed, memory after spaced training (and not massed training) is selectively impaired in drosophila mutant for Fragile X gene.

3.3 Discussion

3.3.1 dFmr1 is disrupted in Fragile X Drosophila melanogaster model.

Fragile X is caused by the lack of FMRP being present. FMRP has been implicated in many processes such as synaptic plasticity as well as learning and memory defects observed in FXS. The models of FX that we used over the course of my work are lacking FMRP, and therefore provide an excellent model to study Fragile X Syndrome. (Fig.3-2).

3.3.2 AKT-mTOR pathway is misregulated in Fragile X Syndrome

Fragile X syndrome is the most common heritable form of mental retardation and a leading genetic cause of autism (Hagerman and Hagerman, 2013). A hallmark characteristic of *Fmr1* knockout mice is the overactivation of group I mGluR signaling. Activation of this group I mGluRs promote mTOR signaling (Hou and Klann, 2004) and drives mTOR-dependent protein synthesis (Sharma et al., 2010). Components of the mTOR signaling cascade are present at synapses and have been shown to mediate synaptic plasticity via protein synthesis (Huber et al., 2000; Sharma et al., 2010; Illiff et al., 2013). Others have demonstrated that mTOR signaling is overactivated in the hippocampus of FX mice, by assessing levels of different kinase proteins, such as AKT and p-AKT, along with the phosphorylation of mTOR downstream targets such as S6K (Sharma et al., 2010). In addition to the mTOR

pathway feeding down into AKT, Insulin pathway dysregulation leads to enhanced p-AKT levels in Drosophila larval brains (Callan et al., 2012). *Drosophila melanogaster* is an effective model organism for interrogating Fragile X Syndrome, particularly concerning the learning and memory phenotypes seen in FX patients (Bolduc et al., 2008). Memory encoding and storage occurs via carefully regulated interactions of neuronal networks of the nervous system. It has been previously demonstrated that there are different forms of Long-Term Memory (LTM). There is an LTM that is considered protein synthesis dependent memory (space training), due to the fact it requires protein synthesis to take place in order for a stronger memory to be formed (Goodwin et al., 1997; Yin et al., 1995; Bolduc et al., 2008). Our work shows that AKT-mTOR signaling is over-activated in the cortex of FX Drosophila by western blot analysis (Fig. 3-3).

3.3.3 AKT levels are similar in WT and FX Drosophila melanogaster brain protein samples while p-AKT levels are highers within FX compared to WT.

FX results in impairments in protein synthesis-dependent form of memory, with no discernable effects on protein synthesis independent memory (Bolduc et al., 2008). We know of no previous research investigating different targets within the AKT-mTOR pathway after these different forms of memory training have been able to take place. Data regarding different targets in the AKT axis of the Insulin Pathway has been collected. One of the first targets looked at was one of the phosphorylated forms of AKT, p-AKT^{Ser505}, which is homologous to an active form of AKT in mice

and humans, p-AKT^{Ser473}. AKT contains two phosphorylation sites that lead to it performing its action, Ser ^{473} and Thr^{308} (Bolukbasi et al., 2012; Moon and Chung, 2013; Wang et al., 2014). This could result in the second band that appears when western blot analysis is performed on the Drosophila (Fig. 3-3A). It can be seen that similar results to previous research have been obtained (Sharma et al., 2010). Results have shown higher levels of p-AKT within the heads of untrained *fmr1*^{3} flies and no significant difference in the AKT levels between mutant and wild-type untrained flies (Fig3-3). This is consistent with the theory that over activation of AKT-mTOR pathway is a result of Fragile X Syndrome.

3.3.4 AKT protein levels do not change after long-term memory training while p-AKT levels are sensitive to memory training procedures.

Further investigation was required and the AKT level differences in FXS and WT flies that had undergone training for LTM testing were looked at (Fig.3-3). As far as we know, looking at the levels of these different targets within the AKT-mTOR pathway after LTM training has taken place has never been explored before. Unphosphorylated AKT remained the same between WT and FX flies and did not change following training (Fig.3-3). This suggests that any differences seen in other targets of the pathway are due to the effects of the phosphorylated form, p-AKT. However, following memory training that the p-AKT levels of FXS flies return to normal levels that do not differ significantly from levels found in WT flies (Fig.3-3). This means that in WT flies the p-AKT levels have increased after training while in

FX flies the p-AKT levels decrease after training compared to untrained FX flies. This could demonstrate that signaling pathways adjacent to the AKT-mTOR pathway may add to the excess protein synthesis taking place in FX, particularly after memory training has taken place.

3.3.5 Protein levels of p70S6K are elevated in FX Drosophila melanogaster.

A common target to look at in order to assess levels of protein synthesis is p70S6K. This target is downstream of many initiators of protein synthesis (Fig.3-1), which therefore makes it a logical target to assess activation of protein synthesis pathways. p70S6K is a serine/threonine kinase that acts downstream of PIP3 and phosphoinositide-dependent kinase-1 in the PI3 kinase pathway (Bhattacharya et al., 2012). mTOR is also in a pathway downstream of AKT. AKT is typically activated upon stimulation of a cell with a growth factor (such as IGF-1). AKT then activates mTOR (by inhibiting the Tsc complex), leading to p70S6K activation. Previous research has looked at p70S6K in a Fragile X mouse model, and found no variation in the levels of p70S6K in FX when compared to WT controls (Sharma et al., 2010; Osterweil et al., 2010; Hoeffer et al., 2012). In FX flies, the learning and memory defects seen were due to the excess protein synthesis taking place. A potent protein synthesis inhibitor, cyclohexamide, was given to FX flies prior to learning and memory tests. This resulted in a significant improvement in learning as well as protein synthesis dependent memory (Bolduc et al., 2008). This suggests that excess protein synthesis causes the learning and memory defects seen in Fragile X. When

investigating the levels of p70S6K in untrained WT and FX flies to see if excess protein synthesis was taking place, I found a significant increase in the amount of p70S6K in FX UT flies when compared to WT flies (Fig.3-4). This result is contradictory to previous findings (Table 3-1) however this finding does suggest that there is excess protein synthesis taking place in the Fragile X model and provides possible treatments of this syndrome.

3.3.6 Levels of p70S6K remain elevated after Space Training but appear normal after Mass Training.

Due to the fact that FX flies perform poorly in protein synthesis dependent memory tasks but perform normally on protein synthesis independent memory tasks we investigated the levels of p70S6K were in both of these conditions. After spaced training there were significantly higher levels of p70S6K in FX flies when compared to WT flies (Fig.3-4), suggesting that excess protein synthesis is the cause for the defect in the memory scores. Interestingly, after massed training there was no difference between FX and WT flies (Fig.3-4). This supports the theory that in protein independent memory formation, there is no difference in the levels of p70S6K between FX and WT flies.

3.3.7 Future Plans

These results demonstrate that key targets within the AKT pathway are misregulated in Fragile X Syndrome. In the future experiments I would like to determine why there is an excess of p-AKT in untrained flies however once memory

training takes place there is no difference between Fragile X and Wild-type flies. This could be done by extracting proteins immediately after both spaced and massed training have taken place instead of waiting 24 hours where protein synthesis could be occurring. In addition I would like to administer a protein synthesis inhibitor to the flies before training, train them on spaced and massed protocols then extract protein from them. This could demonstrate if protein synthesis is in fact taking place in massed trained flies since those levels should not change even after protein synthesis inhibitor has been applied. In addition I would like to utilize confocal microscopy to determine if there are localization differences between Wild-type and Fragile X flies when looking at the targets AKT, p-AKT and p70S6K that may lead to the memory defects seen in Fragile X Syndrome. Finally I would like to obtain antibodies for Insulin Receptor and Insulin Receptor Substrate (Chico) to observe if there are differences in those protein levels in Fragile X compared to Wild-type.

Figure 3-1

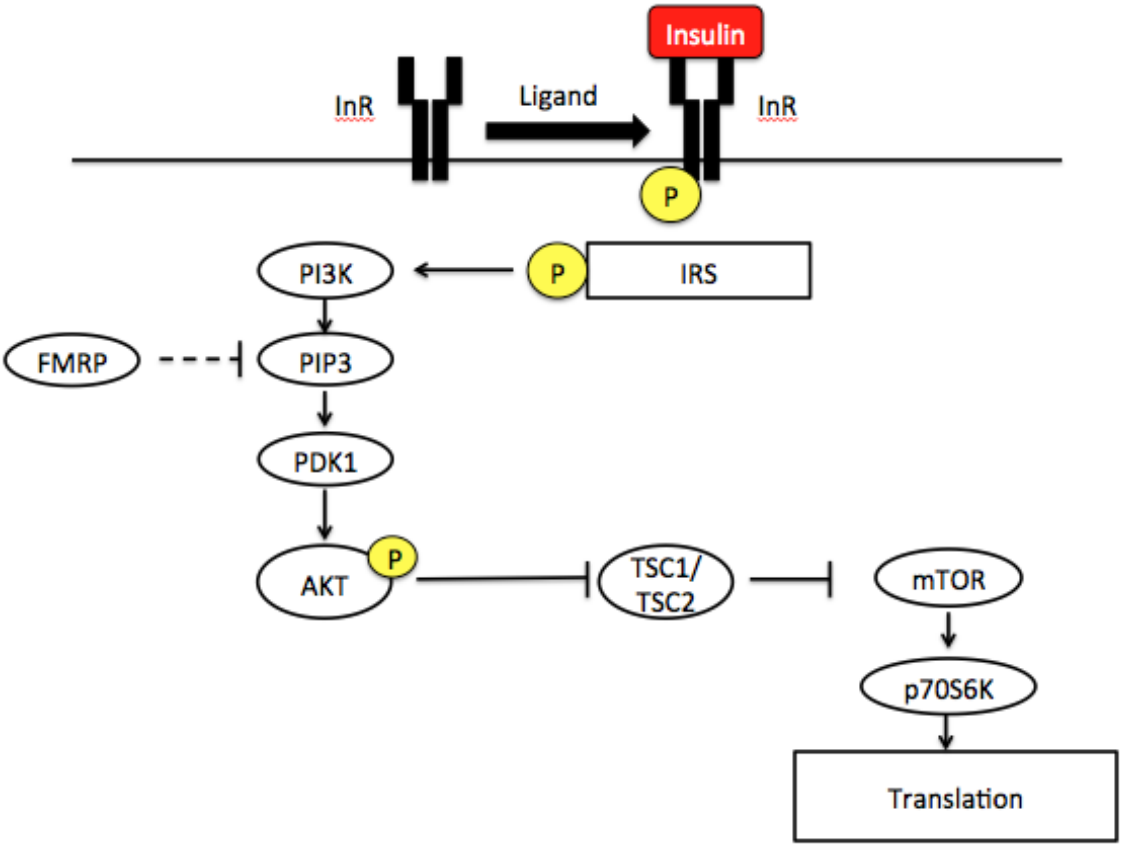
Insulin
InR
Ligand
InR
P
PI3K
P
IRS
FMRP
PIP3
PDK1
AKT
P
TSC1/
TSC2
mTOR
p70S6K
Translation

Figure 3-1. Schematic representation of the AKT axis of the Insulin receptor (InR) signaling pathway. Binding of insulin or Insulin-like growth factor (IG-F) to the insulin receptor (InR) leads to its phosphorylation and downstream phosphorylation of the insulin receptor substrate (IRS). IRS in turn activates the phosphoinositol-3-kinase (PI3K)/AKT pathway. The activation of AKT leads to global increase in protein synthesis.

Table 3-1

Author:	Liu, Huang and Smith, 2012	Hoeffer/ Tessone et al, 2012	Min/Yuskatis/ Jope et al, 2009	Callan/Zarnescu et al, 2012	Sharma/Zukin et al, 2010	Osterweil/Bear et al, 2010
Method/ Protein:	WB: Adult Mouse Hippocampus	WB: Human Brain Sections	WB: Adult Mouse Brains (tested Striatum, Cortex, Cerebellum, Hippocampus)	IF: Drosophila Larval Brains (fmr1 RNAi in Glia or Neurons)	WB: Mouse Hippocampus and Cortex (4-6 weeks)	WB: Cultured Mouse Hippocampus slices
p-Akt (S473)	UP	UP	ND	UP 12-18h ALH	UP	Normal
p-Akt (T308)	ND	ND	ND	ND	UP	ND
Akt	Normal	Normal	ND	ND	Normal	Normal
p70s6k	Normal	Normal	ND	ND	Normal	Down

Table 3-1. Results obtained by previous groups looking at the AKT-mTOR pathway protein levels in Fragile X model.

Figure 3-2

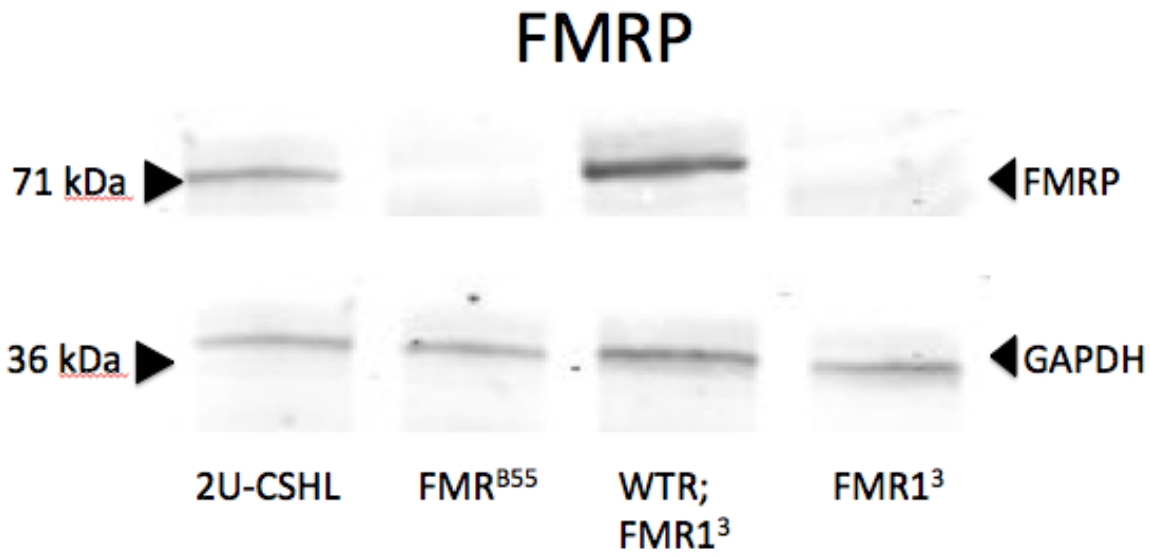

Figure 3-2. Neurogenetic characterizations of disruptions of *Fmr1*. A) Western Blot analysis from adult heads of wild-type (2U-CSHL, WTR; $FMR1^{3}$) flies and $Fmr1^{3}$ [FMR1(3)] or $Fmr1^{B55}$ (FMR1B55) homozygous mutants. Using an anti-dFMRP 5A11 primary antibody, no FMRP was discernable in $Fmr1^{3}$ or $Fmr1^{B55}$, compared to that in wild-type flies (dark upper band). N=6

Figure 3-3

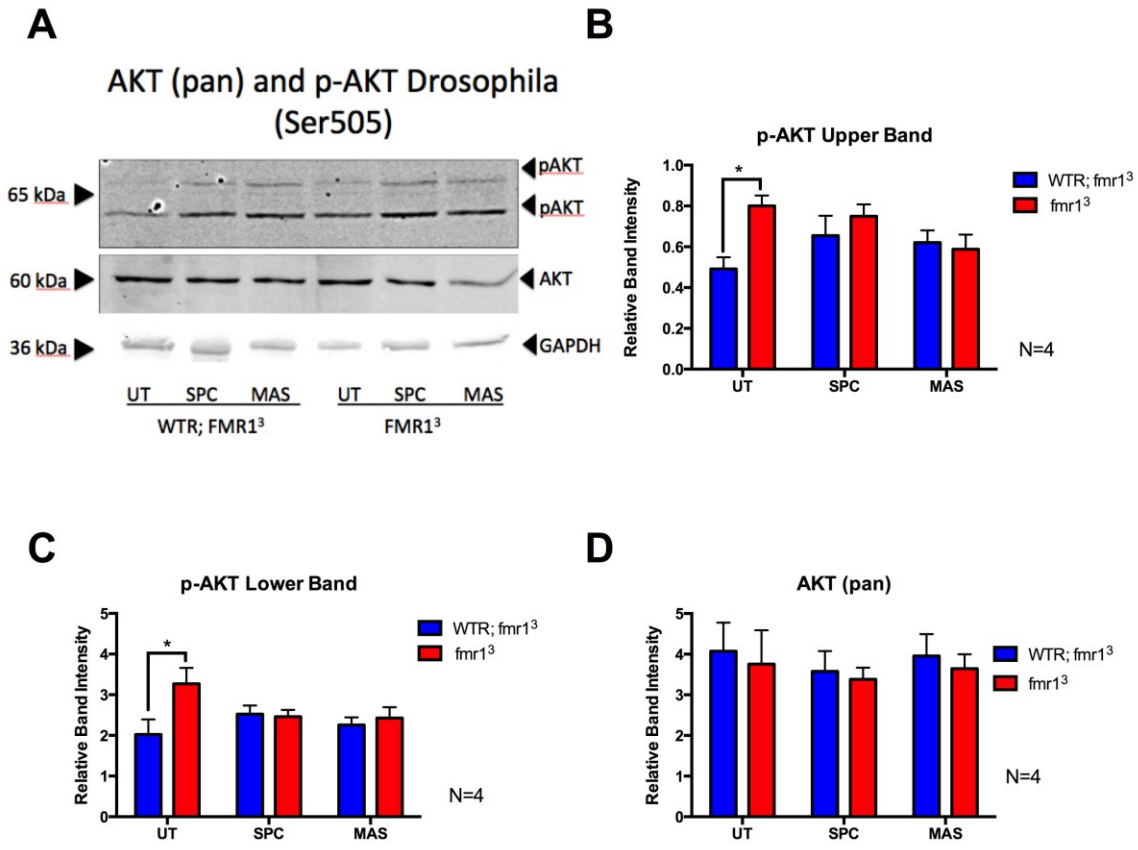
A
AKT (pan) and p-AKT Drosophila (Ser505)
65 kDa
60 kDa
36 kDa
pAKT
pAKT
AKT
GAPDH
UT SPC MAS UT SPC MAS
WTR; FMR1³
FMR1³
B
p-AKT Upper Band
Relative Band Intensity
WTR; fmr1³
fmr1³
N=4
UT
SPC
MAS
C
p-AKT Lower Band
Relative Band Intensity
WTR; fmr1³
fmr1³
N=4
UT
SPC
MAS
D
AKT (pan)
Relative Band Intensity
WTR; fmr1³
fmr1³
N=4
UT
SPC
MAS

Figure 3-3. AKT phosphorylation is enhanced in the brains of untrained fragile x Drosophila, but returns to normal levels after memory training. Representative Western blots (A) showing relative abundance of p-AKT (C+D) and total AKT (B) in lysates from whole Drosophila brains. 1-3 days old flies' mutant for dfmr1, $fmr1^{3}$, along with their genetic controls containing a genomic rescue fragment for *dfmr*1, WTR; $dfmr1^{3}$. Western blots were probed with antibodies to p-Ser505-AKT and total AKT. Phosphorylation of AKT is basally enhanced in UT flies but not SPC or MAS trained flies. (two-way ANOVA, Upper band *p=0.0140, Lower band *p=0.0167) N=4.

Figure 3-4

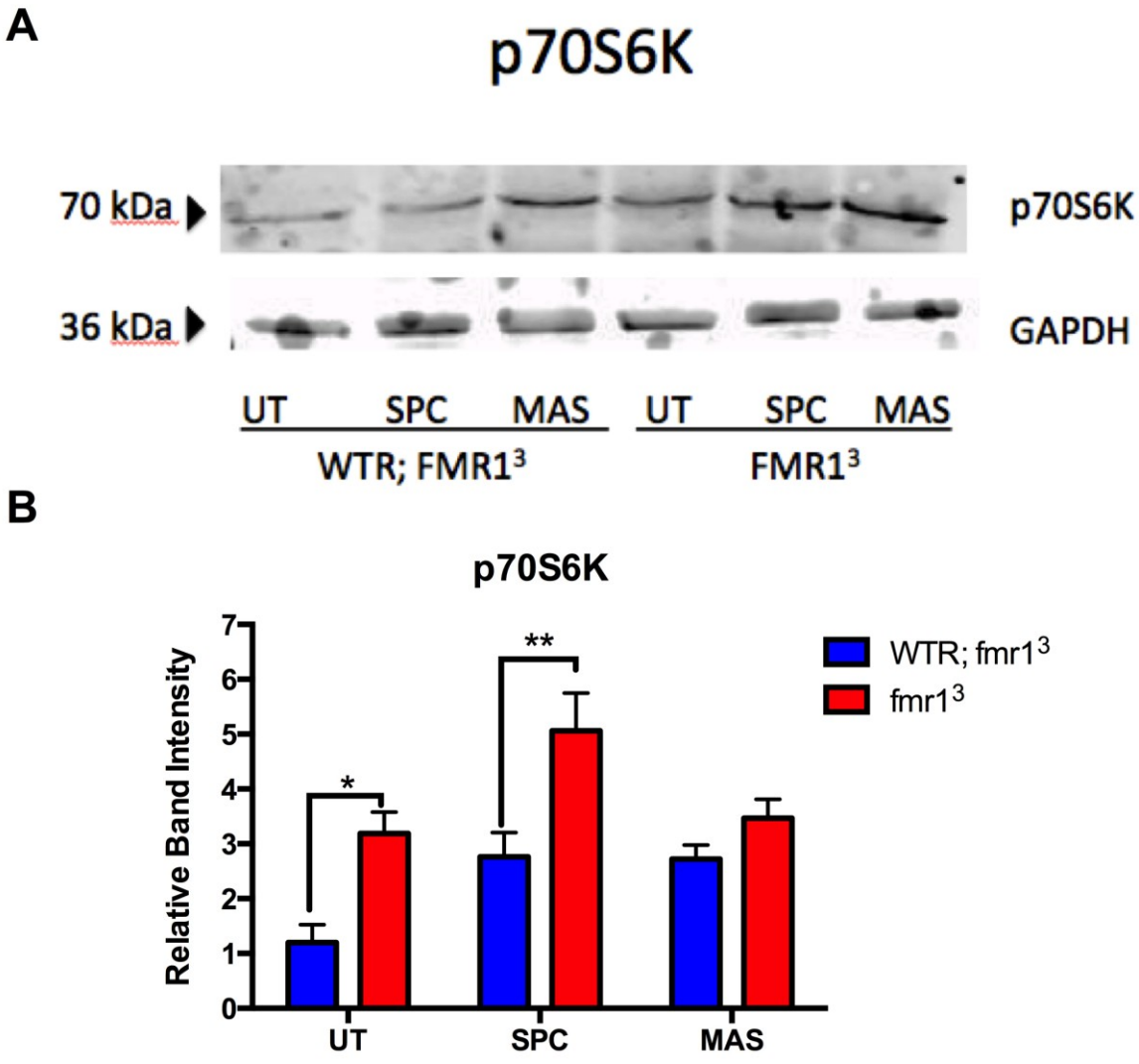
A
p70S6K
70 kDa
p70S6K
36 kDa
GAPDH
UT
SPC
MAS
UT
SPC
MAS
WTR; FMR1³
FMR1³
B
p70S6K
Relative Band Intensity
0
1
2
3
4
5
6
7
*
**
UT
SPC
MAS
WTR; fmr1³
fmr1³

Figure 3-4. p70S6K level is enhanced in the cortex of Fragile X Drosophila. Representative Western blots showing relative abundance of p70S6K in lysates of whole brain Drosophila samples. Western blots were probed with an antibody to p70S6K. Levels of p70S6K are enhanced in UT and SPC trained flies but there is no difference between MAS trained flies. (two-way ANOVA, *p=0.0129, **p=0.0042) N=4.

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Chapter 4. INSULIN PATHWAY SIGNALING IS REQUIRED FOR LEARNING AND MEMORY IN DROSOPHILA

4.1 Introduction

In the previous chapter, protein levels of different targets within the AKT-mTOR axis of the Insulin receptor pathway in a Fragile X model were evaluated. Enhanced levels of activating targets within this pathway appear to be prominent features of this syndrome. These proteins possess functions that regulate protein synthesis. As a result their enhanced signaling may contribute to the behavioral phenotype of impaired learning and memory abilities. We know of no published research investigating the resulting learning and memory phenotype when the activator of this pathway, the Insulin receptor, is unable to perform its function.

One of the initiators of the AKT pathway is the binding of a ligand to the Insulin Receptor. The insulin pathway is highly conserved across species, and it aids in the translation of nutritional status into neural stem cell behavior, which moderates neural tissue growth (Callan et al., 2012). Studies in Drosophila have characterized Insulin receptor/Phosphoinositide 3-kinase (Inr/PI3K) signaling as a potent regulator of cell growth. As was discussed in Chapter 1, the Insulin Pathway plays a critical role in regulation of protein synthesis, making it an ideal target for investigation.

In this set of experiments, the effects of misregulation of AKT-mTOR axis of the Insulin receptor pathway were examined. Drosophila that had expression of

initiators of this pathway knocked down, were generated using the UAS-Gal4 transgenic system and then assessed for learning and memory abilities through olfactory classical conditioning. This novel research examines the role that Insulin signaling plays in learning and memory in Drosophila. We also examined the role that Insulin signaling plays in specific structures of the Drosophila brain, namely the Mushroom Body. Inhibition of either the Insulin receptor or the Insulin receptor substrate in all Drosophila neural cells leads to learning and memory impairment. However Inhibition of either of these targets in the MB results in no learning and memory impairment. This narrows down the potential location where Insulin signaling is crucial for memory formation, and supports the concept that Insulin signaling dysregulation plays a prominent role in Fragile X syndrome.

4.2 Results

The purpose of this set of experiments was to assess the memory defect present in mutants of the AKT-mTOR axis of the Insulin Receptor pathway. We reproduced results previously published by Bolduc et al. (2008), where Drosophila mutants for the homologue of Fragile X had a defect selectively in learning and memory after spaced training. Using similar mutants, the *dfmr1* ^{B55} mutant was generated by imprecise excision of a p-element (Inoue et al., 2002). The d*fmr1*^{3} mutant was also generated by imprecise excision of a P-element (Dockendorff et al., 2002). Please see Figure 4-1.

The first RNAi construct was synthesized from published sequence previously shown to work in Schneider2 cells. It covers base pair 569 to 1069 of Fmr1. The first

segment contained 500 bp corresponding to the beginning of the *Fmr1* coding region; the second contained a spacer region consisted of the second exon of *GFP*; the third segment consisted of the reverse-complement sequence from the first segment. This synthesized sequence (Retrogen) then was cloned into a "pBIMBO" vector. Transgenic lines were established by BestGene, using w^{1118} *(isoCJ1)* as the parental stock. A second construct was designed after the Heidelberg RNAi probe ID# BKN27935, targeted region corresponding to base pair 670 to 950 of the Fmr1 gene. Transgenic flies were generated as above.

4.2.1 Drosophila mutant for dfmr1 have defective learning and long-term memory.

We have replicated findings of FX flies having defects in learning and memory as can be seen by figure 4-2. Both of the models for FX have significantly decreased learning capabilities compared to their genetic controls (Fig.4-2A). The long-term memory at 1 day (LTM) after spaced training is impaired in FXS flies compared to WT (Fig.4-2B) however the LTM after massed training is not impaired and there is no significant difference between FXS and WT flies (Fig.4-2C). Both the fmr^{B55} and $fmr1^{3}$ along with their genetic controls performed normally on sensory assays that test their abilities to smell the odors presented during training (Fig.4-3B). Moreover both mutants and their genetic controls performed normally in shock avoidance assay (Fig.4-3A). This demonstrates that the impaired learning and memory scores are not due to any sensory impairment.

4.2.2 Drosophila expressing RNAi against dfmr1 have defective learning and long-term memory.

Next, we used previously published transgenic flies with UAS- FMR RNAi (Bolduc et al., 2008). These flies showed defects in learning and memory after mushroom body expression (Bolduc et al., 2008). I therefore tested the effect of pan-neuronal expression to see what impact pan-neuronal knockdown of FMRP would have on learning and memory. I found behavioral scores similar to those in flies after selective mushroom body expression suggesting that the mushroom bodies play a key role in altering this behavior. Alternatively, the mushroom bodies could be the final step of processing in relation to fragile X. Therefore, this experiment does not rule out the possibility of other structures being involved. Similar results were observed with pan neuronal expression (using Elav GAL4) of the 2 transgenic lines (Fig.4-4, Fig.4-6).

The learning abilities of RNAiFMR$^{2\text{-}1}$ flies are impaired and significantly lower than WT and control flies (Fig. 4-4A). Based on the results these flies have impairments in protein synthesis dependent memory tasks (Fig. 4-4B) but perform similar to WT flies when tested on protein synthesis independent memory (Fig.4-4C). The RNAi knockdown of FMRP leads to the same learning and memory phenotype seen in FX mutant flies. The RNAiFMR$^{2\text{-}1}$ along with their genetic controls performed normally on sensory assays that test their abilities to smell odors presented during training and feel foot-shocks (Fig.4-5). Therefore, the impaired learning and memory scores are not due to any sensory impairment.

Using a second transgenic RNAi line I wanted to confirm the validity of the RNAi phenotype so I repeated the same experiments. FMR^{1-7} confirmed previous findings. The learning abilities of these RNAi flies are impaired and significantly lower than WT and control flies (Fig. 4-6A). Based on the results these flies have impairments in protein synthesis dependent memory tasks (Fig. 4-6B) but perform similar to WT flies when tested on protein synthesis independent memory (Fig.4-6C). This confirms that RNAi knockdown of FMRP lead to the same learning and memory phenotype seen in FX mutant flies. The $RNAiFMR^{1-7}$ along with the genetic controls performed normally on sensory assays that test their abilities to smell odors presented during training and feel foot-shocks (Fig.4-7). This demonstrated that using the UAS-Gal4 system can be used to replicate a Fragile X learning and memory phenotype when FMRP is the target of RNA interference.

4.2.3 Drosophila Insulin-like receptor is required in learning and memory

Earlier work has shown that hyperactive AKT signaling is seen in Fragile X mice models (Sharma et al., 2010). The AKT pathway hyperactivity may be related to hyperactivity of the insulin-signaling pathway (Callan et al., 2012). One of the key components of the pathway is the Insulin Receptor. The Drosophila homolog to the Insulin Receptor is called the Insulin-like receptor, performing the same function as the Insulin receptor in a mammalian model.

To investigate the role that the Insulin-like Receptor plays in learning and memory we utilized the UAS-Gal4 system (Brand and Perrimon, 1993). We made use of the UAS-GAL4 system to knockdown the translation and therefore overall expression of the Insulin-Like Receptor (IR). Using a pan-neuronal driver, Elav-GAL4, we targeted the translation of Insulin-Like Receptor proteins by using RNA interference (RNAi) flies 992. This knocks down the expression of Insulin Receptor, preventing its ability to perform its function. We found that these RNAi flies are impaired and score significantly lower than WT and control flies in immediate recall (Fig.4-8A). Based on the results these flies have impairments in protein synthesis dependent forms of memory (Fig.4-8B) but perform similar to WT flies when tested for protein synthesis independent form of memory (Fig.4-8C). Insulin-Like Receptor $RNAi^{992}$ as well as their genetic controls present with normal shock avoidance and avoidance to odors (OCT or MCH) used in the classical conditioning experiments (Fig.4-9).

4.2.4 Drosophila insulin receptor substrate is required for learning and memory.

Next we tested the role of the Insulin Receptor Substrate (IRS), known as Chico in Drosophila. Chico has been shown to play a role in longevity (Clancy et al., 2001) but has not been previously involved in learning and memory, making it a very interesting target of investigation. This set of experiments could potentially narrow down the mechanism by which enhanced protein synthesis is seen in Fragile X syndrome.

Using the UAS-GAL4 system yielded a knockdown of the translation and therefore overall expression of the Insulin Receptor Substrate (IRS, Chico). Using a pan-neuronal driver, Elav-GAL4, we targeted the translation of Chico proteins by using RNA interference (RNAi) flies 7777 and 7776. This should specifically knock down Chico throughout all neurons in the Drosophila nervous system, thereby inhibiting its function. The learning abilities of these RNAi flies are impaired and significantly lower than WT and each of the control group flies (Fig3-10A). Further investigation demonstrated that ChicoRNAi^{7777} flies have a defect in protein synthesis dependent memory (Fig.4-10B) but perform similar to WT flies when tested on protein synthesis independent memory (Fig.4-10C).

Chico RNAi^{7777} as well as their genetic controls present with normal shock avoidance and avoidance to avoidance to odors (OCT or MCH) used in the classical conditioning experiments (Figs.4-11A, 4-11B). We repeated these experiments using a second transgenic RNAi line to ensure the validity of our findings. Therefore the same experiments were repeated using ChicoRNAi^{7776} yielding similar results (Fig.4-12, Fig.4-13).

This provides the first evidence that the Insulin receptor substrate (Chico) is required in learning and memory in a *Drosophila melanogaster* model. Furthermore, the learning and memory phenotype is not due to any tactile or olfactory impairment.

4.2.5 Drosophila insulin receptor substrate is not required in the mushroom bodies for normal learning and memory.

To our knowledge, the tissue specific expression of both Insulin-like receptor and Chico has not been investigated in a learning and memory fashion. The Mushroom Body in Drosophila is known to play a role in olfactory learning and memory (Bolduc et al., 2008), making it a logical target to observe the role that Insulin signaling plays in it. There was no significant effect on learning or memory by expressing the transgenic RNAi lines against insulin receptor or insulin receptor substrate in mushroom bodies neurons (Fig.4-14). This is in contrast to the previously demonstrated role of FMRP in mushroom bodies and suggests a more complex neuronal circuit.

4.3 Discussion

4.3.1 Fragile X Drosophila melanogaster have impaired learning and memory capabilities.

As was demonstrated by previous research groups, FX flies have decreased abilities in both Learning and Memory (McBride et al., 2005; Bolduc et al., 2008). Earlier work attributed this defect in Learning and Memory to excess protein synthesis taking place due to the lack of FMRP (Bolduc et al., 2008). When FX flies were given a protein synthesis inhibitor this lead to a rescuing of the learning and memory defect (Bolduc et al., 2008). We have replicated these findings in two separate Drosophila models of FX, $fmr1^{B55}$ and $fmr1^{3}$, as can be seen by figure 4-2.

The protein synthesis dependent form of memory after space training is impaired in FMR^{B55} flies compared to WT as well as it was impaired in *fmr1*^{3} when comparing those to their genetic control containing a genomic rescue fragment for *dfmr1*, WTR; d*fmr1*^{3}, however the protein synthesis independent memory after mass training is not impaired and there is no significant difference between FXS and WT flies (Fig.4-2). The decrease in Performance Index score is not due to the FX flies having any sort of sensory impairment. All groups as well as their genetic controls exhibit normal shock avoidance and avoidance to odors (OCT or MCH) used in the classical conditioning experiments. This provides evidence that FMRP is required for the proper formation of memories, whether they are short-term memories or long-term. This lack of FMRP leads to excess protein synthesis taking place which causes the memory impairments in FX models (Bolduc et al., 2008; Banerjee et al., 2010).

4.3.2 Pan-neuronal knockdown of FMRP in Drosophila melanogaster produces a learning and memory phenotype similar to Fragile X flies.

To produce the same memory impairment phenotype as FX using a different methodology we used the UAS-GAL4 system to knockdown the translation and therefore overall expression of FMRP. Using a pan-neuronal driver, Elav-GAL4, we targeted the translation of FMRP by using RNA interference (RNAi) flies $FMR^{2\text{-}1}$ and for confirmation we generated a second line, $FMR^{1\text{-}7}$. This should knock down the expression of FMRP mimicking the FX mutant flies. The learning abilities of these RNAi flies are impaired and significantly lower than WT and control flies (Fig.4-4A).

Based on our findings the FMR^{2-1} flies have impairments in protein synthesis dependent memory (Fig.4-4B) but perform similar to WT flies when tested for protein synthesis independent memory (Fig.4-4C). FMR^{2-1} as well as their genetic controls demonstrate normal shock avoidance and avoidance to odors (OCT or MCH) used in the classical conditioning experiments (Fig.4-5). This demonstrates that RNAi knockdown of FMRP leads to the same learning and memory phenotype seen in FX mutant flies (Bolduc et al., 2008). To enhance the validity of my results we repeated the same experiments utilizing a different line of RNAi flies. FMR^{1-7} are targeted to knockdown FMRP, similar to FMR^{2-1}. This yielded similar results, impairment in the learning and memory capabilities (Fig.4-5). There were additionally no impairments seen in the sensory control tests (Fig.4-6), leading to the conclusion that the learning and memory defects seen are not due to a sensory impairment in feeling the foot-shock or smelling the different odors. This provides strong evidence that FMRP regulation on processes such as protein synthesis (Krueger and Bear, 2011), are crucial for the proper formation of memory. Furthermore, a lack of FMRP leads to a dysregulation of numerous different pathways.

4.3.3 Pan-neuronal knockdown of the Insulin-like receptor leads to impairments in learning and memory in Drosophila melanogaster.

One of the activators of the AKT pathway is the binding of Insulin-like peptides to the Insulin Receptor of Drosophila. Studies in Drosophila have

characterized Insulin receptor/Phosphoinositide 3-kinase (Inr/PI3K) signaling as a potent regulator of cell growth (Callan et al., 2012). Inhibiting Inr/PI3K signaling phenocopies the cellular and organismal effects of starvation, whereas activating this pathway bypasses the nutritional requirement for cell growth, causing starvation sensitivity at the organismal level. Drosophila insulin-like peptides (Dilps) promote tissue growth through the single Insulin-like Receptor. Binding of this ligand to the Insulin Receptor leads to a promotion of tissue growth through the activation of PI3K/AKT pathway (Callan et al., 2012). FMRP plays a key role in the modulation of the Insulin pathway in a Drosophila model. The lack of FMRP can lead to negative outcomes for the organism (Callan et al., 2012). The downstream targets of the Insulin Receptor are targets within the AKT-mTOR pathway, such as AKT, p-AKT^{Ser473} and p70S6K. These targets in the AKT-mTOR pathway are dysregulated in FX (Sharma et al., 2010, Bhattacharya et al., 2012). To our knowledge, the role that the Insulin-like receptor plays in learning and memory has not been explored. Utilizing the UAS-Gal4 system, we knocked down the Insulin-like Receptor at a pan-neuronal level within the Drosophila nervous system. Knocking down the expression of, Elav>Insulin-like receptor $RNAi^{992}$, led to learning impairments (Fig.4-8A). We then investigated if this was purely a short-term memory defect or if long-term memory was impacted as well. The Elav>Insulin-like receptor $RNAi^{992}$ flies have impairments in memory after space training has taken place (Fig.4-8B) but perform similar to control group flies when tested after mass training (Fig.4-8C). This supports the theory that the Insulin pathway plays an important role in protein synthesis and memory formation. When FMRP is reduced there is an over activation

of downstream targets of Insulin receptors which leads to cellular defects as well as behavior defects in learning and memory (Callan et al., 2012). Now there is evidence that an absence of Insulin signaling also leads to behavioral defects. Therefore, a balancing act must occur, whereby a range between no signaling and too much signaling results in the proper functionality of the organism.

4.3.4 Pan-neuronal knockdown of Insulin receptor substrate (Chico) leads to learning and memory impairment in Drosophila melanogaster.

Due to the fact that the receptor is an upstream target, we targeted downstream, looking at other targets to see if we could pinpoint the critical step in this pathway for memory formation. Again using the UAS-Gal4 system we targeted the Insulin Receptor Substrate, which in the fly model is known as Chico. We crossed a pan-neuronal driver with an RNAi responder for Chico which resulted in, Elav>Chico RNAi^{7777}, to knockdown the translation of the RNA encoding for Chico in Drosophila. The first form of memory we looked at was immediate recall memory, referred to as learning, and we found an interesting result. The lack of Chico within the Drosophila model resulted in a learning impairment when comparing the mutant to the genetic control groups (Fig.4-10A). To see if this phenomenon was only a short-term memory issue or possibly a long-term memory issue as well we put the mutant flies along with their genetic controls through Space training as well as Mass training. After these experiments we saw that the Elav>Chico RNAi^{7777} flies have impairments after space training (Fig.4-10B) but perform similar to control group

flies when tested after mass training (Fig.4-10C). This demonstrates that even when the Insulin receptor is intact there can be defects observed when the Insulin Receptor Substrate aka Chico does not perform properly. The impairment does not appear to be very severe as compared to Fragile X flies (Fig. 4-2). This could be a result of other pathways such as mGluR (Bear et al., 2004) or the cAMP pathway (Berry-Kravis et al., 1998) in addition to the Insulin Signaling pathways are disrupted in Fragile X and the additive effects of all these disrupted pathways leads to a more severe impairment. The dysregulation within the Insulin pathway/AKT-mTOR pathway may take place further downstream from the Receptor at a place such as the Receptor substrate. We further confirmed this finding by using another Chico RNAi line, Chico RNAi^{7776}. The first form of memory we looked at again was immediate recall memory, referred to as learning, and we found a very similar result. We found that the lack of Chico within the Drosophila model resulted in a learning impairment when comparing the mutant to the genetic control groups (Fig.4-12A). We needed to see if this impairment was exclusively a short-term memory, i.e., learning, issue or if it carried over to LTM as well. After the same experiments as previously described we saw that the Elav>Chico RNAi^{7776} flies have impairments in protein synthesis dependent memory (Fig.4-12B) but perform similar to control group flies when tested for protein synthesis independent memory (Fig.4-12C). This provides even stronger evidence that the Insulin pathway dysregulation seen in FX models (Callan et al., 2012) is making a large contribution to the learning and memory defects seen in Fragile X Syndrome.

4.3.5 Mushroom Body specific knockdown of Insulin-like receptor and Insulin Receptor Substrate results in no impairment of Learning or Memory in Drosophila melanogaster.

We had determined that a pan-neuronal knockdown of Insulin-like Receptor or Chico leads to an impairment in learning and memory. We wished to determine where in the brain the impairments in learning and memory originated. To do this we utilized the UAS-Gal4 system with a tissue specific driver, OK107-Gal4. This driver is specific to the Mushroom Body in the Drosophila brain. The developmental effect seen in FX is in part due to the malformation of the Mushroom Body (MB) (Bolduc et al., 2008), a large structure present in each side of the brain, which is thought to be important for the formation of LTM. In the fly model of FXS, there is an abnormal fusion between the MB from each hemisphere (Bolduc et al., 2008). We set up an RNAi group for Insulin-like Receptor, OK107>Insulin-Like Receptor RNAi^{992}, and an additional group for Chico RNAi in the MB, OK107>Chico RNAi^{7777}. The proper genetic controls for these two groups were included as well (Chico RNAi^{7777}/+ and Insulin-Like Receptor RNAi^{992}/+). Learning showed no impairment when either Insulin-like receptor or Chico is knocked down in the Mushroom body of Drosophila (Fig.3-14A). In addition, LTM of these flies was not different between the mutants and the control groups after both spaced and mass training (Fig.3-14B, C). This demonstrates that Insulin-like receptor and Chico are not causing defects in memory within the mushroom body but their target of action are somewhere else in the nervous system of Drosophila.

4.3.6 Future Plans

These results demonstrate that Insulin signaling is required for Learning and Memory. In the future I would like to make use of the UAS-Gal4 system to try and further narrow down where in the Drosophila brain the Insulin Signaling is required. I could use Feb170-Gal4, which is a driver specific for the Central Complex in Drosophila, to drive RNAi against Chico and Insulin-like Receptor to determine if that structure is where is the learning and memory defect is localized. Continuing with the UAS-Gal4 system I would like to see if there is an acute effect of RNAi against Chico and Insulin-like Receptors. Using Gal80 which is a driver that is activated by high temperatures I can look at the effect of knocking down these two targets in adulthood as compared to them being knocked down during development. This will demonstrate whether or not these two targets are required during development for proper learning and memory function. Finally I also would like to use non-RNAi methods to confirm the findings I have obtained here. Generation of mutants for both Chico and Insulin-like Receptor would be required but this would add additional strength to the observation that Chico and Insulin-like signaling are required for proper learning and memory formation. Crossing the mutants for Chico and Insulin-like Receptor with a Fragile X fly could result in a rescue of the learning and memory defect seen, this would demonstrate that over activation of Insulin signaling is a cause of the learning and memory defect.

Figure 4-1

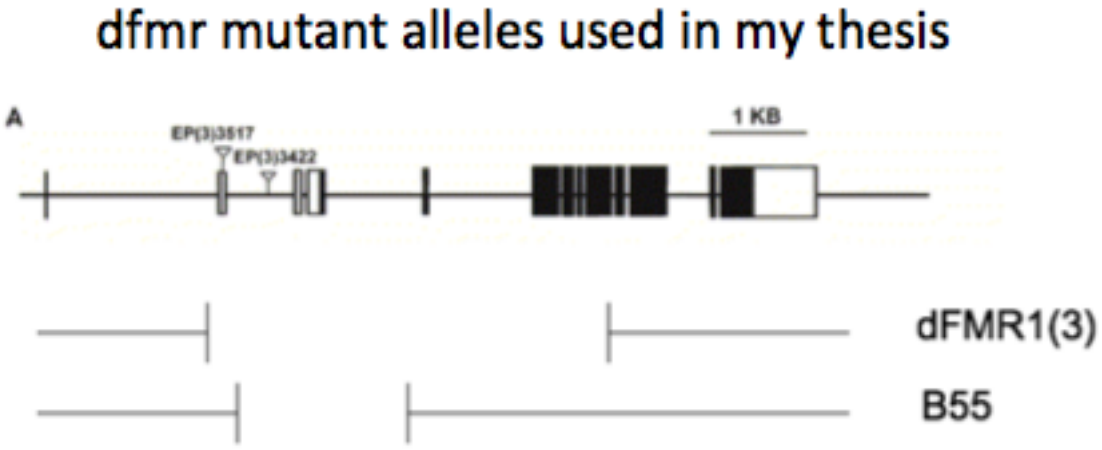

dfmr mutant alleles used in my thesis
A
EP(3)3517
EP(3)3422
1 KB
dFMR1(3)
B55

Figure 4-1. Characterization of the *dfmr1* Deletion Mutants d*fmr1*^{3} and d*fmr*^{B55}. The deleted genomic region of the d*fmr1*^{3} and d*fmr*^{B55} chromosome is shown. The d*fmr1* locus is positioned in a small region on the cytological location 85F11-12, on the right arm of the third chromosome. There is no other gene that overlaps in the d*fmr1* locus.

Figure 4-2

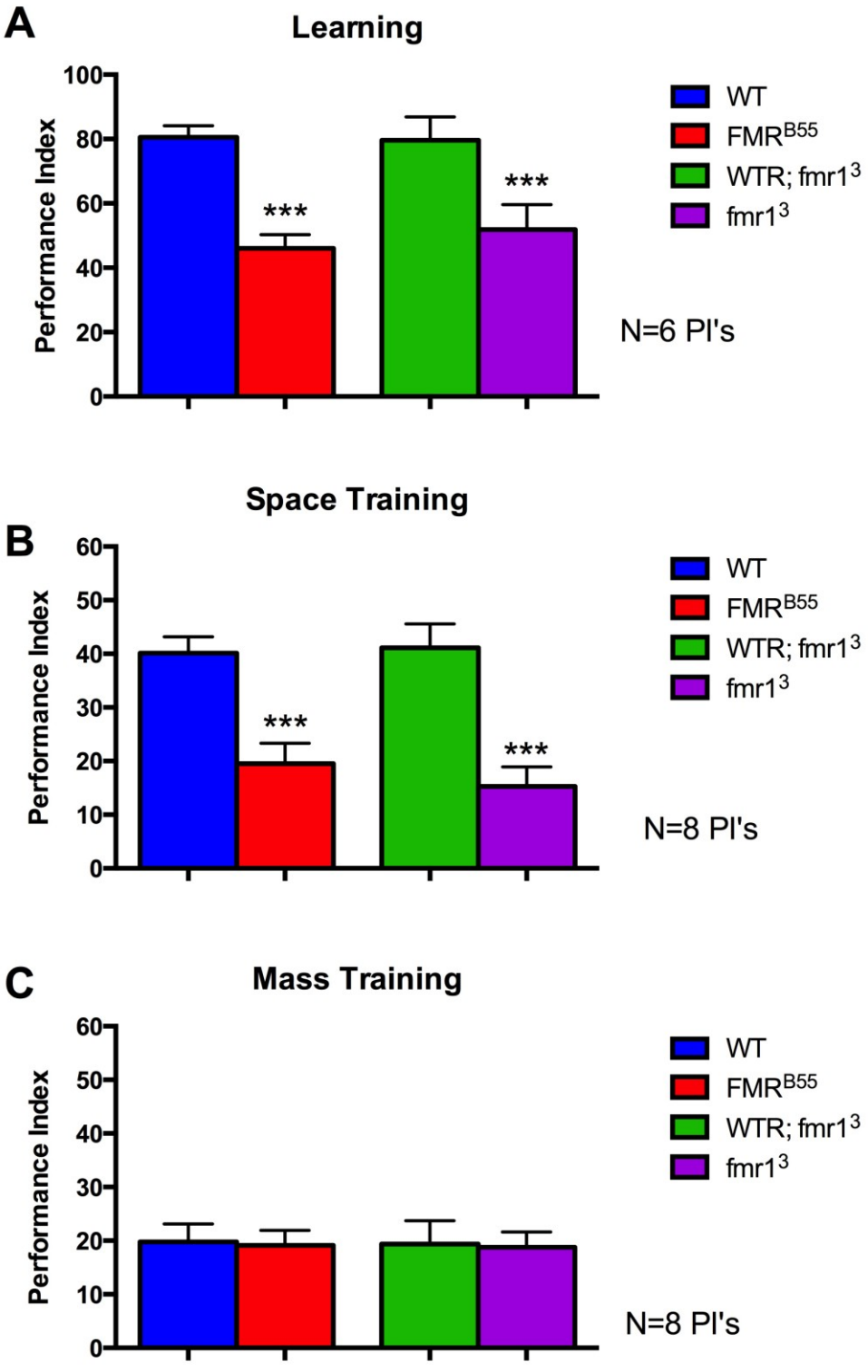
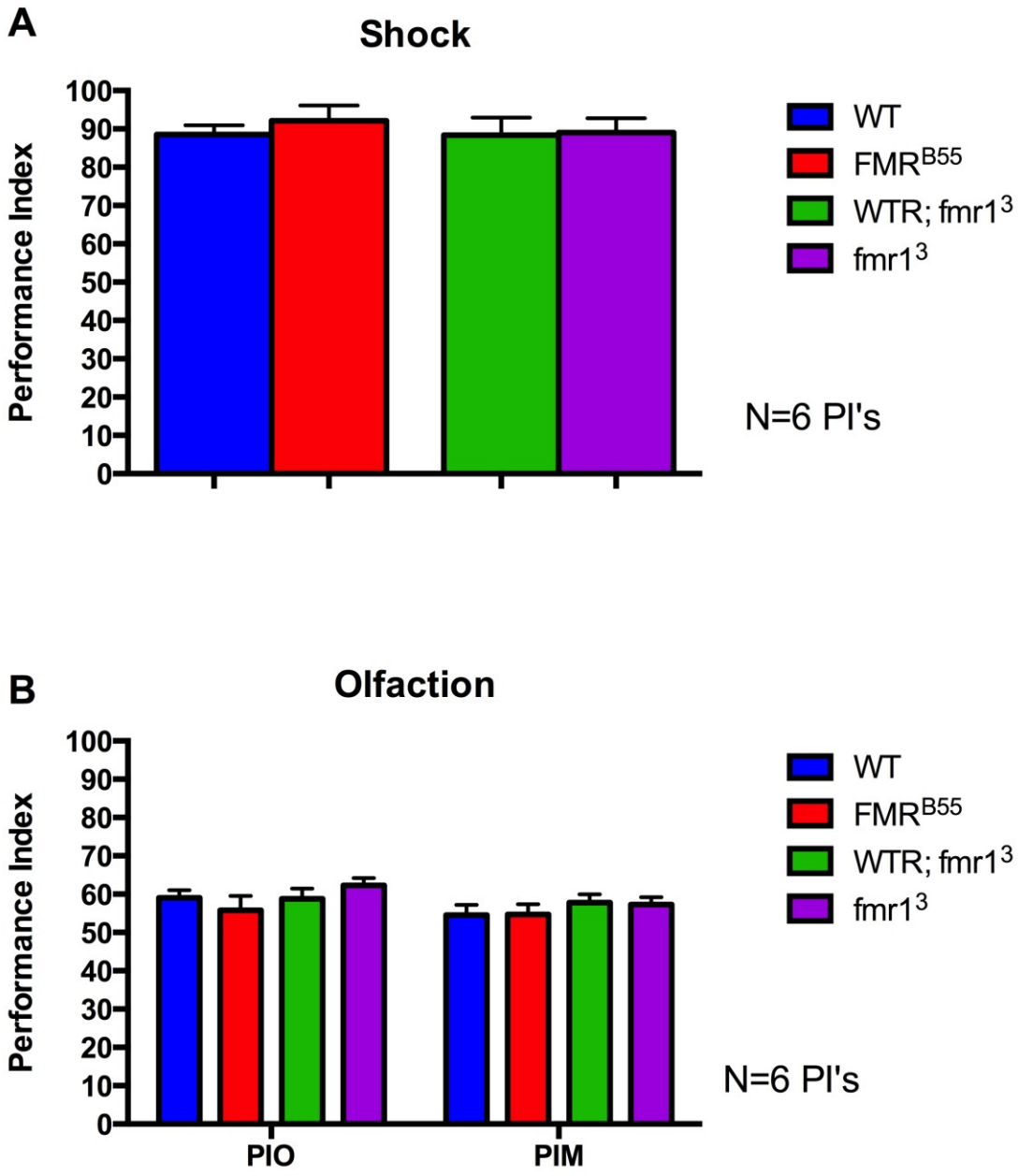

A
Learning
Performance Index
100
80
60
40
20
0
***
***
WT
FMR^B55
WTR; fmr1^3
fmr1^3
N=6 PI's
B
Space Training
Performance Index
60
50
40
30
20
10
0
***
***
WT
FMR^B55
WTR; fmr1^3
fmr1^3
N=8 PI's
C
Mass Training
Performance Index
60
50
40
30
20
10
0
WT
FMR^B55
WTR; fmr1^3
fmr1^3
N=8 PI's

Figure 4-2. Absence of Drosophila FMRP leads to defects in Learning and Long-Term Memory. A) Immediate olfactory conditioning memory, named learning, was significantly lower in Drosophila mutants for dfmr1 (FMR^{B55} and $fmr1^{3}$). No effect was observed in their genetic controls (WT) containing a genomic rescue fragment for dfmr1 (WTR; $dfmr1^{3}$). (One-way ANOVA, Tukey's post hoc test) FMR^{B55} p=0.0002, $Fmr1^{3}$ p=0.00012; N = 6 PIs per group. **B)** One-day memory after spaced training was significantly reduced in mutants for dfmr1 (FMR^{B55} and $fmr1^{3}$) compared to their genetic controls. (One-way ANOVA, Tukey's post hoc test) FMR^{B55} p=0.00016, $Fmr1^{3}$ p=0.00023; N = 8 PIs per group. **C)** One day memory after massed training did not differ between groups. (One-way ANOVA, Tukey's post hoc test) p=0.9465 N = 8 PIs per group. All graphs depict mean ± s.e.m.

Figure 4-3


A
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Performance Index
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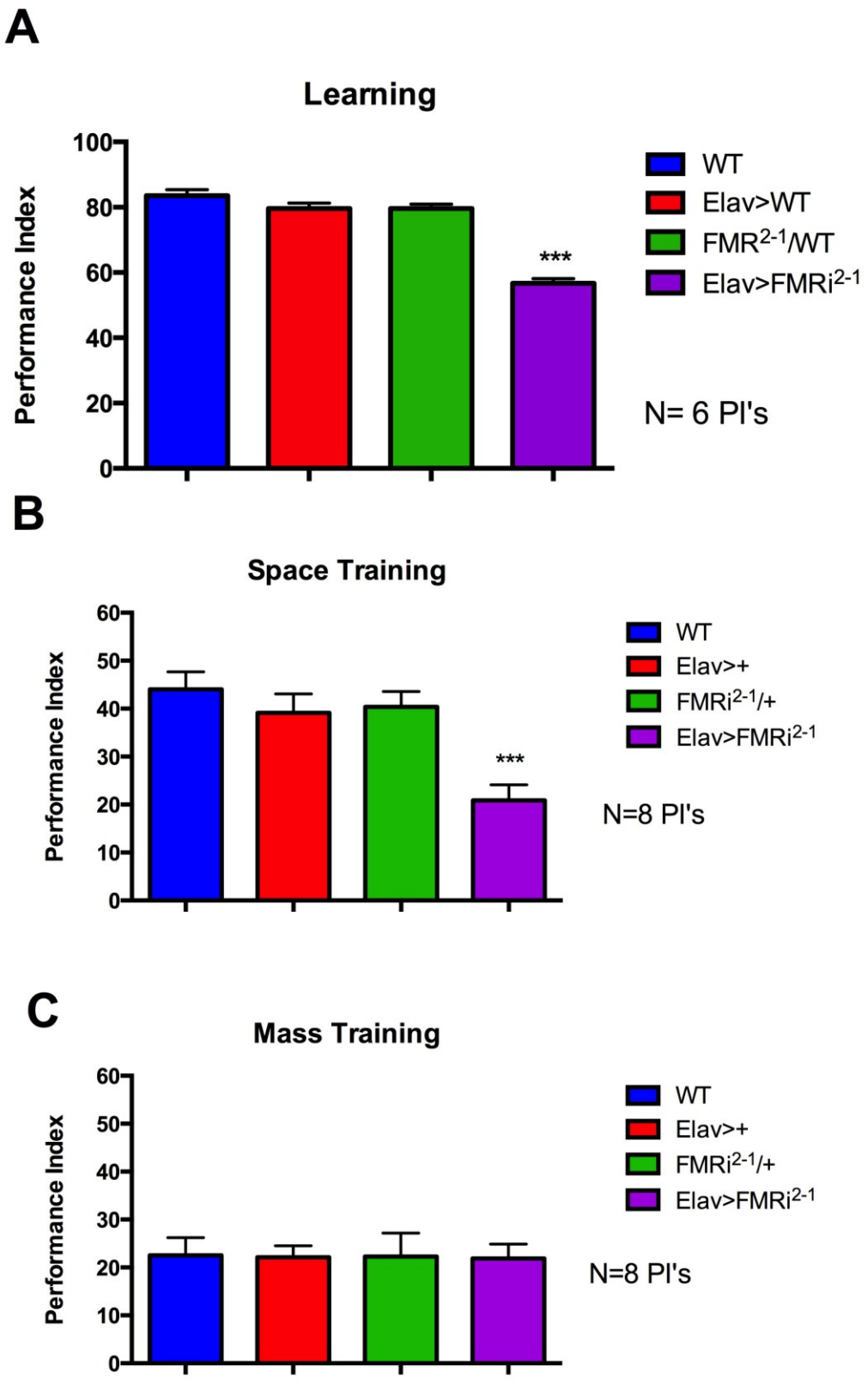
0
WT
FMR^B55
WTR; fmr1^3
fmr1^3
N=6 PI's
B
Olfaction
Performance Index
100
90
80
70
60
50
40
30
20
10
0
PIO
PIM
WT
FMR^B55
WTR; fmr1^3
fmr1^3
N=6 PI's

Figure 4-3. Absence of Drosophila FMRP has no impact on sensory function of Drosophila melanogaster. A) 1-3 days old Drosophila mutants for dfmr1 (FMR^{B55} and $fmr1^{3}$) and their genetic controls (WT) containing a genomic rescue fragment for dfmr1 (WTR; $dfmr1^{3}$) present normal shock avoidance. (One-way ANOVA, Tukey's post hoc test) p=0.2832; N=6 PI per genotype. B) 1-3 days old Drosophila mutants for dfmr1 (FMR^{B55} and $fmr1^{3}$) and their genetic controls (WT) containing a genomic rescue fragment for dfmr1 (WTR; $dfmr1^{3}$) present with normal avoidance to the odors (OCT= PIO or MCH= PIM) used in the classical conditioning experiments. (One-way ANOVA, Tukey's post hoc test) p=0.7857; N=6 PI per genotype. All graphs depict mean ± s.e.m.

Figure 4-4


A
Learning
Performance Index
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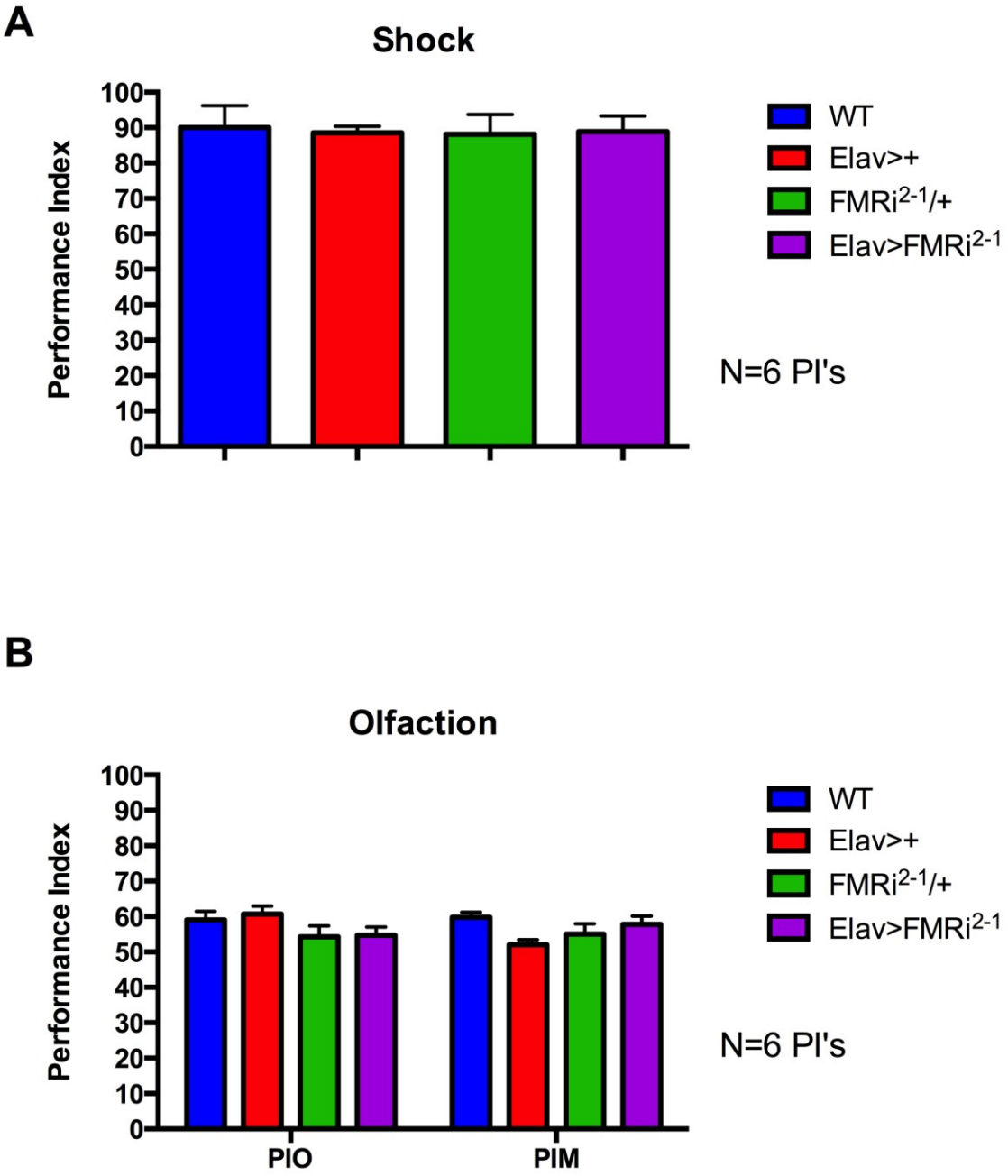
40
20
0
***
WT
Elav>WT
FMR2-1/WT
Elav>FMRi2-1
N= 6 PI's
B
Space Training
Performance Index
60
50
40
30
20
10
0
***
WT
Elav>+
FMRi2-1/+
Elav>FMRi2-1
N=8 PI's
C
Mass Training
Performance Index
60
50
40
30
20
10
0
WT
Elav>+
FMRi2-1/+
Elav>FMRi2-1
N=8 PI's

Figure 4-4. Knockdown of Drosophila FMRP leads to defects in Learning and Long-Term Memory. A) Learning was significantly defective transgenic flies expressing Fmr1 RNAi$^{2-1}$ pan neuronally. Indeed, Elav Gal4> UAS-FMR RNAI$^{2-1}$ performed significantly lower than the appropriate genetic control groups WT, UAS-FMRRNAi$^{2-1}$/+, Elav Gal4>+. (One-way ANOVA, Tukey's post hoc test) P=0.00043 N = 6 PIs per group. **B)** One-day memory after spaced training was significantly reduced (One-way ANOVA, Tukey's post hoc test) ($P < 0.001$) in Elav Gal 4; FMR-RNAi $^{2-1}$ compared with appropriate genetic controls. N = 8 PIs per group. **C)** One day memory after massed training did not differ between groups. (One-way ANOVA, Tukey's post hoc test) N = 8 PIs per group. All graphs depict mean ± s.e.m.

Figure 4-5


A
Shock
Performance Index
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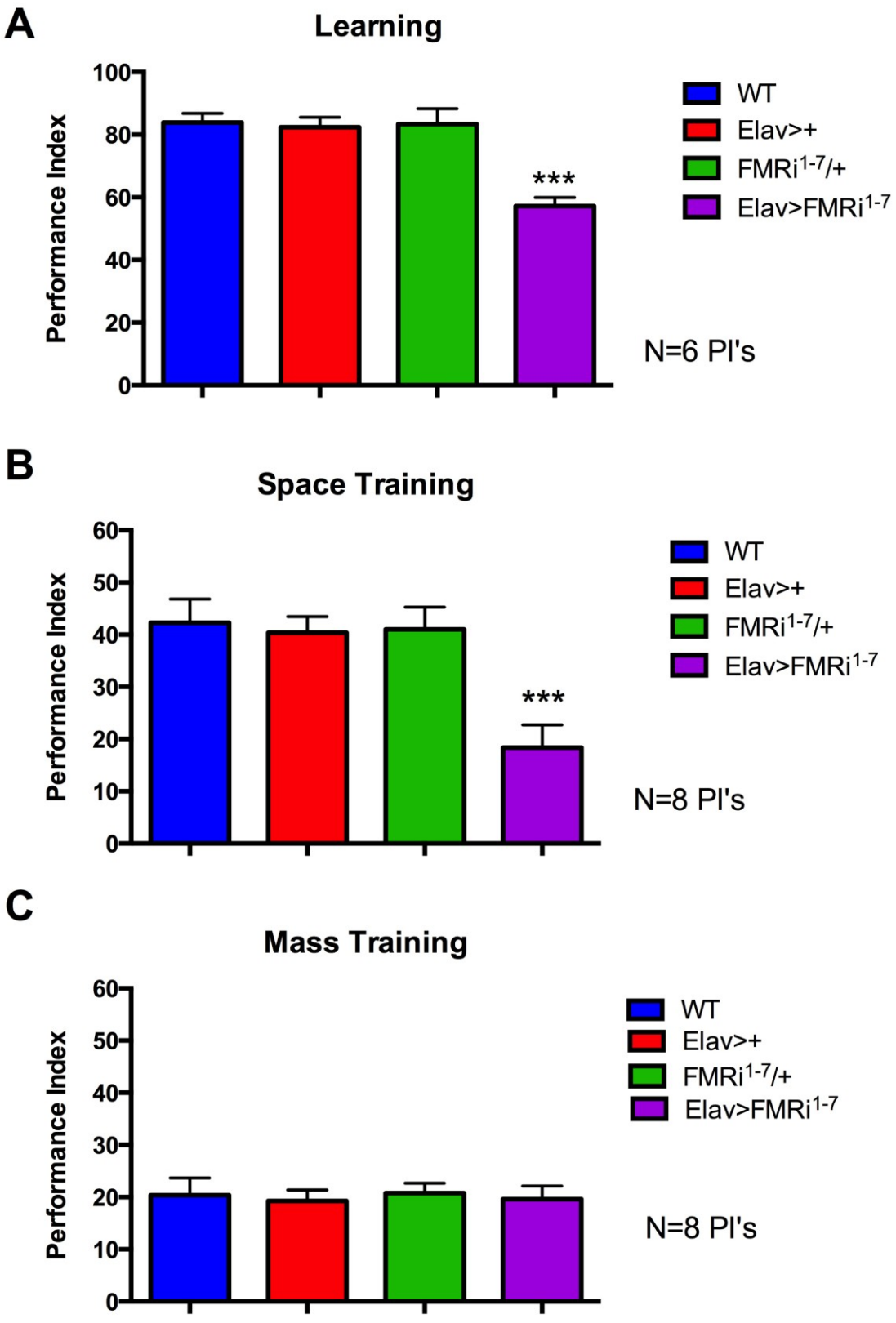
WT
Elav>+
FMRi2-1/+
Elav>FMRi2-1
N=6 PI's
B
Olfaction
Performance Index
100
90
80
70
60
50
40
30
20
10
0
PIO
PIM
WT
Elav>+
FMRi2-1/+
Elav>FMRi2-1
N=6 PI's

Figure 4-5. Knockdown of Drosophila FMRP does not impact the sensory functions of Drosophila melanogaster. A) 1-3 days old Drosophila Elav Gal4> UAS-FMR RNAI$^{2\text{-}1}$ as well as their genetic controls WT, UAS-FMR RNAI$^{2\text{-}1}$ /+, Elav>+, present with a normal shock avoidance. (One-way ANOVA, Tukey's post hoc test) p=0.98; N=6 PI per genotype. B) 1-3 days old Drosophila Elav Gal4> UAS-FMR RNAI$^{2\text{-}1}$ as well as their genetic controls WT, UAS-FMR RNAI$^{2\text{-}1}$ /+, Elav>+, present with normal avoidance to the odors (OCT= PIO or MCH= PIM) used in the classical conditioning experiments. (One-way ANOVA, Tukey's post hoc test) p=0.8596; N=6 PI per genotype.

Figure 4-6


A
Learning
Performance Index
100
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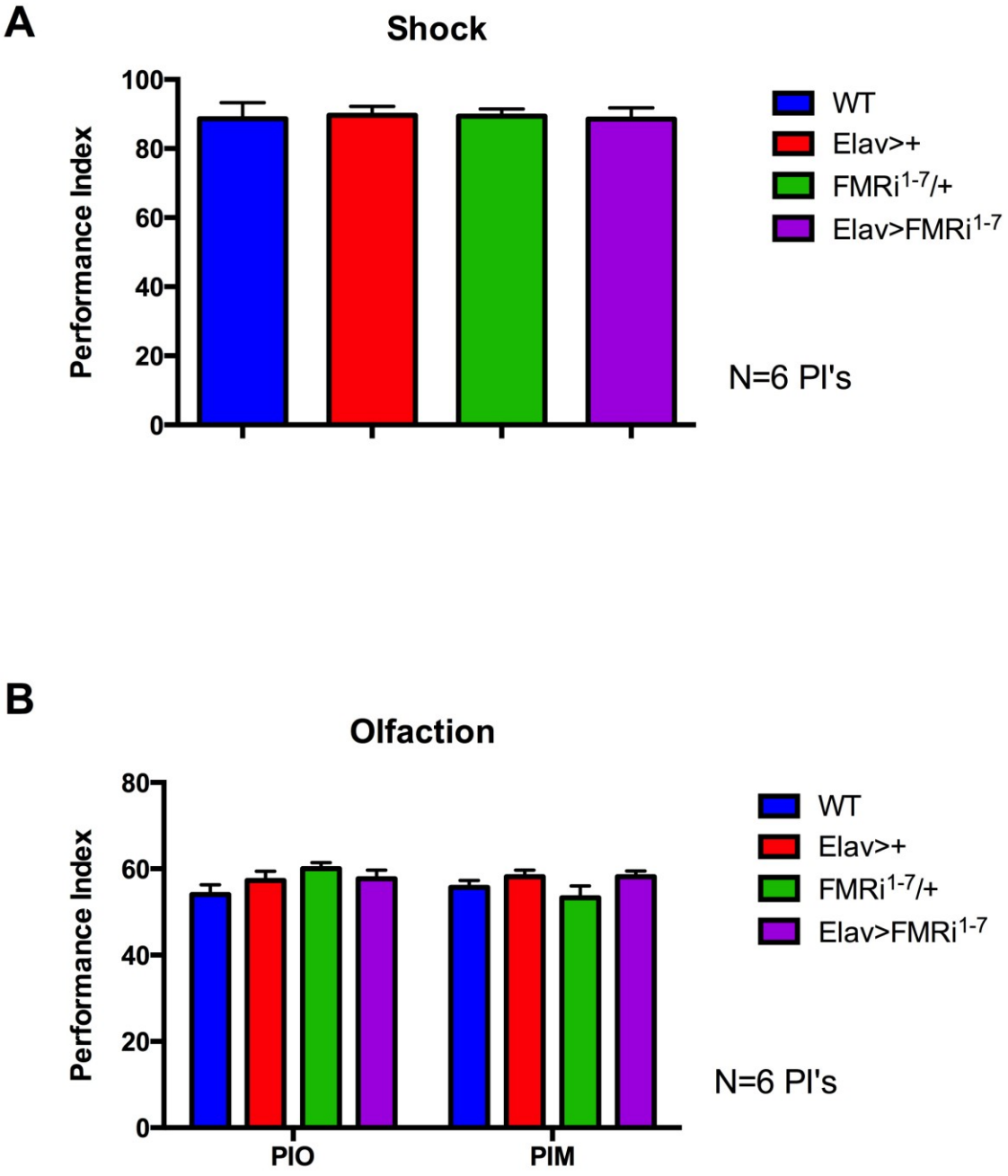
WT
Elav>+
FMRi1-7/+
Elav>FMRi1-7
N=6 PI's
B
Space Training
Performance Index
60
50
40
30
20
10
0
***
WT
Elav>+
FMRi1-7/+
Elav>FMRi1-7
N=8 PI's
C
Mass Training
Performance Index
60
50
40
30
20
10
0
WT
Elav>+
FMRi1-7/+
Elav>FMRi1-7
N=8 PI's

Figure 4-6. Knockdown of Drosophila FMRP leads to defects in Learning and Long-Term Memory. A) Learning was significantly defective transgenic flies expressing Fmr1 RNAi2-1 pan neuronally. Indeed, Elav Gal4; UAS-FMR RNAI$^{1-7}$ performed significantly lower than the appropriate genetic control groups WT, UAS-FMRRNAi$^{1-7}$/+, Elav Gal4>+. (One-way ANOVA, Tukey's post hoc test) $p<0.001$ N = 6 PIs per group. **B)** One-day memory after spaced training was significantly reduced ($P < 0.001$) in Elav Gal 4; FMR-RNAi $^{1-7}$ compared with appropriate genetic controls (One-way ANOVA, Tukey's post hoc test) (N = 8 PIs per group). **C)** One day memory after massed training did not differ between groups. (One-way ANOVA, Tukey's post hoc test) $p=0.6241$; N = 8 PIs per group. All graphs depict mean ± s.e.m.

Figure 4-7


A
Shock
Performance Index
100
80
60
40
20
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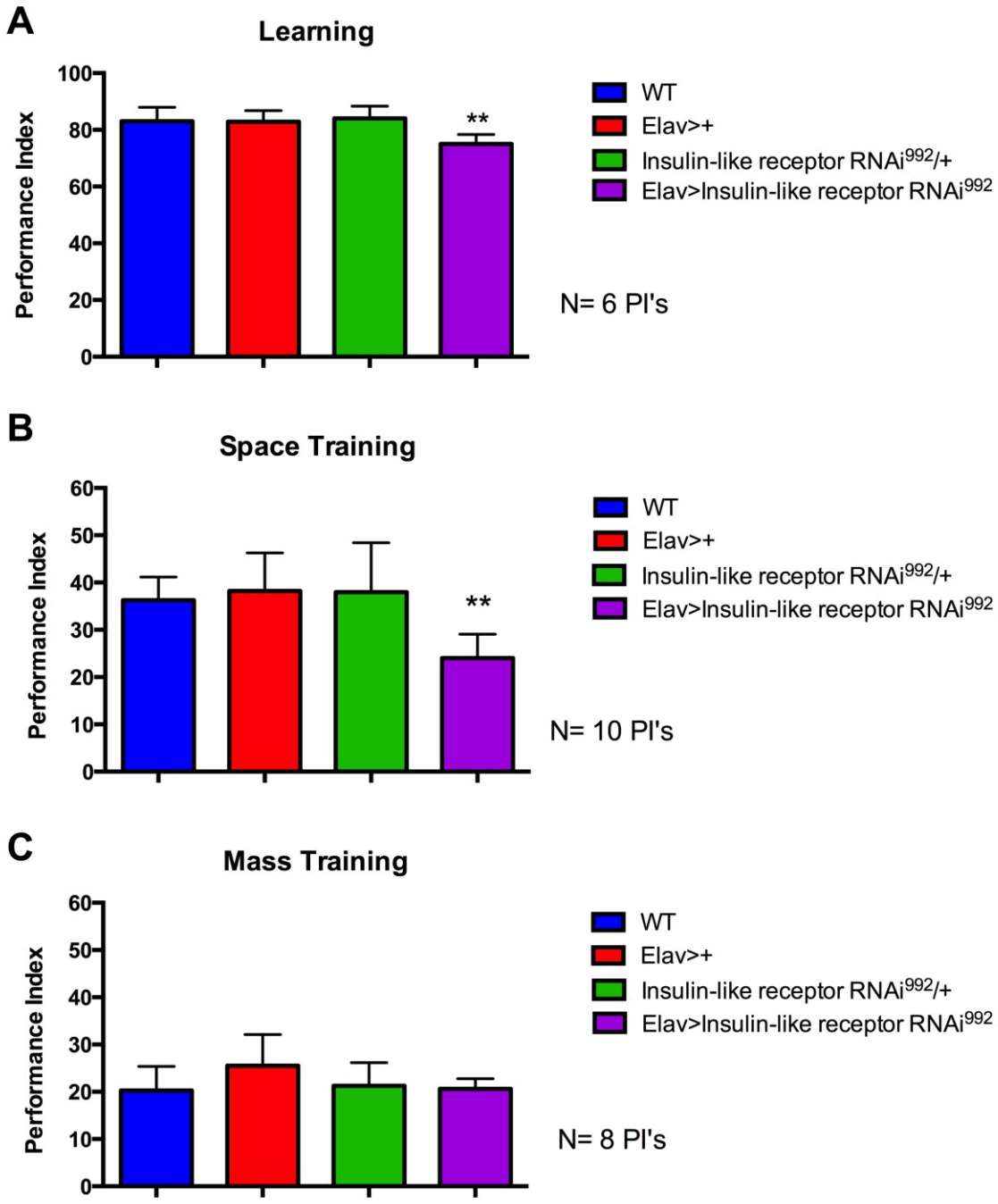
WT
Elav>+
FMRi1-7/+
Elav>FMRi1-7
N=6 PI's
B
Olfaction
Performance Index
80
60
40
20
0
PIO
PIM
WT
Elav>+
FMRi1-7/+
Elav>FMRi1-7
N=6 PI's

Figure 4-7. Knockdown of Drosophila FMRP does not impact the sensory functions of Drosophila melanogaster. A) 1-3 days old Drosophila Elav Gal4> UAS-FMR RNAI$^{1-7}$ as well as their genetic controls WT, UAS-FMR RNAI$^{1-7}$ /+, Elav>+, present with a normal shock avoidance. (One-way ANOVA, Tukey's post hoc test) p=0.9161; N=6 PI per genotype. B) 1-3 days old Drosophila Elav Gal4> UAS-FMR RNAI$^{1-7}$ as well as their genetic controls WT, UAS-FMR RNAI$^{1-7}$ /+, Elav>+, present with normal avoidance to the odors (OCT= PIO or MCH= PIM) used in the classical conditioning experiments. (One-way ANOVA, Tukey's post hoc test) p=0.6381; N=6 PI per genotype.

Figure 4-8


A
Learning
Performance Index
100
80
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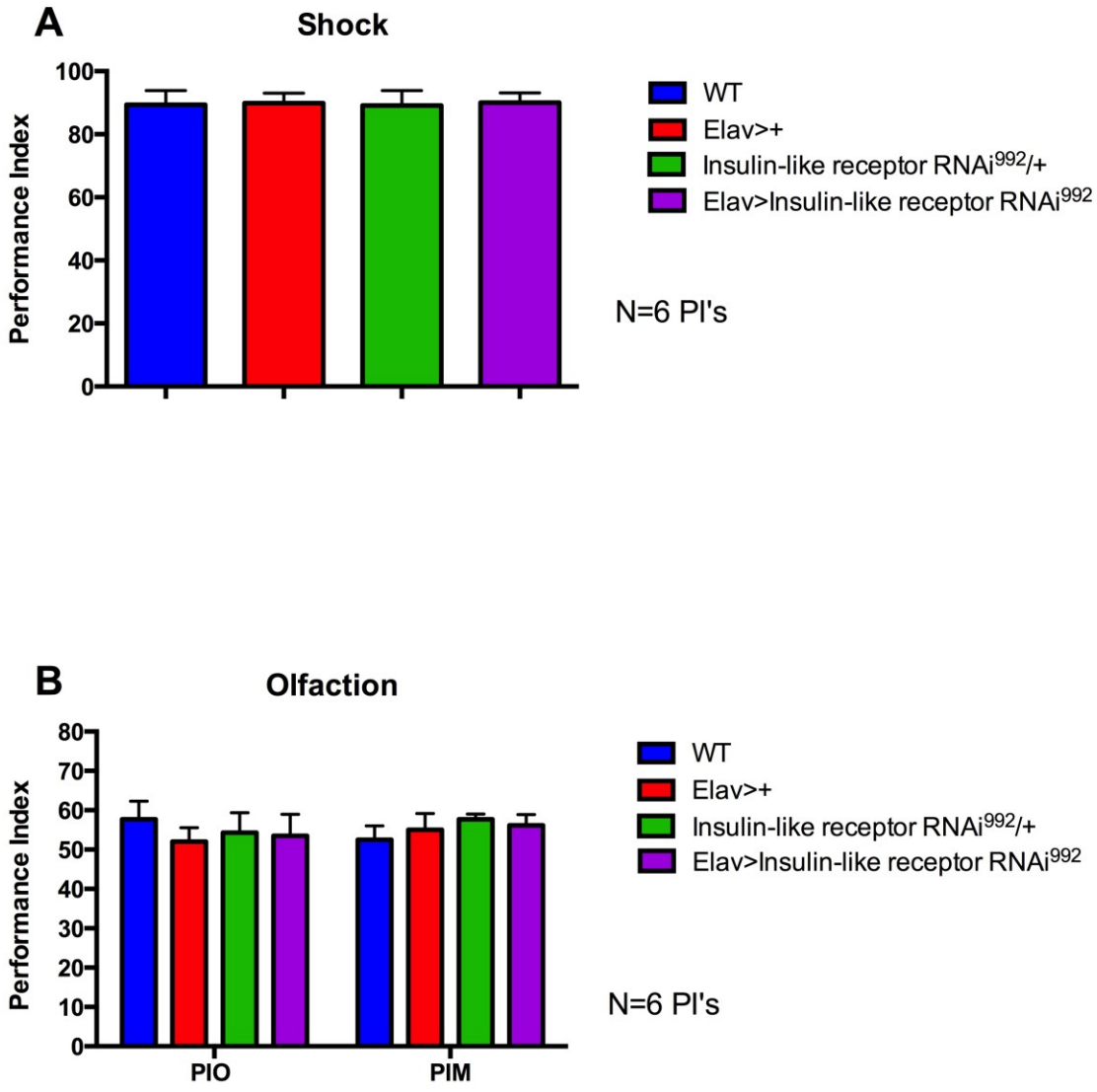
WT
Elav>+
Insulin-like receptor RNAi992/+
Elav>Insulin-like receptor RNAi992
N= 6 PI's
B
Space Training
Performance Index
60
50
40
30
20
10
0
**
WT
Elav>+
Insulin-like receptor RNAi992/+
Elav>Insulin-like receptor RNAi992
N= 10 PI's
C
Mass Training
Performance Index
60
50
40
30
20
10
0
WT
Elav>+
Insulin-like receptor RNAi992/+
Elav>Insulin-like receptor RNAi992
N= 8 PI's

Figure 4-8. Knockdown of Drosophila Insulin-Like Receptor leads to defects in Learning and Long-Term Memory. A) Immediate olfactory conditioning memory, named learning, was significantly lower in Elav>Insulin-Like Receptor RNAi^{992} versus WT, Insulin-Like Receptor RNAi^{992} /+, Elav>+. (One-way ANOVA, Tukey's post hoc test) p= 0.0044; N = 6 PIs per group. B) One-day memory was significantly reduced in Elav>Insulin-Like Receptor RNAi^{992} compared with controls WT, Insulin-Like Receptor RNAi^{992} /+, Elav>+. (One-way ANOVA, Tukey's post hoc test) p=0.0011; N = 10 PIs per group. C) One day memory after massed training did not differ between the groups. (One-way ANOVA, Tukey's post hoc test) p=0.1517; N = 8 PIs per group. All graphs depict mean ± s.e.m.

Figure 4-9


A
Shock
Performance Index
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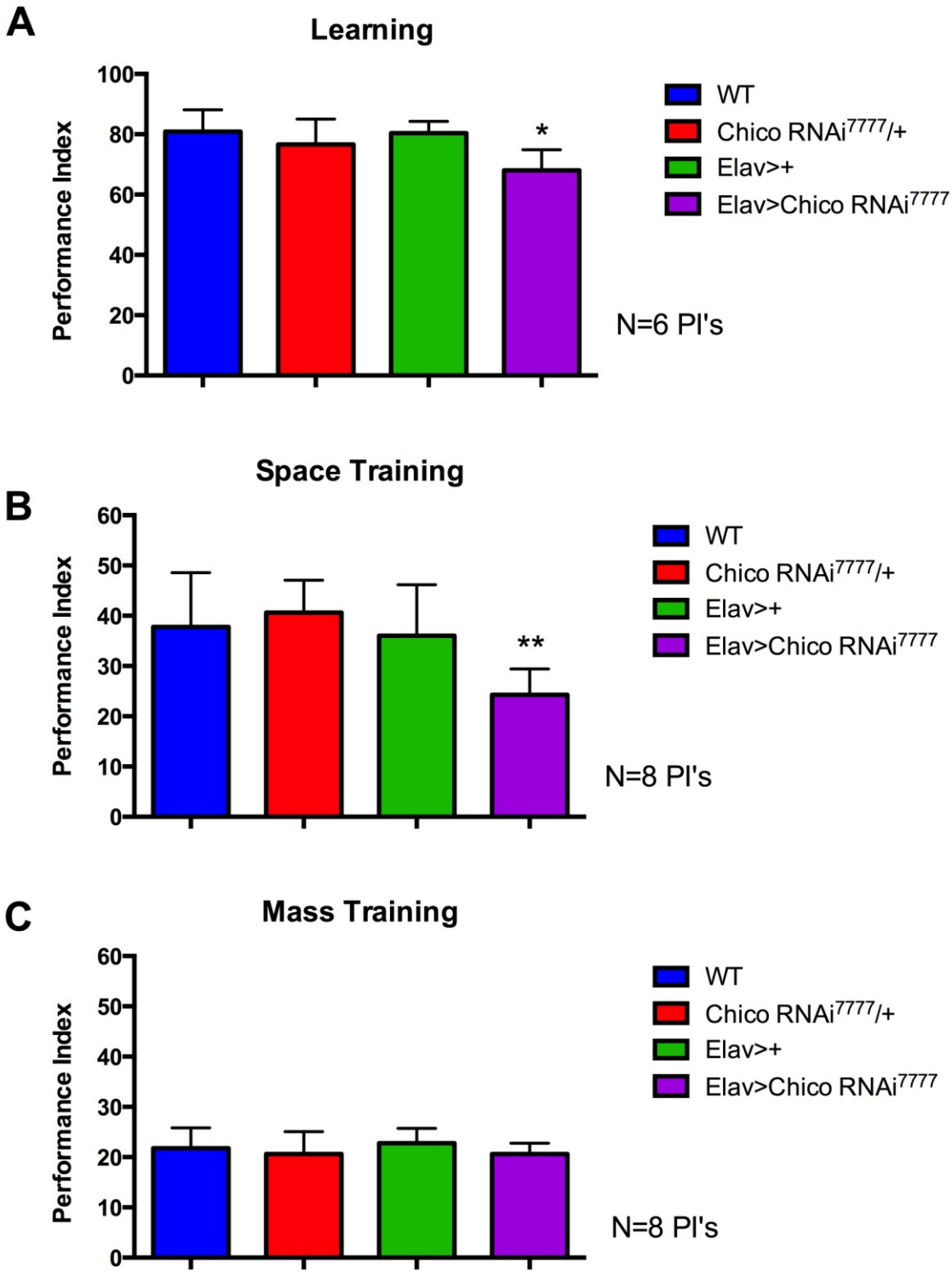
WT
Elav>+
Insulin-like receptor RNAi992/+
Elav>Insulin-like receptor RNAi992
N=6 PI's
B
Olfaction
Performance Index
80
70
60
50
40
30
20
10
0
PIO
PIM
WT
Elav>+
Insulin-like receptor RNAi992/+
Elav>Insulin-like receptor RNAi992
N=6 PI's

Figure 4-9. Knockdown of Insulin-Like Receptor does not impact the sensory functions of Drosophila melanogaster. A) 1-3 days old Drosophila mutant Insulin-Like Receptor RNAi^{992} as well as their genetic controls WT, Insulin-Like Receptor RNAi^{992} /+, Elav>+, present with a normal shock avoidance. (One-way ANOVA, Tukey's post hoc test) p=0.98; N=6 PI per genotype. B) 1-3 days old Drosophila mutant Insulin-Like Receptor RNAi^{992} as well as their genetic controls WT, Insulin-Like Receptor RNAi^{992} /+, Elav>+, present with normal avoidance to the odors (OCT= PIO or MCH= PIM) used in the classical conditioning experiments. (One-way ANOVA, Tukey's post hoc test) p=0.8596; N=6 PI per genotype.

Figure 4-10


A
Learning
Performance Index
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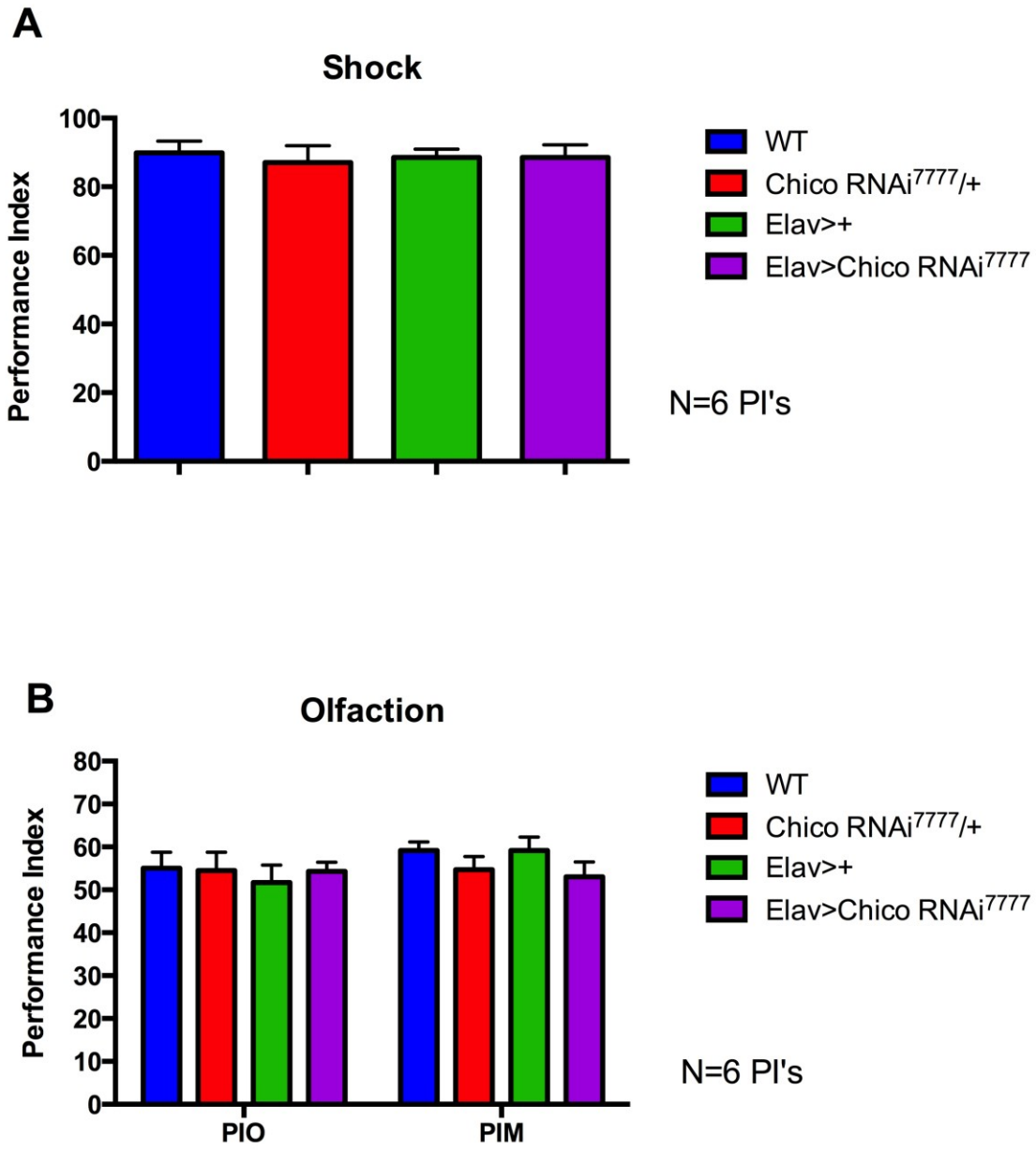
WT
Chico RNAi7777/+
Elav>+
Elav>Chico RNAi7777
N=6 PI's
B
Space Training
Performance Index
60
50
40
30
20
10
0
**
WT
Chico RNAi7777/+
Elav>+
Elav>Chico RNAi7777
N=8 PI's
C
Mass Training
Performance Index
60
50
40
30
20
10
0
WT
Chico RNAi7777/+
Elav>+
Elav>Chico RNAi7777
N=8 PI's

Figure 4-10. Knockdown of Drosophila Chico leads to defects in Learning and Long-Term Memory. A) Immediate olfactory conditioning memory, named learning, was significantly lower in Elav>Chico RNAi^{7777} versus WT, Chico RNAi^{7777} /+, Elav>+. (One-way ANOVA, Tukey's post hoc test) p=0.0137; N = 6 PIs per group. B) One-day memory was significantly reduced in Elav>Chico RNAi^{7777} compared with controls WT, Chico RNAi^{7777} /+, Elav>+. (One-way ANOVA, Tukey's post hoc test) p=0.0034; N = 8 PIs per group. C) One day memory after massed training did not differ. (One-way ANOVA, Tukey's post hoc test) p=0.5784; N = 8 PIs per group. All graphs depict mean ± s.e.m.

Figure 4-11


A
Shock
Performance Index
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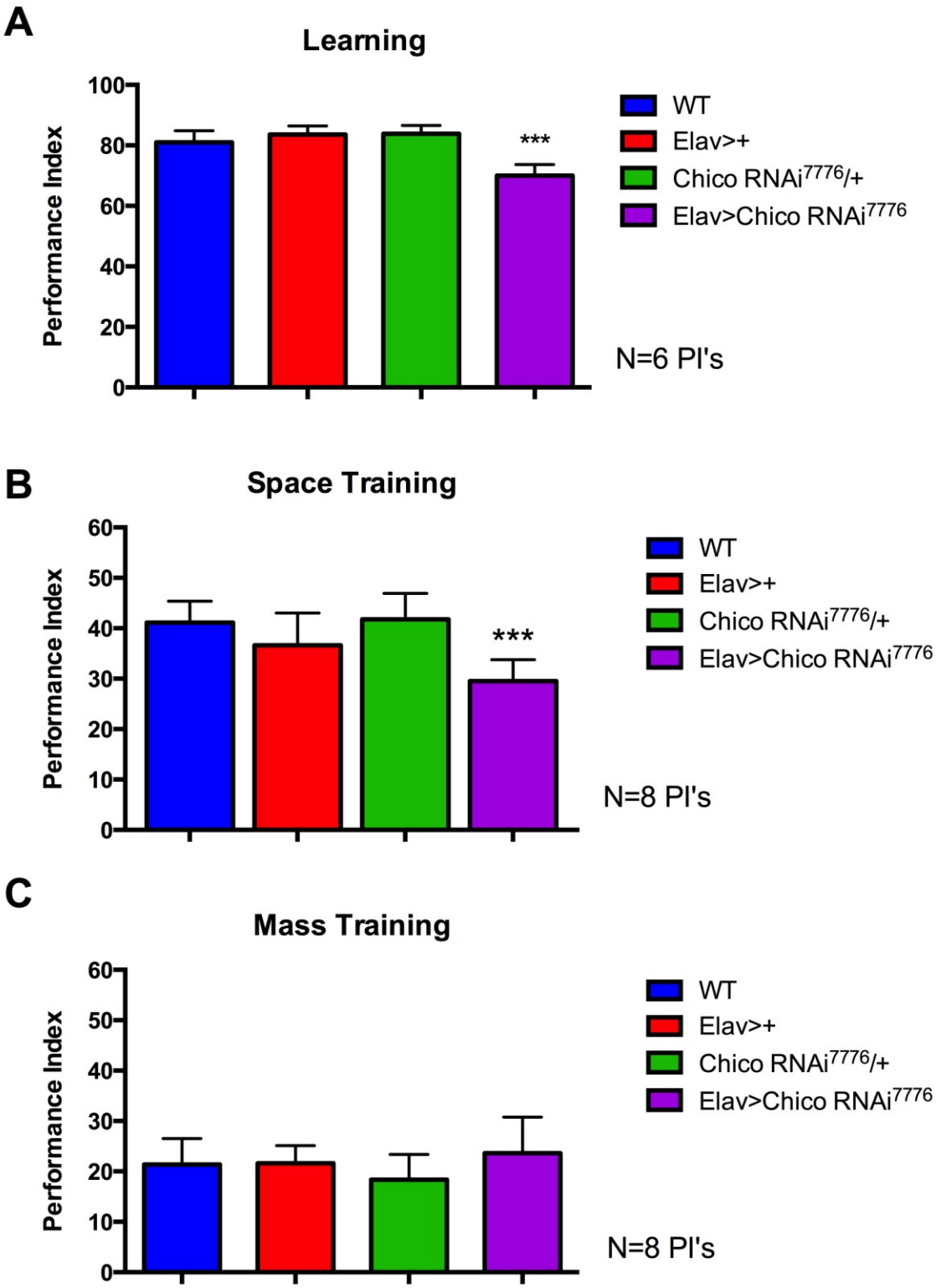
WT
Chico RNAi7777/+
Elav>+
Elav>Chico RNAi7777
N=6 PI's
B
Olfaction
Performance Index
80
70
60
50
40
30
20
10
0
PIO
PIM
WT
Chico RNAi7777/+
Elav>+
Elav>Chico RNAi7777
N=6 PI's

Figure 4-11. Knockdown of Drosophila Chico does not impact the sensory functions of Drosophila melanogaster. A) 1-3 days old Drosophila mutant Elav>Chico RNAi^{7777} versus WT, Chico RNAi^{7777} /+, Elav>+, present with a normal shock avoidance. (One-way ANOVA, Tukey's post hoc test) p=0.6356; N=6 PI per genotype B) 1-3 days old Drosophila mutant Elav>Chico RNAi^{7777} versus WT, Chico RNAi^{7777} /+, Elav>+, present with normal avoidance to the odors (OCT= PIO or MCH= PIM) used in the classical conditioning experiments. (One-way ANOVA, Tukey's post hoc test) p=0.9462; N=6 PI per genotype.

Figure 4-12


A
Learning
Performance Index
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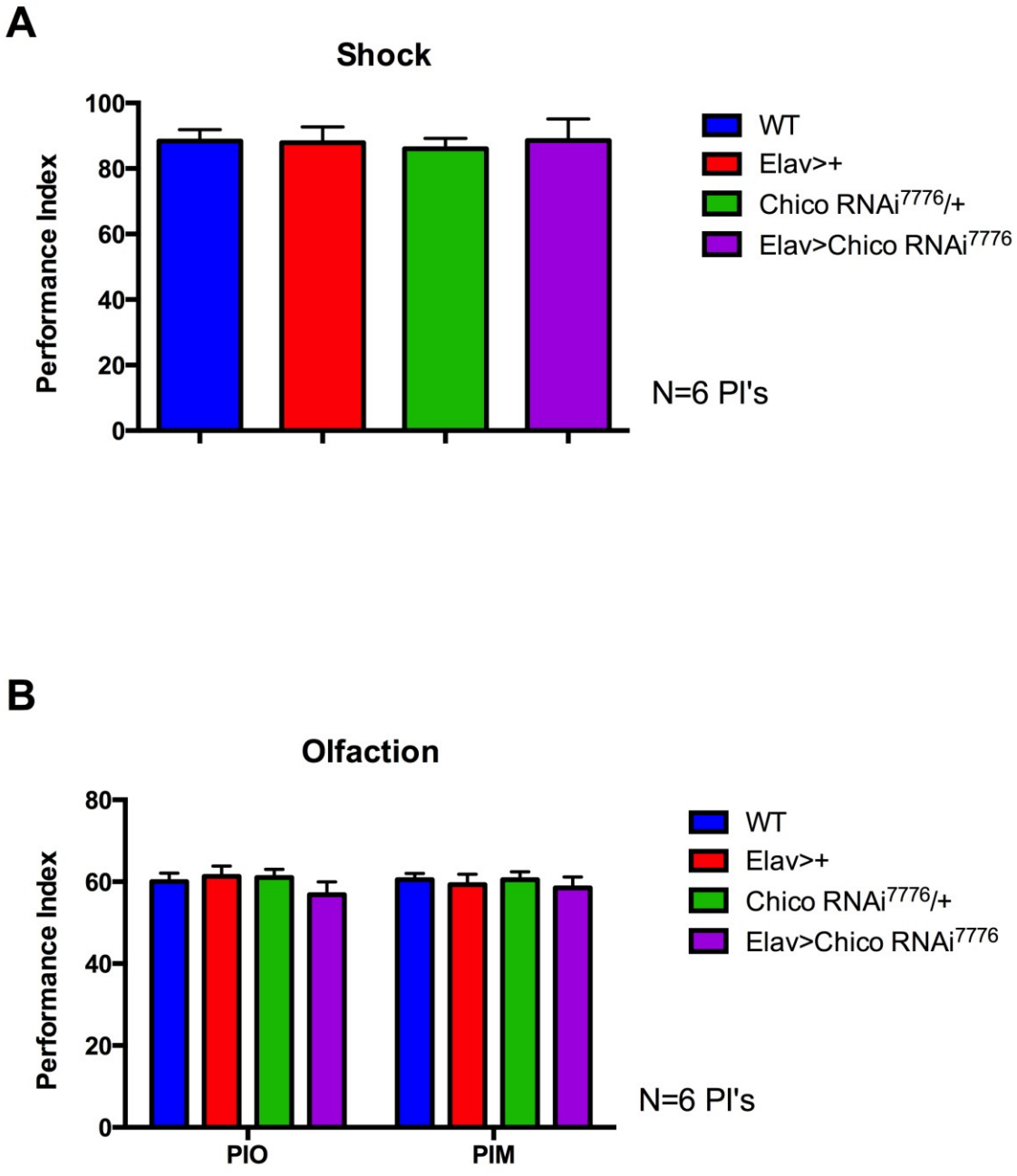
WT
Elav>+
Chico RNAi7776/+
Elav>Chico RNAi7776
N=6 PI's
B
Space Training
Performance Index
60
50
40
30
20
10
0
***
WT
Elav>+
Chico RNAi7776/+
Elav>Chico RNAi7776
N=8 PI's
C
Mass Training
Performance Index
60
50
40
30
20
10
0
WT
Elav>+
Chico RNAi7776/+
Elav>Chico RNAi7776
N=8 PI's

Figure 4-12. Knockdown of Drosophila Chico leads to defects in Learning and Long-Term Memory. A) Immediate olfactory conditioning memory, named learning, was significantly lower in Elav>Chico RNAi^{7776} versus the control groups, WT, Chico RNAi^{7776} /+, Elav>+. (One-way ANOVA, Tukey's post hoc test) $p<0.0001$; N = 6 PIs per group. B) One-day memory was significantly reduced in Elav>Chico RNAi^{7776} compared with controls WT, Chico RNAi^{7776} /+, Elav>+. (One-way ANOVA, Tukey's post hoc test) $p=0.00019$; N = 8 PIs per group. C) One day memory after massed training did not differ. (One-way ANOVA, Tukey's post hoc test) $p=0.2918$; N = 8 PIs per group. All graphs depict mean ± s.e.m.

Figure 4-13


A
Shock
Performance Index
100
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WT
Elav>+
Chico RNAi7776/+
Elav>Chico RNAi7776
N=6 PI's
B
Olfaction
Performance Index
80
60
40
20
0
PIO
PIM
WT
Elav>+
Chico RNAi7776/+
Elav>Chico RNAi7776
N=6 PI's

Figure 4-13. Knockdown of Drosophila Chico does not impact the sensory functions of Drosophila melanogaster. A) 1-3 days old Drosophila mutant Elav>Chico RNAi^{7776} versus WT, Chico RNAi^{7776} /+, Elav>+, present with a normal shock avoidance. (One-way ANOVA, Tukey's post hoc test) p=0.7900; N=6 PI per genotype B) 1-3 days old Drosophila mutant Elav>Chico RNAi^{7776} versus WT, Chico RNAi^{7776} /+, Elav>+, present with normal avoidance to the odors (OCT= PIO or MCH= PIM) used in the classical conditioning experiments. (One-way ANOVA, Tukey's post hoc test) p=0.5485; N=6 PI per genotype.

Figure 4-14

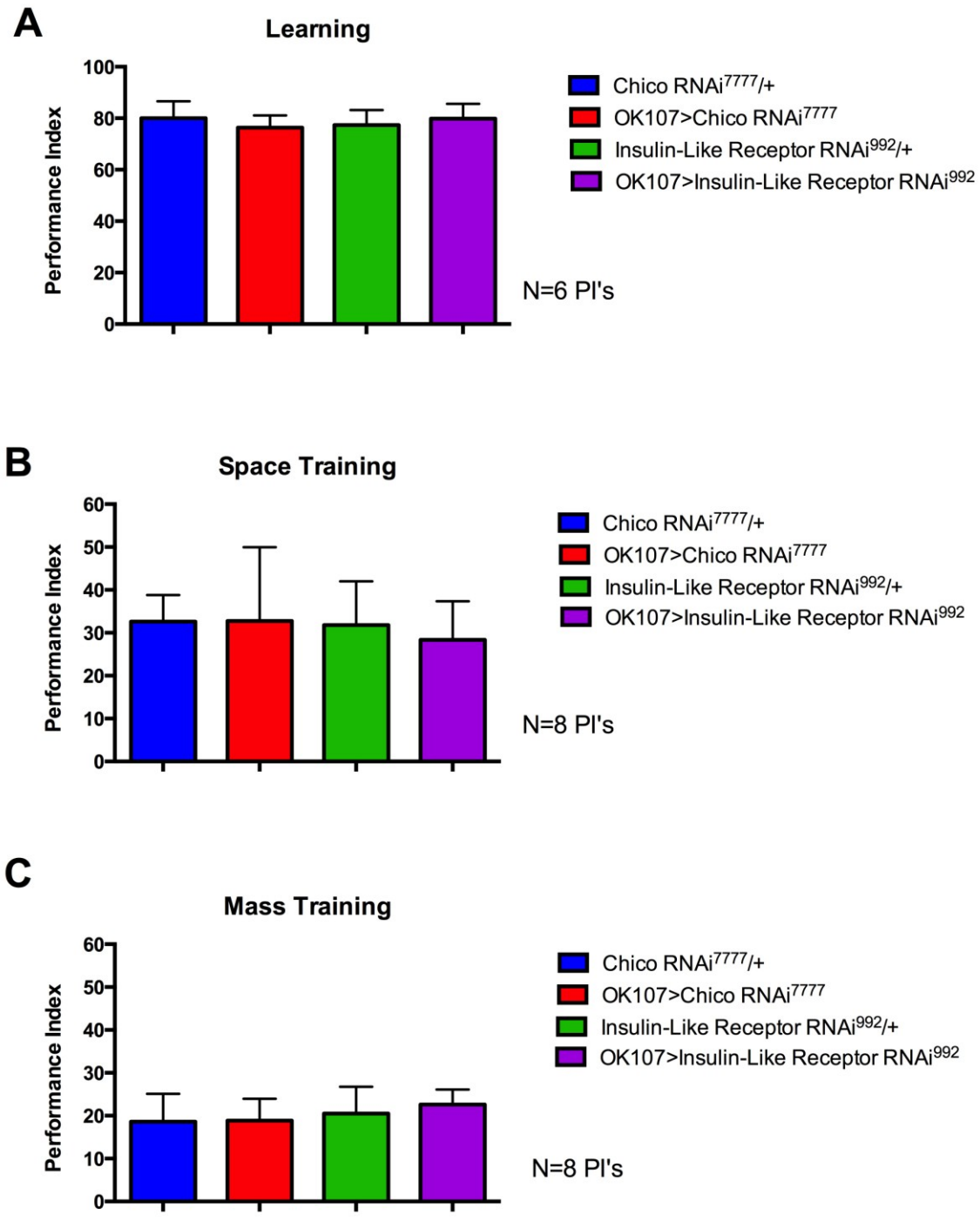
A
Learning
Performance Index
100
80
60
40
20
0
Chico RNAi7777/+
OK107>Chico RNAi7777
Insulin-Like Receptor RNAi992/+
OK107>Insulin-Like Receptor RNAi992
N=6 PI's
B
Space Training
Performance Index
60
50
40
30
20
10
0
Chico RNAi7777/+
OK107>Chico RNAi7777
Insulin-Like Receptor RNAi992/+
OK107>Insulin-Like Receptor RNAi992
N=8 PI's
C
Mass Training
Performance Index
60
50
40
30
20
10
0
Chico RNAi7777/+
OK107>Chico RNAi7777
Insulin-Like Receptor RNAi992/+
OK107>Insulin-Like Receptor RNAi992
N=8 PI's

Figure 4-14. Mushroom Body specific knockdown of Drosophila Chico and Insulin-like receptor does not impact Learning and Long-Term Memory. A) Immediate olfactory conditioning memory, named learning, was similar between all groups, Chico RNAi^{7777}/+, OK107>Chico RNAi^{7777}, Insulin-Like Receptor RNAi^{992}/+, OK107>Insulin-Like Receptor RNAi^{992}. (One-way ANOVA, Tukey's post hoc test) p=0.6255; N = 6 PIs per group. B) One-day memory was similar between all groups, Chico RNAi^{7777}/+, OK107>Chico RNAi^{7777}, Insulin-Like Receptor RNAi^{992}/+, OK107>Insulin-Like Receptor RNAi^{992} (One-way ANOVA, Tukey's post hoc test) p=0.8547; N = 8 PIs per group. C) One day memory after massed training did not differ. (One-way ANOVA, Tukey's post hoc test) p=0.4486; N = 8 PIs per group. All graphs depict mean ± s.e.m.

4.5 Bibliography

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Chapter 5. INVESTIGATE A PHARMACOLOGICAL APPROACH TO RESCUE THE FRAGILE X PHENOTYPE BASED ON DRUGS TARGETING THE AKT AXIS OF THE INSULIN RECEPTOR PATHWAY.

5.1 Introduction

In the previous chapters we have looked at the role that the Insulin pathway plays in learning and memory as well as the levels of different targets within this pathway in a FX model. The implications of dysregulating the Insulin pathway were demonstrated in a learning and memory setting, this lead to the hypothesis that if we could modulate this pathway in FX there could be a therapeutic avenue to explore. As a result our observation of excessive AKT signaling and p70S6K in brains of Drosophila mutant for *fmr1* combined with the role of the insulin receptor we hypothesized that down-regulating insulin signaling could be useful in rescuing memory in Drosophila FX mutants. We used the same mutants tested in the first part of the behavior section (Chapter 3). We started by testing a commonly used oral hypoglycemiant drug, metformin. Metformin mechanism of action is still unknown.

4.1.1 The Insulin Pathway regulates AKT

As discussed in chapter 1, one of the initiators of the AKT pathway is the binding of a ligand to the Insulin Receptor. The insulin pathway is highly conserved across species, and it aids in the translation of nutritional status into neural stem cell

behavior, which moderates neural tissue growth (Callan et al., 2012). Studies in Drosophila have characterized Insulin receptor/Phosphoinositide 3-kinase (Inr/PI3K) signaling as a potent regulator of cell growth. Inhibiting Inr/PI3K signaling phenocopies the cellular and organismal effects of starvation, whereas activating this pathway bypasses the nutritional requirement for cell growth, causing starvation sensitivity at the organismal level. Drosophila insulin-like peptides (Dilps) promote tissue growth through the single InR. Binding of this ligand to the Insulin Receptor leads to a promotion of tissue growth through the activation of PI3K/AKT pathway (Callan et al., 2012). Research has demonstrated that FMRP plays a key role in the modulation of the Insulin pathway in a Drosophila model and that lack of FMRP can lead to negative outcomes for the organism (Callan et al., 2012).

5.2 Results

Considering our observation of excessive AKT signaling and p70S6K in brains of drosophila mutant for fmr1 combined with the role of the insulin receptor we decided to see if downregulating insulin signaling could be useful in rescuing memory in Drosophila FX mutants. We used the same mutants tested in the first part of the behavior section (Chapter 4). We started by testing a commonly used oral hypoglycemiant drug, metformin.

Experiments have been performed to determine which dose of a drug called Metformin could potentially improve learning and memory scores. Using a dose of 1mM has, proven most effective at improving the learning capabilities of FX mutant flies (Fig.5-1).

We tested the effect Metformin has on the learning abilities of *fmr1*^{3} mutant flies and found that there was significant improvement in the learning scores of *fmr1*^{3} flies compared to *fmr1*^{3} flies that were given vehicle treatment (Fig.5-2A). The drug did not have an effect on the learning scores of *fmr1*^{3} wild-type rescue flies, both the drug and vehicle groups performing on the same level (Fig.5-2A). The 1mM concentration of Metformin significantly improved in the memory scores of *fmr1*^{3} flies after space training compared to *fmr1*^{3} flies that were administered vehicle (Fig.5-2B). The drug had no effect on the *fmr1*^{3} wild-type rescue flies that were tested at the same time (Fig.5-2B). There was no significant difference between the different groups in memory scores after mass training, all performing around the normal scores (Fig.5-2C). *fmr1*^{3} as well as their genetic controls containing a genomic rescue fragment for d*fmr1* (*fmr1* WTR; d*fmr1*^{3}) present with a normal shock avoidance with vehicle and 1 mM Metformin treatment. In addition they also present with normal avoidance to the odors (OCT or MCH) used in the classical conditioning experiments (Fig.5-3).

To try and produce the same phenotype as FX but using a different method we made use of the UAS-GAL4 system to knockdown the translation and therefore overall expression of FMRP. Using a pan-neuronal driver, Elav-GAL4, we targeted the translation of FMRP by using RNA interference (RNAi) flies FMR$^{2\text{-}1}$. This knocks down the expression of FMRP mimicking the absence seen in FX mutant flies. We then administered the 1mM dose of Metformin to these FMR^{RNAi} flies and the control groups. This concentration of Metformin significantly improved the

learning scores of FMR RNAi$^{2\text{-}1}$flies compared to FMR RNAi$^{2\text{-}1}$ flies that were administered vehicle (Fig.5-4A). The drug had no effect on the control group flies that were tested at the same time (Fig.5-4A). We continued and tested the effect Metformin has on the protein synthesis dependent memory abilities of FMR RNAi$^{2\text{-}1}$ mutant flies and found that there was significant improvement in the memory scores of FMR RNAi$^{2\text{-}1}$ flies compared to FMR RNAi$^{2\text{-}1}$ flies that were given vehicle treatment (Fig.5-4B). The drug did not have an effect on the memory scores of control group flies, both the drug and vehicle groups performing on the same level (Fig.5-4B). There was no difference in the protein synthesis dependent memory assay between the different groups (Fig.5-4C).

The rescuing effect of Metformin was exciting and this lead to us using the UAS-Gal4 system again but this time using the OK107-Gal4 Mushroom Body specific driver to knock down the translation of FMRP in the Mushroom Body. These flies were given 1mM dose of Metformin and this resulted in a significant improvement in protein synthesis dependent form of memory (Fig.5-5). This allowed for greater insight into where Metformin is performing its mechanism of action.

We were able to conduct experiments to determine which dose of a drug called Rolipram that can improve learning and memory scores. Using a dose of 50μM has proven most effective at improving the long-term memory capabilities of FX mutant flies (Fig.5-6). We continued and tested the effect Rolipram has on the learning abilities of *fmr1*^{3} mutant flies and found that there was significant

improvement in the learning scores of *fmr1*^{3} flies compared to *fmr1*^{3} flies that were given vehicle treatment (Fig.5-7A). The drug did not have an effect on the learning scores of *fmr1*^{3} wild-type rescue flies, both the drug and vehicle groups performing on the same level (Fig.5-7A). The 50μM concentration of Rolipram significantly improved in the protein synthesis dependent memory scores of *fmr1*^{3} flies compared to *fmr1*^{3} flies that were administered vehicle (Fig.5-7B). The drug had no effect on the *fmr1*^{3} wild-type rescue flies that were tested at the same time (Fig.5-7B). Rolipram did not have an effect on the protein synthesis independent form of memory as well (Fig.5-7C).

In order to ensure that the improvements were specific to memory improvements we performed experiments to test the olfactory acuity and shock reactivity of the flies given the drug and vehicle. d*fmr1*^{3} as well as their genetic controls containing a genomic rescue fragment for dfmr1 (WTR; d*fmr1*^{3}) present with a normal shock avoidance with vehicle and 50μM Rolipram treatment. In addition they also present with normal avoidance to the odors (OCT or MCH) used in the classical conditioning experiments (Fig.5-8).

5.3 Discussion

5.3.1 Metformin treatment rescues Learning and Memory phenotype seen in Fragile X Syndrome

In a field such as Intellectual Disability research it is very hard to come up with a cure to a particular disease due to the fact that the majority of them are developmental in nature. Nonetheless, our lab and others now have shown that genes

involved in ID are required during development but also acutely in cognitive functioning. This opens the door to development of treatments aimed at treating cognitive symptoms. To this end we wanted to try different pharmacological agents to see if they could improve any of the cognitive defects seen in Fragile X. Metformin is thought to act on AMPK, which is downstream of the cAMP-PKA pathway. It has been shown to directly increase AMPK activity, increasing PTEN expression and ultimately leading to the inhibition of mTOR activity. We found that metformin treatment rescued immediate recall memory, referred to as learning, in *dfmr1* mutants trained to associate a shock with an odor stimulus (Fig. 5-2A). Similarly, we found that metformin treatment rescued LTM^{d} (1-day) olfactory memory in d*fmr1* mutants subjected to spaced training with the odor stimulus and shock but had no effect on wild-type control flies (Fig. 5-2B). We replicated previous results where d*fmr1* mutant flies showed no defects in olfaction or shock sensitivity (Bolduc et al., 2008), but also observed that metformin did not exert its effect via enhanced olfaction or shock reactivity (Fig. 5-3), indicating that metformin rescues cognitive rather than sensory defects. The rescue of olfactory learning and long-term memory after spaced training was replicated in flies with pan-neural RNAi knockdown of d*fmr1*. Furthermore, we showed that the rescue of 1day memory was specific to the protein synthesis dependent form of memory as there was no effect of metformin in d*fmr1* mutant trained with mass training (LTM^{i}) (Fig. 5-2C). The degree of rescue is similar to what was previously obtained with mGluR antagonist MPEP and protein synthesis inhibitors cycloheximide and puromycin (Bolduc et al., 2008).

We wanted to try and determine where in the Drosophila brain metformin was performing its mechanism of action. The developmental effect seen in FX is in part due to the malformation of the Mushroom Body (Bolduc et al., 2008), a large structure present in each side of the brain, which is thought to be important for the formation of LTM. In the fly model of FXS, there is an abnormal fusion between the MB from each hemisphere (Bolduc et al., 2008). To this end we targeted RNA interference in the mushroom body of Drosophila using the OK107-Gal4 driver that drove the knockdown of FMRP using UAS FMR$^{1\text{-}7}$. After these mutant flies were given an acute dose of metformin and exposed to the space training protocol we saw a rescue of the memory defect seen in FX (Fig. 5-5). This helps to narrow down the site of action where metformin is able to exert its effect. These results show that treatment with a drug known to target the insulin signaling pathway rescues two forms of memory in d*fmr1* mutant flies and gives us greater insight into where exactly this treatment is exerting its effects.

5.3.2 PDE-4 Inhibitor, Rolipram, rescues Learning and Memory phenotype seen in Fragile X Syndrome.

The objective of this work was to examine the efficacy of pharmacologically inhibiting PDE-4 activity to correct synaptic plasticity impairments in the fly and mouse models of Fragile X syndrome. The *Drosophila* Fragile X model recapitulates the most debilitating aspect of the disease in humans, namely impaired cognitive function. In our further dissection of the proteins involved in the mGluR signaling cascade, we identified PDE-4 as a potential substrate whose inhibition may be

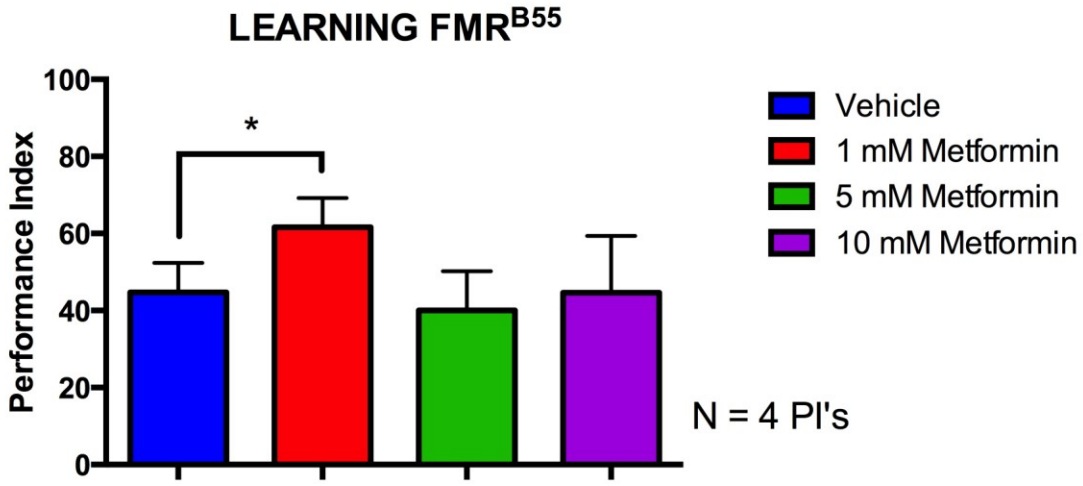
beneficial in restoring proper intracellular signaling in the Fragile X model. Based on the fly data, tissue culture work, and samples from humans afflicted with Fragile X syndrome, we speculated that the suppressed cAMP levels previously identified (Berry-Kravis and Sklena, 1993; Berry-Kravis et al., 1995; Berry-Kravis and Ciurlionis, 1998; McBride et al., 2005; Kelley et al., 2007) may impact the learning and memory process. PDE-4 inhibition should increase cAMP signaling by preventing the breakdown of cAMP that is produced during synaptic stimulation. Fragile X flies acutely treated in adulthood with PDE-4 inhibitors, demonstrated intact immediate recall and short-term memory, validating PDE-4 inhibition as a potential novel therapeutic target for the treatment of synaptic plasticity impairments in Fragile X. This finding adds to the growing body of literature demonstrating that pharmacologic treatment initiated in adulthood may have efficacy for the treatment of cognitive disorders that are already present in childhood as was first demonstrated in animal models of Fragile X (McBride et al., 2005).

5.3.3 Future Plans

Future experiments that should be done for this group of results could include doing Western Blot analysis on AKT pathway targets (Chapter 3) to observe if Metformin does anything to alter the levels of these proteins. This would shed light on the mechanism by which Metformin performs its action and allow us to understand this drug much better. Levels of these targets are elevated in Fragile X therefore after being given Metformin the Fragile X levels of these targets should return closer to wild-type levels.

In order to better understand the mechanism by which Rolipram's positive effects are occurring I would utilize tissue specific drivers in the UAS-Gal4 system to narrow down the area of the brain that medicinal effects are taking place. Using OK107-Gal4 and Feb170-Gal4 would tell me if Rolipram was performing its mechanism in the Mushroom Body or Central Complex respectively. Finally I would also like to perform Western Blot analysis on Fragile X flies after they have been given Rolipram to see if AKT pathway targets are impacted by this drug (Chapter 3). This will demonstrate the mechanism by which Rolipram is performing its therapeutic action.

Figure 5-1


LEARNING FMR^B55
Performance Index
100
80
60
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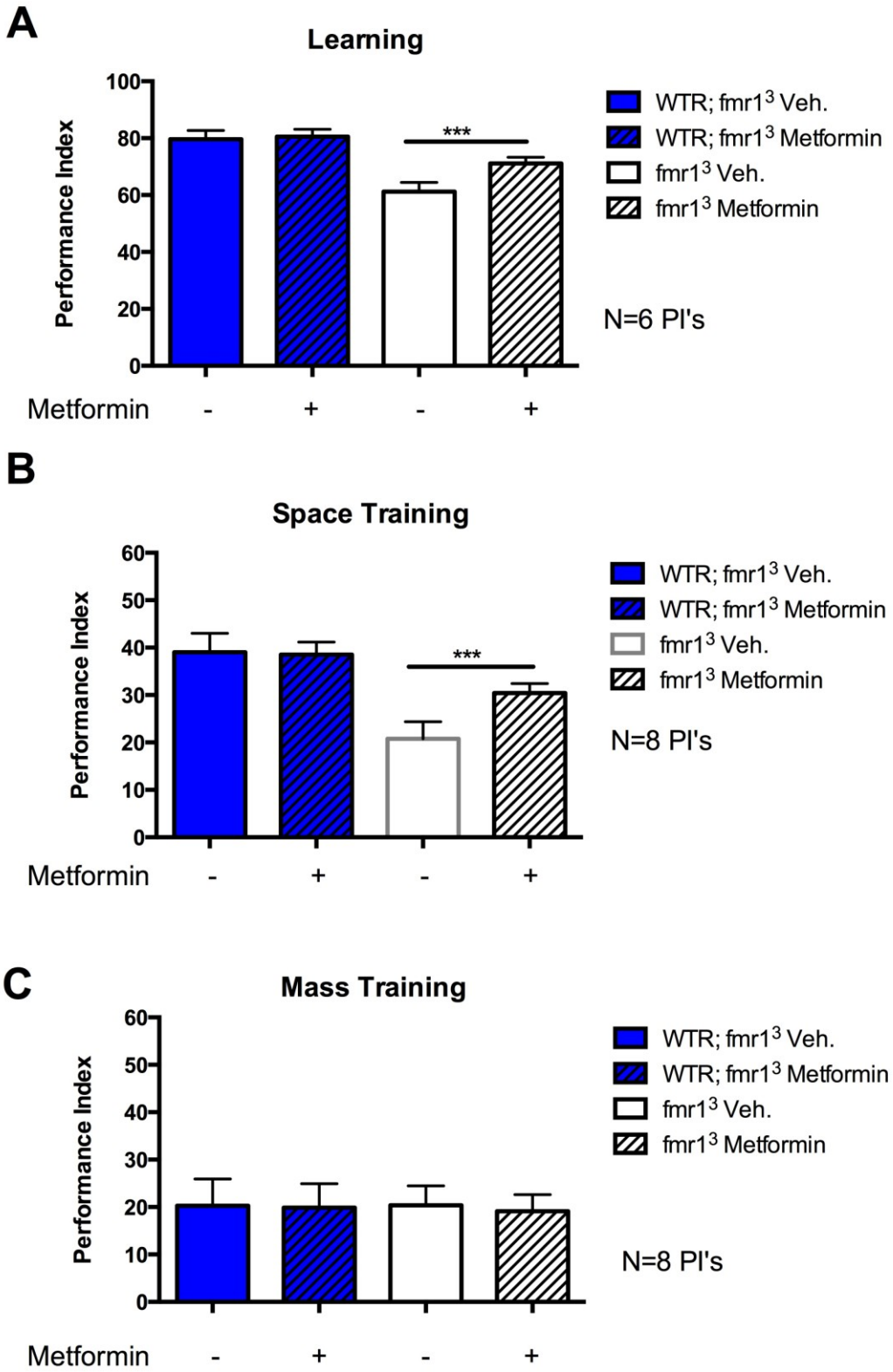
20
0
*
Vehicle
1 mM Metformin
5 mM Metformin
10 mM Metformin
N = 4 PI's

Figure 5-1. 1mM dose of Metformin improves Learning capabilities of FX Drosophila.

Drosophila model of Fragile X were given different amounts of Metformin, 1mM, 5mM and 10mM in order to determine which concentration produced the greatest effect. FMR^{B55} flies given 1mM dose of Metformin perform significantly better in Learning compared to Vehicle and the 5mM and 10mM doses. (One-way ANOVA, Tukey's post hoc test) $p=0.0136$; $N=4$ PI per treatment. All graphs depict mean ± s.e.m.

Figure 5-2


A
Learning
Performance Index
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Metformin - + - +
WTR; fmr1 3 Veh.
WTR; fmr1 3 Metformin
fmr1 3 Veh.
fmr1 3 Metformin
N=6 Pl's
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Space Training
Performance Index
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Metformin - + - +
WTR; fmr1 3 Veh.
WTR; fmr1 3 Metformin
fmr1 3 Veh.
fmr1 3 Metformin
N=8 Pl's
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Mass Training
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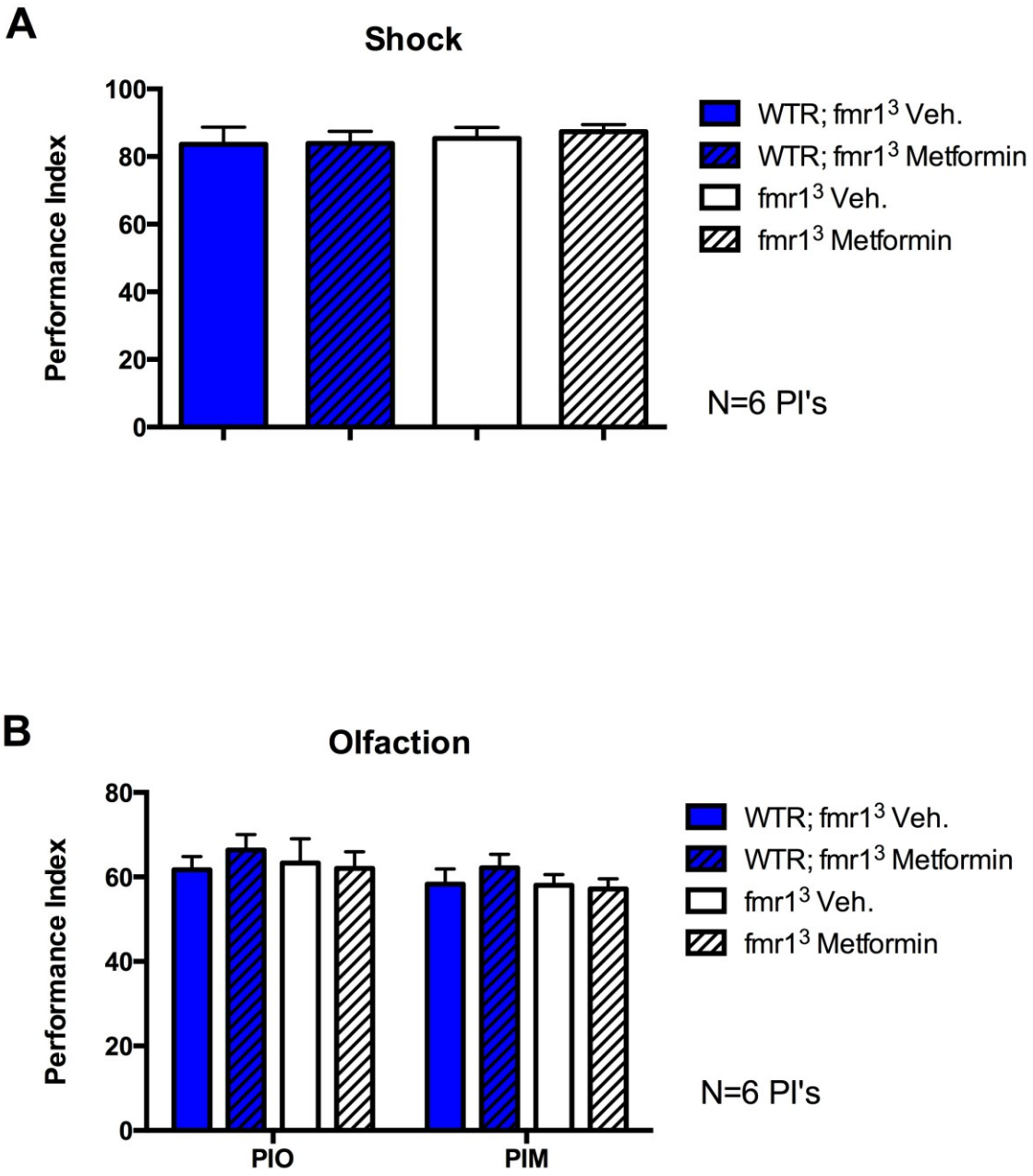
Metformin - + - +
WTR; fmr1 3 Veh.
WTR; fmr1 3 Metformin
fmr1 3 Veh.
fmr1 3 Metformin
N=8 Pl's

Figure 5-2. Metformin rescues the learning and memory defects observed in dfmr1 mutants. A) Immediate olfactory conditioning memory, named learning, was significantly improved in Drosophila mutant $dfm1^3$ ($dfmr1^3$) after being administered a 1mM dose of Metformin ($p = 0.00098$). No effect was observed in their genetic controls containing a genomic rescue fragment for dfmr1 (WTR; $dfmr1^3$). (One-way ANOVA, Tukey's post hoc test) N = 6 PIs per group. **B)** One day memory after spaced training is significantly improved in Drosophila mutant $dfm1^3$ ($dfmr1^3$) after being administered a 1mM dose of Metformin. No effect was observed in their genetic controls containing a genomic rescue fragment for dfmr1 (WTR; $dfmr1^3$). (One-way ANOVA, Tukey's post hoc test, $p = 0.00018$) N = 8 PIs per group. C) One day memory after massed training did not differ. (One-way ANOVA, Tukey's post hoc test) N=8 PI's per group. All graphs depict mean ± s.e.m.

Figure 5-3


A
Shock
Performance Index
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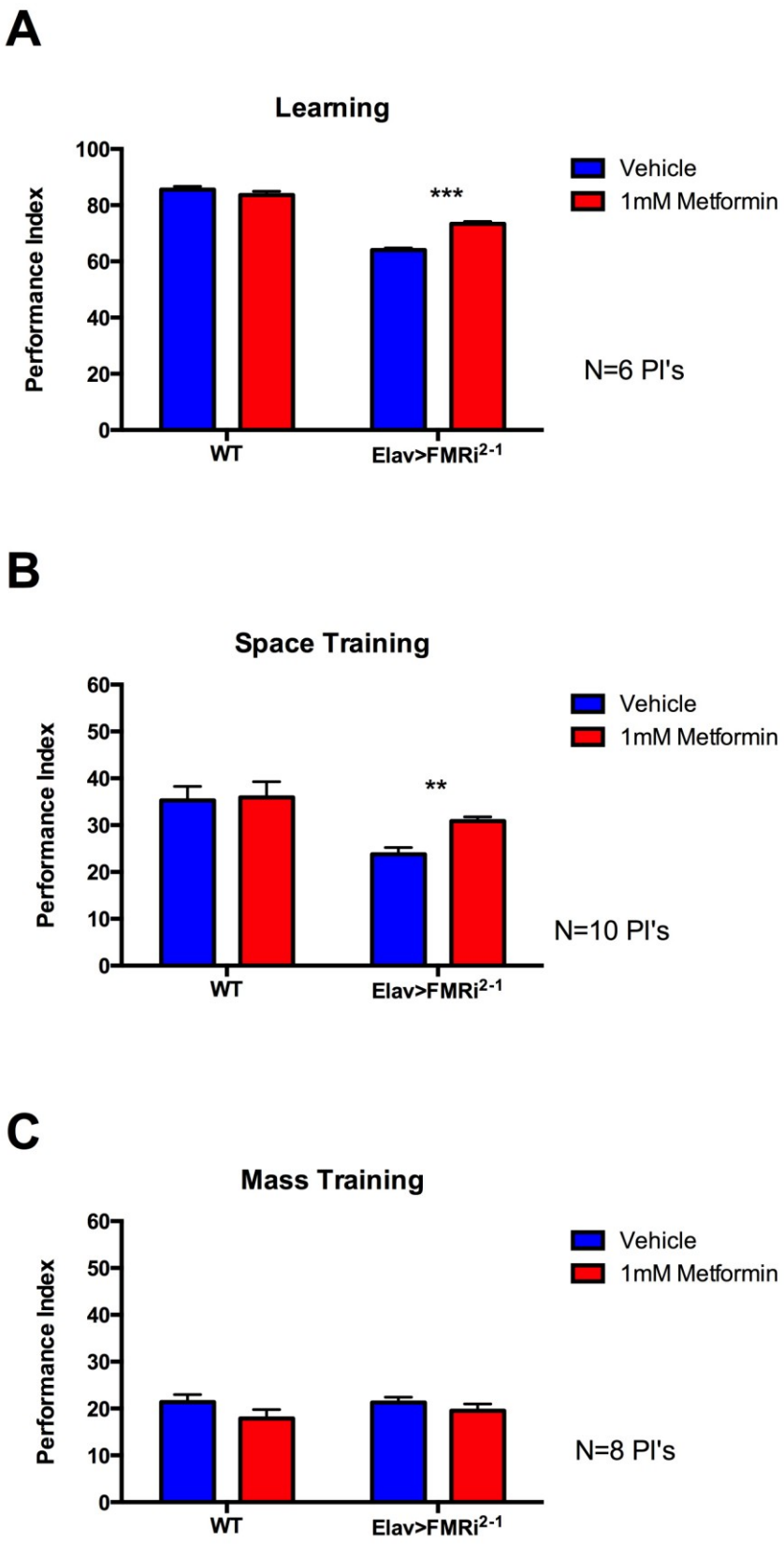
WTR; fmr1 3 Veh.
WTR; fmr1 3 Metformin
fmr1 3 Veh.
fmr1 3 Metformin
N=6 PI's
B
Olfaction
Performance Index
80
60
40
20
0
PIO
PIM
WTR; fmr1 3 Veh.
WTR; fmr1 3 Metformin
fmr1 3 Veh.
fmr1 3 Metformin
N=6 PI's

Figure 5-3. Metformin does not impact the sensory functions of FMR1^{3}. A) 1-3 days old Drosophila mutant dfm1^{3} (dfmr1^{3}) as well as their genetic controls containing a genomic rescue fragment for dfmr1 (WTR; dfmr1^{3}) present with normal shock avoidance with vehicle and 1 mM metformin treatment. (One-way ANOVA, Tukey's post hoc test) p=0.3043; N= 6 PI per genotype. B) 1-3 days old Drosophila mutant dfm1^{3} (dfmr1^{3}) as well as their genetic controls containing a genomic rescue fragment for dfmr1 (WTR; dfmr1^{3}) present with normal avoidance to the odors (OCT= PIO or MCH= PIM) used in the classical conditioning experiments. (One-way ANOVA, Tukey's post hoc test) p=0.9936; N= 6 PI per genotype.

Figure 5-4


A
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Performance Index
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WT
Elav>FMRi2-1
Vehicle
1mM Metformin
N=6 Pl's
B
Space Training
Performance Index
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Elav>FMRi2-1
Vehicle
1mM Metformin
N=10 Pl's
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Mass Training
Performance Index
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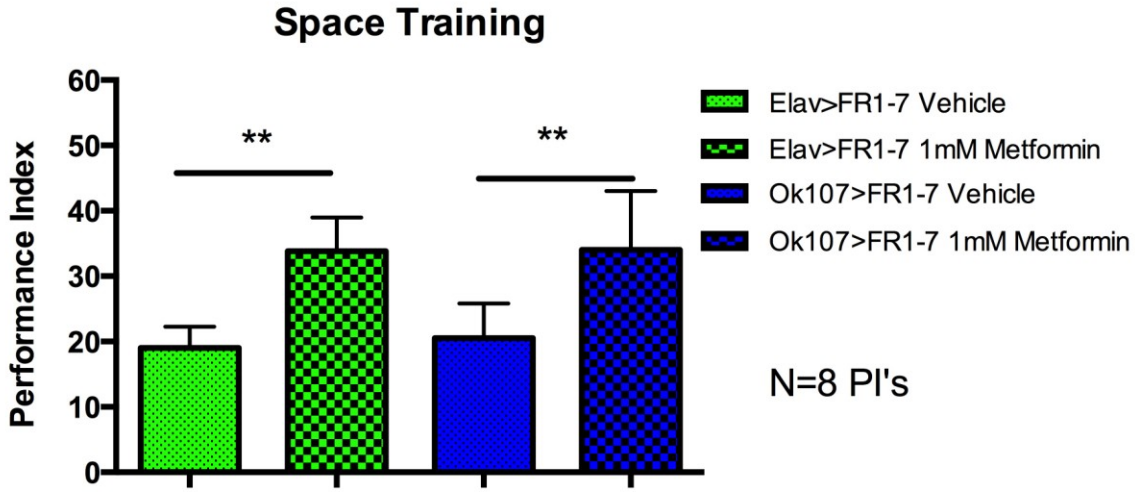
WT
Elav>FMRi2-1
Vehicle
1mM Metformin
N=8 Pl's

Figure 5-4. Metformin rescues learning and memory defects observed after pan neuronal knockdown of dfmrp. **A)** Administration of Metformin at 1mM dose improves learning and long-term memory in Elav Gal4; UAS-FMR RNAI$^{2\text{-}1}$ flies. Learning was significantly improved in Elav Gal 4; RNAi-FMRP after being administered a 1mM dose of Metformin (One-way ANOVA, Tukey's post hoc test, $p = 0.00012$). N = 6 PIs per group. **B)** One day memory was significantly improved in Elav Gal 4; RNAi-FMRP flies after being given a 1mM dose of Metformin the day before training. (One-way ANOVA, Tukey's post hoc test, $p = 0.0015$) n = 8 PIs per group. **C)** One day memory after massed training did not differ. (One-way ANOVA, Tukey's post hoc test) N = 8 PIs per group.

Figure 5-5


Space Training
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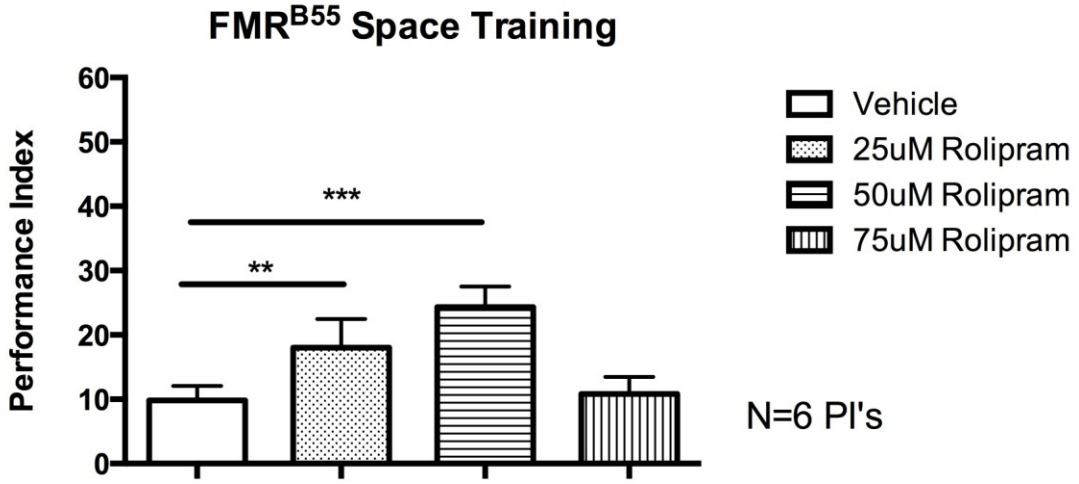
Elav>FR1-7 Vehicle
Elav>FR1-7 1mM Metformin
Ok107>FR1-7 Vehicle
Ok107>FR1-7 1mM Metformin
N=8 PI's

Figure 5-5. Metformin improves memory defects in Drosophila expressing the loss of FMRP in the Mushroom Body. Drosophila expressing loss of FMRP in a pan-neuronal manner (Elav) as well as in a mushroom body specific manner (OK107-GAL4) exhibit impairments in one-day memory. After being given 1mM dose of Metformin there is significant improvement in memory scores for both conditions. (One-way ANOVA, Tukey's post hoc test) Elav ** p=0.002, OK107-GAL4 ** p=0.005; N=6 PI's per group. All graphs depict mean ± s.e.m.

Figure 5-6


FMR^B55 Space Training
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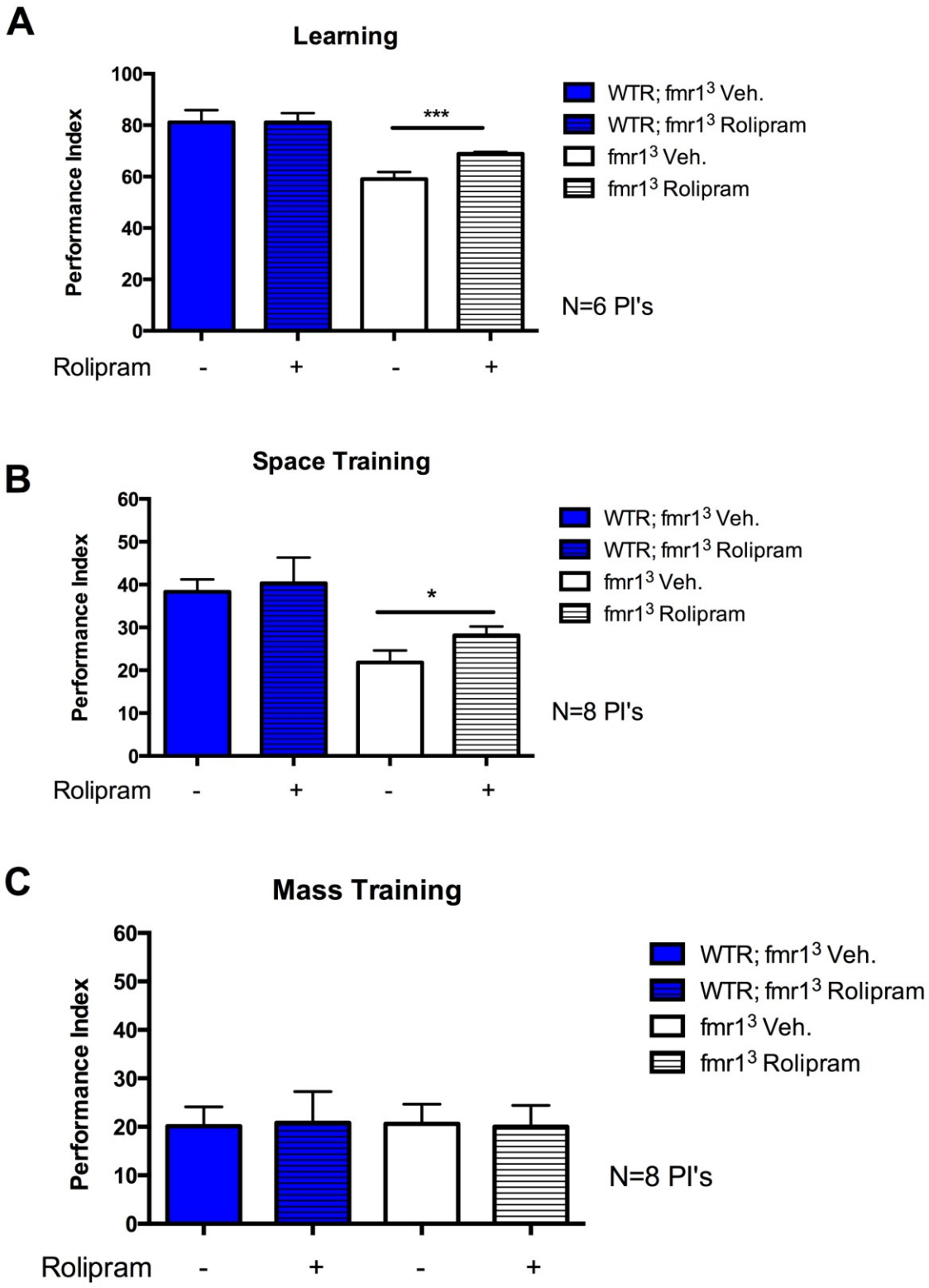
Vehicle
25uM Rolipram
50uM Rolipram
75uM Rolipram
N=6 PI's

Figure 5-6. 50μM dose of Rolipram improves one-day memory capabilities of FX Drosophila.

Drosophila model of Fragile X were given different amounts of Rolipram, 25μM, 50μM and 75μM in order to determine which concentration produced the greatest effect. FMR^{B55} flies given 50μM dose of Rolipram perform significantly better in Learning compared to Vehicle and the 25μM and 75μM doses. (One-way ANOVA, Tukey's post hoc test) ** p=0.0016 *** p=0.000159; N=6 PI per treatment. All graphs depict mean ± s.e.m.

Figure 5-7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Performance Index
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WTR; fmr1^3 Veh.
WTR; fmr1^3 Rolipram
fmr1^3 Veh.
fmr1^3 Rolipram
N=6 PI's
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*
WTR; fmr1^3 Veh.
WTR; fmr1^3 Rolipram
fmr1^3 Veh.
fmr1^3 Rolipram
N=8 PI's
Rolipram - + - +
C
Mass Training
Performance Index
60
50
40
30
20
10
0
WTR; fmr1^3 Veh.
WTR; fmr1^3 Rolipram
fmr1^3 Veh.
fmr1^3 Rolipram
N=8 PI's
Rolipram - + - +

Figure 5-7. Rolipram rescues the learning and memory defects observed in dfmr1 mutants. A) Immediate olfactory conditioning memory, named learning, was significantly improved in Drosophila mutant $dfm1^3$ ($dfmr1^3$) after being administered a 50μM dose of Rolipram ($p = 0.0002$).No effect was observed in their genetic controls containing a genomic rescue fragment for dfmr1 (WTR; $dfmr1^3$). (One-way ANOVA, Tukey's post hoc test) N = 6 PIs per group. **B)** One day memory after spaced training is significantly improved in Drosophila mutant $dfm1^3$ ($dfmr1^3$) after being administered a 50μM dose of Rolipram. No effect was observed in their genetic controls containing a genomic rescue fragment for dfmr1 (WTR; $dfmr1^3$). (One-way ANOVA, Tukey's post hoc test, $p = 0.0381$) N = 8 PIs per group. C) One day memory after massed training did not differ. N=8 PI's per group. All graphs depict mean ± s.e.m.

Figure 5-8

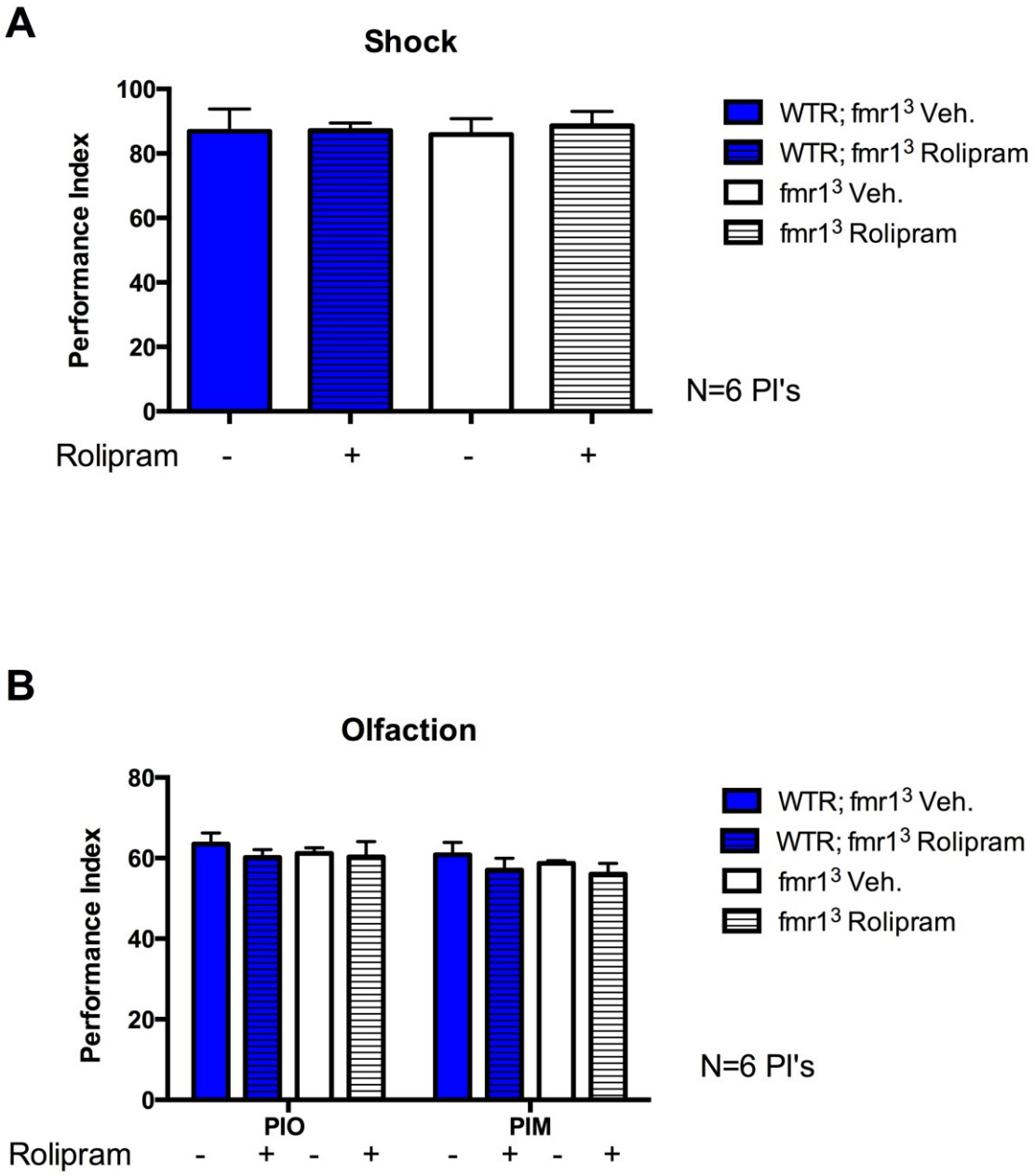
A
Shock
Performance Index
100
80
60
40
20
0
Rolipram - + - +
WTR; fmr1^3 Veh.
WTR; fmr1^3 Rolipram
fmr1^3 Veh.
fmr1^3 Rolipram
N=6 Pl's
B
Olfaction
Performance Index
80
60
40
20
0
PIO
PIM
Rolipram - + - + - + - +
WTR; fmr1^3 Veh.
WTR; fmr1^3 Rolipram
fmr1^3 Veh.
fmr1^3 Rolipram
N=6 Pl's

Figure 5-8. Rolipram does not impact the sensory functions of FMR1^{3}. A) 1-3 days old Drosophila mutant dfm1^{3} (dfmr1^{3}) as well as their genetic controls containing a genomic rescue fragment for dfmr1 (WTR; dfmr1^{3}) present with a normal shock avoidance with vehicle and 50μM dose of Rolipram. (One-way ANOVA, Tukey's post hoc test) p=0.9161; N=6 PI per genotype B) 1-3 days old Drosophila mutant dfm1^{3} (dfmr1^{3}) as well as their genetic controls containing a genomic rescue fragment for dfmr1 (WTR; dfmr1^{3}) present with normal avoidance to the odors (OCT= PIO or MCH= PIM) used in the classical conditioning experiments, with vehicle and 50μM dose of Rolipram. (One-way ANOVA, Tukey's post hoc test) p=0.3680; N=6 PI per genotype.

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Chapter 6. GENERAL CONCLUSIONS

6.1 Conclusions

Fragile X mental retardation syndrome, caused by the absence of fragile x protein (FMRP) is the most common cause of heritable intellectual disability and autism in males. Lack of FMRP has been associated with increases in protein synthesis (Bear et al., 2004; Bolduc et al., 2008) and dysregulation of AKT-mTOR signaling (Sharma et al., 2010) along with dysfunctional signaling of Insulin pathways (Callan et al., 2012). Here we found that different targets within the AKT-mTOR pathway are also dysregulated in a Drosophila model of Fragile X. In addition, we show for the first time the dynamic response of the protein network to classical olfactory memory training. Indeed, after memory training these different protein targets react differently, p-AKT goes to normal levels, AKT does not undergo any change, and finally p70S6K remains higher in FX. This examines the possible causes for the learning and memory defects seen in this disease.

Our work identifies a key role for Insulin signaling in the formation of olfactory memory in Drosophila. Both the Insulin-like receptor and Chico (Insulin receptor substrate) play a role in the formation of memories. Furthermore, we show that the memory defects phenocopy the defects observed in dfmr1 mutants, ie defects in learning and more severe defect in protein synthesis dependent memory.

Finally, our work shows for the first time that affecting the insulin signaling can rescue partially memory defects in d*fmr1* mutant flies. This is very important as it provides a novel avenue for future treatment options.