Cyclic Adenosine Monophosphate (cAMP) and Fragile X Mental Retardation Protein (FMRP) Mediate Avoidance Behaviour in Drosophila

by

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Abstract

Fragile X Syndrome (FXS) is neurodevelopmental disorder caused by a trinucleotide CGG repeat expansion that leads to the methylation and transcriptional silencing of the Fragile X mental retardation 1 (*fmr1*) gene. This results in the loss of Fragile X mental retardation protein (FMRP), an mRNA-binding protein that functions in neuronal mRNA metabolism, namely in the translation of neuronal mRNAs involved in synaptic structure and function. FXS is the most common form of inherited intellectual disability and the largest single genetic cause of autism, affecting 1 in 4000 males and 1 in 8000 females. FMRP plays a crucial role in neural circuit patterning/formation and the regulation of key signalling pathways. FXS is a syndrome is characterized by misregulation of protein synthesis and dysfunction within multiple signalling pathways. Here we implicate two signalling pathways underlying Fragile X Syndrome neuropathology, the cAMP signalling pathway and the Wnt signalling pathway, in cognitive defects.

In chapter 2 and 3, we show that FMRP is required for Drosophila stress odour (dSO) avoidance behaviour; dSO is an odour emitted when flies are subjected to electrical or mechanical stress, elicits an innate avoidance behavioural response by *Drosophila.* Our results suggest that FMRP is required developmentally, specifically in the mushroom body for higher-order processing, in the establishment of neuronal networks and in the regulation of the cAMP signalling cascade that mediates *Drosophila* stress odour (dSO) sensory processing and avoidance behaviour in *Drosophila*. Furthermore we show that cyclic adenosine monophosphate (cAMP) is required for avoidance and identify the cAMP cascade as a key signalling pathway underlying avoidance behaviour dysfunction in FXS. Through pharmacological intervention targeting the misregulated cAMP pathway we show that avoidance behaviour can be rescued in FXS flies, demonstrating the ability to ameliorate a developmental abnormality.

In chapter 4 we show that over-expression of armadillo/β-catenin results in learning and long-term memory defects and likely contributes to FXS pathology. Furthermore we demonstrate that FXS flies exhibit a learning reversal and long-term memory reversal defect, which may be a result of abnormal armadillo/β-catenin expression resulting in synaptic function and remodeling defects.

Preface

This thesis is original work by Alaura Androschuk. No part of this thesis has been previously published. Chapter 2 and 3 of this thesis are included in manuscript entitled “Information Processing by the Olfactory System in Drosophila Requires FMRP” currently under review for publication. All research represented in the manuscript was conducted by myself, including data collection and analysis, and manuscript composition.

Dedication

This thesis is dedicated to my sister, Tara and my friend, Reilly.
Thank you for your endless support.

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List of Abbreviations

AC	Adenylate cyclase
AKAPs	A kinase anchoring proteins
Akt	Protein kinase B
AL	Antennal lobe
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
aNSC	Adult neural stem cell
APC	Adenomatous polyposis coli
APL	Anterior paired lateral
ASD	Autism spectrum disorders
ATP	Adenosine triphosphate
AU	Adenylate-uridylate
Ca^{2+}	Calcium
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNG	Cyclic nucleotide-gated
CNS	Central nervous system
$CO_{2(g)}$	Carbon dioxide
CREB	cAMP response element-binding protein
CS+	Conditioned stimulus
CS-	Unconditioned stimulus
CYFIP1	Cytoplasmic FMRP-interacting protein
DC0	Drosophila cAMP-dependent protein kinase 1
dfmr1^{3}*WTR*	dfmr1^{3} flies containing a wild-type rescue transgene
dnc^{1}	Dunce
DPM	Dorsal paired medial
dSO	Drosophila stress odour
Dvl	Dishevelled
E	Emitter
eIF4F	Eukaryotic initiation factors 4F
eIF4F	eIF4A-eIF4G-eIF4E
eIF4A-eIF4G-eIF4E	Eukaryotic translation initiation factors 4A-eukaryotic translation initiation factors 4G-

	eukaryotic translation initiation factors 4E
EPAC	Exchange protein directly activated by cAMP
FMRP	Fragile X Mental Retardation Protein
FXS	Fragile X Syndrome
FXOPI	Fragile X-related primary ovarian insufficiency
FXTAS	Fragile X-associated tremor/ataxia syndrome
Fz	Frizzled receptor
GABA	-Aminobutyric acid
GEF	Guanine-nucleotide-exchange factors
GPCR	G protein-coupled receptor
Gr21a	Gustatory receptor 21a
Gr63a	Gustatory receptor 63a
GSK-3β	Glycogen synthase kinase-3β
IBMX	3-isobutyl-1-methylxanthine
ID	Intellectual disability
IGF-1r	Insulin-like growth factor 1 receptor
InS	Insulin signalling
IQ	Intelligence quotient
JNK	c-Jun-N-terminal kinase
K^+	Potassium
KH	hnRNP K homology
LH	Lateral horn
LiCl	Lithium chloride
LN	Local interneurons
LTD	Long-term depression
LTP	Long-term potentiation
MAP1b	Microtubule-associated protein 1B
MB	Mushroom body
MCH	3-Methylcyclohexanol
mGluR	Metabotropic glutamate receptors
miRNA	microRNA
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein
mTOR	Mechanistic/mammalian target of rapamycin
NDF	N-terminal domain region
NFACT	Nuclear factor of activated T cells
NGS	Normal goat serum

NMJ	Neuromuscular junction
NMDAR	N-methyl-D-aspartate receptor
OCT	3-Octanol
ORNs	Olfactory receptor neurons
OR	Odorant receptor
PB	Processing bodies
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with TritonX-100
PCR	Polymerase chain reaction
PDE	Phosophodiesterase
PFA	Paraformaldehyde
PI	Performance index
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PN	Projection neurons
Pre-mRNA	Precursor mRNA
R	Responder
Rac1	Ras-related C3 botulinum toxin substrate 1
RGG Box	Arginine/glycine-rich RNA-binding motif
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNPs	Ribonucleoprotein particles
rnRNP-K	Ribonucleoprotein K homology
rut^{1}	Rutabaga
Ror2	Orphan receptor tyrosine kinase
Ryk	Tyrosine kinase-like receptor
SG	Stress granule
sLNvs	Small ventrolateral neurons
SSRIs	Serotonin reuptake inhibitors
Tcf/Lef	T-cell factor/lymphoid enhancer factor
UTR	Unranslated region
WT	Wild-type
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
6020	dnc^{1}/Dunce

8-CPT	8-(4-Chlorophenylthio)adenosine 3’-,5’-cyclic monophosphate sodium salt
9404	rut[1]/Rutabaga

CHAPTER 1-Introduction to Fragile X Syndrome (FXS)

1.1 Fragile X Syndrome (FXS)

Fragile X Syndrome (FXS) is the most common form of inherited intellectual disability, with an incidence of 1:4000 males and 1:8000 females (*1*). FXS is caused by the loss of the Fragile X Mental Retardation protein (FMRP) due to the presence of CGG trinucleotide repeat expansion in the 5'-untranslated promoter region of the *FMR1* gene, resulting in methylation and transcriptional silencing of the gene (*2, 3*). The CGG repeat is highly polymorphic, with unaffected individuals possessing 6-60 repeats with 30 being the most common (*2*). Individuals harbouring 60-200 repeats are considered to have the premutation allele. The full mutation results when the repeat number is greater than 200, and leads to the hypermethylation and silencing of *FMR1 (4).* Full mutations are completely penetrant in men, and 50% penetrant in women (*5*). Premutation alleles are unstable when transmitted to offspring, which can give rise to repeat expansions outside of the permutation range and may give rise to the full mutation in women (*5*). Premutation carriers may have Fragile X-associated tremor/ataxia syndrome (FXTAS) a late-onset neurodegenerative disease that is characterized by progressive cerebellar gait ataxia, intention tremor, and cognitive decline (*6*). Premutations can also result in Fragile X-related primary ovarian insufficiency (FXOPI) in which carriers experience the onset of menopause at or before the age of 40 (*7, 8*).

1.2 Fragile X Mental Retardation Protein (FMRP)

Fragile X Mental Retardation Protein (FMRP) is an RNA-binding protein involved in mRNA regulation. FMRP interactions with RNA are mediated through four RNA-binding motifs; two ribonucleoprotein K homology (hnRNP-K) domains (KH domains; KH1 and KH2), an arginine/glycine-rich RNA-binding motif (RGG box), and an RNA-binding domain located in the N-terminal domain region of the protein (NDF) (*9-13*). FMRP recognizes secondary RNA structures on target mRNAs, 'kissing complexes' and G-quadruplexes, which mediate binding to KH domains and the RGG box respectively (*12, 14*).
A isoleucine to asparagine substitution at residue 304 in the KH2 domain of FMRP disrupts interactions with secondary RNA structures and associations with polyribosomes, resulting in a severe form of FXS

(*13, 15, 16*). Loss of FMRP's RNA-binding capability and association with polyribosomes suggests that loss of translational regulation by FMRP results in FXS.

FMRP expression is ubiquitous throughout the CNS, where it forms part of messenger ribonucleoprotein (mRNP) complexes than can associate with polyribosomes in an RNA-dependent manner (*9, 16*). Phosphorylation of FMRP may modulate translational state of FMPR-associated polyribosomes as unphosphorylated FMRP is associated with actively translating polyribosomes and phosphorylated FMRP is associated with stalled polyribosomes (*17*). FMRP regulates translation by inhibiting the initiation and elongation phases of translation and through the RNA interference (RNAi) pathway. During translation FMRP inhibits the recruitment of translation initiation machinery or cause ribosome stalling effectively inhibiting initiation and elongation of translation respectively (*18, 19*). FMRP binds to the 3' UTR of target mRNAs where it mediates binding of complementary microRNA (miRNA) and associates with the RNA-induced silencing complex (RISC) where translation is blocked through the degradation of the miRNA-RISC bound mRNA (*20*).

FMRP binds to 5% of brain mRNAs including its own mRNA (*13, 21, 22*). Many mRNA targets of FMRP are involved in neuronal development, structure, and function. FMRP contains a nuclear localization signal and a nuclear export signal and is thought to bind target mRNA in the nucleus to form a RNP complex that is transported to the cytoplasm, and transports target mRNAs to dendritic sites for translation in response to activation of group 1 metabotropic glutamate receptors (mGluR) (*23-25*).

1.3 Molecular Manifestations of Fragile X Syndrome (FXS)

FXS has emerged as a disorder of cognitive impairment resulting excess protein synthesis leading to immature dendritic spine architecture and synaptic dysfunction (*26*). FMRP is required for the proper development and wiring of neural circuits (*27*). FMRP functions in neurogenesis, axonal development, synaptogenesis, and activity-dependent modifications, including axon pruning and synaptic plasticity (*28*). FMRP regulates a number of mRNAs throughout the neuron, and controls their localization and translation in dendrites. As many FMRP target mRNAs encode proteins crucial for neuronal structure and function, the loss of regulation of FMRP target mRNAs results in the impairment of neuronal structure and

function leading to the formation of long, thin, and immature synaptic spines and defects in synaptic plasticity (*29*). In FXS the loss of FMRP results in the misregulation of target mRNAs and altered protein expression (*22*).

In addition, to abnormalities in neuronal circuits, loss of FMRP has been associated with the dysregulation of key signalling pathways, including mGluR, cyclic adenosine monophosphate (cAMP), mammalian/mechanistic target of rapamycin (mTOR), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and protein kinase B (Akt). FMRP regulates translational pathways activated by mGluR signalling (*30*). Loss of translational repression by FMRP results in aberrant mGluR signalling (*31*). In absence of FMRP, translation of FMPR target mRNAs is constitutively elevated and unresponsive to mGluR signalling; global protein synthesis is elevated by approximately 20% (*32*). mGluR couples to the mTOR pathway to regulate translation. In absence of FMRP, mTOR is elevated (*33*). PI3K is also a target of FMRP and downstream target of mGluR signalling, and is up-regulated resulting in elevated PI3K signalling to the mTOR pathway (*33*). A downstream target of PI3K and mTOR is Akt, which is also elevated in absence of FMRP. This misregulation of the mTOR/PI3K/Akt signalling cascade contributes to the pathogenesis of FXS (*34*). FMRP can act as both a translational repressor and activator (*35*). Loss of FMRP results in an elevation or decline of proteins whose function is required for normal neuronal functioning.

1.4 Clinical Manifestations of Fragile X Syndrome (FXS)

FXS is a neurodevelopmental disorder caused by the silencing of the *FMR1* gene located on the long-arm of the X chromosome; Xq27.3 (*36*). FXS affects all ethnicities and socio-economic levels, with a prevalence of 1 in 4000 males and 1 in 8000 females (*36*).

Clinical physical and behavioural manifestations can vary widely. Individuals with FXS exhibit varying degrees of developmental delay, intellectual disability, and behavioural and emotional difficulties. Males are usually more severely affected than females as FXS is an X-linked disorder (*37*).

Among the typical dysmorphic features associated with FXS are a long and narrow face, with a prominent jaw and ears, a broad forehead, and high palate (*38*). This feature usually appears in boys after 10 years of age. Affected males also have macroorchidism (*39*). Lax joints, flat feet, and hypotonia are also common (*40*). Females with FXS display similar physical features as males, but to a lesser degree (*37*).

Behavioural problems include speech and language difficulties, motor delays, hyperactivity, attention deficits, tactile defensiveness, and intellectual and learning disabilities (*41*). Intellectual disability (ID) describes impairments in brain function that causes intellectual limitations and functional defects in adaptive behaviours required for daily living with onset before 18 years. ID is characterized by an intelligence quotient (IQ) of less than 70; where an IQ of 50-70 represents mild ID, 35-50 represents moderate ID, and less than 35 represents severe ID. Individuals with FXS exhibit ID with an IQ of 20-60 (*42*).

Autistic-like behaviours such as hand-biting, hand-flapping, and poor eye contact are also common (*40*). Anxiety, mood disorders, and aggressive behaviour can also occur (*39*). Approximately one-third of males diagnosed with FXS are also diagnosed with autism, two-thirds meet the criteria for an autism spectrum disorder, and one-quarter suffer from epilepsy (*43-45*). Severity of ID in females with the full mutation is dependent on the proportion of activated normal *FMR1* alleles and expression of FMRP (*46*). Females with the full mutation may exhibit social anxiety and avoidance, mood liability, and depression, although behavioural and emotional characteristics are highly variable (*39, 47*).

Results from a survey of adults with FXS show that in adulthood, 44% of women with FXS demonstrated a high level of independence where one-third of women lived independently, or with a spouse/romantic partner, and required no assistance with daily living activities (*48*). Women also achieved a moderate level of education, receiving a high school diploma or better, and were successfully employed full or part-time. In contrast only 9% of men achieved a high level of independence, with the majority of men requiring a considerable amount of assistance with daily activates (*48*). The majority of men did not achieve a moderate level of education, most did not have a high school diploma, and only one-fifth had full-time jobs (*48*). Furthermore men had formed fewer friendships and less than half participated in

leisure activities. Women reported forming a large number of friendships and participated in multiple leisure activities (*48*).

FXS can be clinically diagnosed in children as young as 3 years old, but due to variability in clinical presentation, diagnosis of FXS requires both a differential diagnosis and genetic testing (*49-51*). Molecular diagnosis utilizes the combination of PCR and Southern Blot analysis to detect mutations; testing displays 99% sensitivity (*50*).

Interventions for FXS are symptom oriented, aimed at ameliorating symptoms to improve quality of life. Current treatments for FXS involve a combination of supportive therapies such as occupational therapy, speech therapy, individualized educational plans, and pharmacological intervention to address attention deficits, anxiety, or aggressive behaviors, as well as treatment of associated medical issues (*52*). Pharmacological interventions typically include stimulants, selective serotonin reuptake inhibitors (SSRIs), alpha-antagonists, and antipsychotics to treat hyperactivity and attention deficits, mood disorders, anxiety, obsessive-compulsive behaviours, and aggressive behaviours (*53*). Efficacy and side effects of pharmacological interventions vary greatly, and may not always be effective. Clinical assessment indicates that use of psychopharmacological treatment appears to be ameliorate behavioural symptoms in 50-70% of FXS patients, however only 40% of people felt use of psychopharmacological treatment significantly helped. Furthermore 20% of people reported that psychopharmacological treatment was not helpful (*53*). There remains a need for better treatments and intervention strategies.

1.5 Drosophila Neurobiology

Despite the substantial evolutionary divergence between humans and flies, Drosophila has proven to be an invaluable model for elucidating the molecular mechanisms underlying neurological disorders (*54*). Drosophila and humans share similarities in key physiological processes including gene expression, synaptogenesis, neurotransmission, and subcellular trafficking (*54*).

The Drosophila genome exhibits a high degree of conservation of genes and signalling pathways. 75% of known human disease genes have related sequences in Drosophila, with 73% of identified ID genes

being conserved in Drosophila (*55-57*). The Drosophila genome also has minimal genetic redundancy compared to mammalian models (*58*). Moreover the Drosophila nervous system exhibits a moderate level of complexity with approximately 100,000 neurons, compared to the approximately 100 billion in humans, that form circuits for distinctive functions and operate on the same fundamental principles as the brains of mammals (*59*). These circuits mediate complex behaviours that show both phenotypic and molecular similarities with humans including circadian rhythm, addiction, sleep, social interactions, and learning and memory (*57, 60-64*).

A variety of genetic tools are readily available to study mechanisms underlying ID. Through the use of the Gal4-UAS expression system, the molecular and behavioural phenotypes resulting from genetic manipulations can be characterized (*65*). The Gal4-UAS expression system utilizes a yeast derived transcriptional activator, Gal4, that binds to the UAS enhancer element to drive expression of the gene of interest immediately downstream of the UAS enhancer element (*65, 66*). Gene function can be manipulated through misexpression, loss of function, or gain of function, and the temporal and spatial requirements of a gene can be determined. Furthermore modifiers, enhancers or suppressors, of disease genes can also be identified through genetic screens. The use of Drosophila as a model also provides a quick and inexpensive means for high throughput screening for drug screening, due to generation of relatively large quantities of genetically identical progeny (*66, 67*).

FXS in the most common inherited ID in humans. Drosophila FMRP exhibits close homology with human FMRP in that it displays conserved structure with 56% conserved amino acid identity, RNA-binding properties, tissues and subcellular expression patterns, and conserved functional role as a translation repressor (*68, 69*). The Drosophila FXS model also has a more prominent phenotype than FXS mouse models, where both behavioral and neuronal phenotypes are subtle (*70*). In FXS mouse models cognitive and learning defects are mild, and defects in learning are not always observed and appear to be highly dependent on genetic background (*71, 72*). In contrast, FXS fly models display robust defects in learning and memory, as well as characteristic structural defects in neurons observed in humans, but not mouse models (*1, 70*). Other key pathological features of FXS have been successfully demonstrated in flies

including, abnormalities in synaptic structure and function, defects in courtship behaviour, social interaction impairments, as well as circadian rhythm defects (*63, 64, 73-76*).

Use of Drosophila for investigation into pathological mechanisms underlying FXS provides distinct advantages. Drosophila are relatively inexpensive to rear, have a short life span, produce large numbers of identical progeny, display both genetic and behavioural homology, and have a range of tools available for the spatial-temporal regulation of gene expression. The use of the FXS fly model has provided novel mechanistic insight into the disease (*70*).

1.6 Thesis Aims

FMRP functions in the establishment and maintenance of neuronal networks underlying complex cognitive functions, the loss of which results in dysfunction within neural circuits that modulate cognitive functions. This thesis examines how the loss of FMRP results in defects in avoidance behaviour as well as defects in learning reversal and long-term memory reversal.

In chapter 2 we determine how loss of FMRP affects avoidance behaviour. FMRP functions in nearly all aspects of neural circuitry crucial for information processing. First we utilized FXS mutants to characterize avoidance behaviour in absence of FMRP. We then used tissue specific drivers to knockdown or overexpress FMRP to determine the spatial and temporal requirements of FMRP in mediating avoidance behaviour.

In chapter 3 we examined the role of cAMP in mediating avoidance behaviour. In absence of FMRP, the cAMP pathway is mis-regulated and has been implicated in FXS pathology (*77-79*). We utilized cAMP mutants to demonstrate that disruption of cAMP homeostasis results in defects in avoidance behaviour. Moreover we wanted to determine if there was a genetic interaction between cAMP and FMRP in mediating avoidance behaviour. Our results show that avoidance behaviour defects caused by loss of FMRP can be ameliorated through pharmacological intervention targeting the dysregulated cAMP signalling pathway.

In chapter 4 we examine the effect of the overexpression of Armadillo/β-catenin in learning and long-term memory using the classical Pavlovian olfactory conditioning paradigm. We also utilize a modified classical Pavlovian olfactory conditioning paradigm to assay for learning reversal and long-term memory reversal defects in FXS flies and discuss how the overexpression of Armadillo/β-catenin may be implicated in learning reversal and long-term memory reversal defects observed in FXS flies.

CHAPTER 2-Fragile X Mental Retardation Protein (FMRP) is Required for Drosophila Stress Odourant (dSO) Avoidance Behaviour is Drosophila

2.1 Introduction

Drosophila rely on chemosensory cues to mediate key regulatory and adaptive behaviours. Chemosensory cues can be processed in the olfactory system, which processes volatile odours, or the gustatory system, which processes odours through direct contact (*80*). Drosophila stress odour (dSO), an odourant emitted when flies are subjected to electrical or mechanical stress, elicits an innate avoidance behavioural response by Drosophila (*81*). Drosophila stress odorant (dSO) processing occurs through the olfactory system, as demonstrated by the surgical removal of antennae resulting in abolished avoidance response (*81*). Conveyance of olfactory information through the olfactory system occurs in a hierarchal manner (Figure 2-1). Antennae are innervated by dendrites of olfactory sensory neurons (ORNs) (*82-84*). ORNs transmit sensory input to the antennal lobe, primary olfactory sensory centre. In the antennal lobe ORNs converge onto glomeruli according to the expression of odourant receptors (*85*). Each glomerulus also consists of projection neurons, the dendrites of which form excitatory synaptic connections with ORNs, and local neurons, which form a network to connect glomeruli laterally (*86, 87*). Local neurons cannot participate in electrical signal transduction as they lack axons, however they can regulate signal transduction through the release of the neurotransmitter □-aminobutyric acid (GABA) (*88, 89*). Release of GABA results in the hyperpolarization of projection neurons and local neurons and decreased firing rates (*88, 90, 91*). Within a glomerulus an odour-evoked depolarization of ORNs and projection neurons inhibits neurotransmission in other glomeruli mediated by GABA release of local neurons (*90, 92*). Projection neurons send axonal projections to the mushroom body (MB) where they form synapses with MB Kenyon cells (*93, 94*). Synaptic connections formed between MB Kenyon cells and cholinergic boutons of antennal lobe are organized into microglomeruli (*95*). Within each microglomeruli, Kenyon cells form multiple connections with antennal lobe projection neuron axons and GABAergic dendrites from a single anterior paired lateral (APL) neuron that collects output from all Kenyon cell axons (*95-97*).

2.1.1 Role of Fragile X Mental Retardation Protein (FMRP) in Neural Development

Development of neuronal connectivity underlying sensory processing within olfactory systems requires an intricate series of precisely coordinated events during development. FMRP is mainly localized to the cytoplasm and can be found throughout the entire neuron including in the cell body, dendrites, and in low levels in the nucleus (*1, 16, 25, 98*). FMRP is required during neurogenesis, axon pathfinding, synaptogenesis, activity-dependent synaptic pruning, and use-dependent circuit remodeling for the establishment of neural circuitry.

Neurogenesis

Neurogenesis is the process by which neural stem cells produce progenitor cells that give rise to all the neurons in the CNS (*99*). FMRP regulates neural stem cell differentiation, proliferation, and maintenance (*100, 101*). During development, stem cells enter a phase of quiescence marked by inactivity in which cells exit the cell cycle, and do not reenter until prompted. FMRP may modulate stem cell exit from quiescence by initiating signalling PI3K/Akt signalling (*100, 102*). Furthermore FMRP is required to establish the correct number of neurons and glia within the CNS (*103*).

mRNA Transport, Stability, and Translation

FMRP has three RNA-binding motifs—two hnRNP K homology (KH) domains and a glycine-arginine-rich domain (RGG box) that allows it to function in the metabolism of mRNA (*9, 104*). The KH domains recognize and bind to “kissing-complex” motifs in target RNA (*105*). The RGG box recognizes and binds to G-quartet loops on target RNA (*106*). FMRP plays a crucial role in neural circuit formation by functioning in key mechanisms involved in mRNA export, localization, and transport (*107*).

FMRP forms part of a large messenger ribonucleoprotein (mRNPs) complexes. mRNPs contain mRNA and non-coding RNA . The composition of mRNPs bound to mRNA dictate processing and localization. FMRP functions in mRNA transport by binding to specific mRNPs complexes, which are then subsequently transported along microtubules to translational sites via kinesin and dynein (*108-111*). During transport mRNA translation is repressed in mRNP complexes (*112*). Once localized to appropriate synaptic sites, it has been suggested FMRP releases mRNA from the complex into polyribosomes for

translation (*113, 114*). Loss of FMRP results in reduced mRNP complex motility, directionality, and altered localization of target mRNAs (*115*). FMRP likely functions in mRNA transport in a dose-dependent manner as loss of FMRP results in decreased recovery of mRNP complexes from cytoplasmic pools, while overexpression results in increased recovery of mRNP complexes (*115*). FMRP exhibits affinity for specific mRNA involved in neuronal development and synaptic function (*116*).

In the nucleus, FMRP associates with nuclear export proteins, nuclear mRNA, and pre-mRNA (*117*). FMRP contains nuclear localization and nuclear export signals suggesting that FMRP may function in the transport of mRNA out of the nucleus (*23, 118*).

FMRP regulates the stability of mRNA by promoting or preventing degradation (*119, 120*). Regulation of mRNA within neurons occurs in RNA granules that function in transport, storage, translation, and degradation of mRNA. Processing bodies (PB) and stress granules (SG) are located in the cytoplasm where mRNAs are stored for re-entry into translation or degraded respectively (*121, 122*). FMRP is localized within RNPs, PBs, and SG and shares several characteristics of other mRNA modulators of mRNA stability, including the recruitment of FMRP to target mRNA through AU-rich elements (*123-126*). Although the exact mechanism through which FMRP promotes mRNA stability requires elucidation, it is clear that mRNA profiles are altered in FMRP-deficient cells (*22*). Loss of FMRP results in decreased localization of mRNAs at dendritic and synaptic site for subsequent translation. Defects in mRNA localization has been linked to several neurological disorders including Fragile X Syndrome (*127*).

FMRP is a negative regulator of translation in neurons (*69, 128*). FMRP is found in RNPs and associated with polyribosomes, where the RGG-box and KH domains mediate the binding to G-quartet loops and the “kissing-complex” on target mRNAs (*9, 12, 14, 22, 129*). Translation can be inhibited at the level of initiation by FMRP and by microRNA (miRNA) (*5, 20*). FMRP associates with miRNA that base pairs with mRNA targets in RNA-induced silencing complexes (RISC) leading to the destabilization and translational repression of mRNA (*5, 20, 130*). The RNAi/miRNA pathway has been suggested to be the main mechanism through which FMRP controls translation.

FMRP can also inhibit the initiation of translation by recruiting CYFIP1 (cytoplasmic FMRP-interacting protein) to target mRNAs in cap-dependent translation (*19*). Initiation of cap-dependent translation

requires the formation of the eIF4A-eIF4G-eIF4E (eIF4F) complex and it's subsequent binding to target mRNA and recruitment of translation initiation machinery (*19*). The FMRP-CYFIP1 complex binds to eIF4E to block the formation of the eIF4F complex and prevent translation initiation (*19*).

FMRP has also been proposed to regulate translation during the elongation phase by stalling ribosomes. FMRP contains a conserved serine residue, which when phosphorylated alters the functionality of the protein as phosphorylated FMRP is associated with stalled ribosomes (*17, 18, 131*). Interestingly, only the unphosphorylated form of FMRP is found to associated with components of the miRNA/RNAi pathway (*132*).

The phosphorylation of FMRP is regulated by mechanistic/mammalian target of rapamycin (mTOR) pathway, which also regulates cap-dependent translation (*133*). Phosphorylation of 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1) by mTOR results in the disassociation of 4E-BP1 from the eIF4F complex, allowing initiation of translation (*134*).

In the absence of FMRP the translation of FMRP target mRNAs are no longer repressed resulting in excess protein synthesis.

Neuronal Morphology

The majority of FMRP is associated with polyribosomes and localized to dendrites, axons, and axonal growth cones, suggesting that FMRP regulates the translation of key proteins involved in synaptic structure (*118, 131, 135-137*). FMRP functions in mRNP complexes to transport mRNA from neuronal soma to dendrites and axons, where mRNA is released from the complex and can be translated (*24, 108, 113*).

The establishment of neuronal networks relies on axonal pathfinding. At the end of each growing axon is a growth cone, which is a specialized structure that interprets extracellular guidance cues. Correct targeting of axons relies on spatially and temporally correct expression and interpretation of guidance molecules. FMRP is found in axons and growth cones localized with axon guidance factors (*24, 136, 138, 139*). FMRP is thought to function in axon guidance by regulating local protein synthesis of axon guidance factors within growth cones (*74, 136, 139*). Netrins, semaphorins, slits, and ephrins are guidance molecules that provide chemotrophic cues that direct growth cone motility through cytoskeleton

remodeling (*140-143*). Guidance cues mediate growth-cone trajectories by altering growth cone cytoskeletal dynamics through the assembly, disassembly, and stability of cytoskeletal components, including microtubule and actin, as well as the attachment of the growth cone to the substratum (*144*). Axon guidance molecules also function in other aspects of neuronal network establishment including axon and dendrite branching, neuronal migration, synaptogenesis, and neuronal and axonal degeneration and regeneration (*144*).

Axon growth rate can be regulated by a number of growth factors, cytokines, and morphogens, and rely on secondary messenger systems for modulation, namely as cAMP and cGMP signalling (*145-147*). The mTOR, PI3K, and Akt signalling pathways have all been implicated in mediating guidance cue signalling through their interaction in FMRP (*148, 149*).

FMRP negatively regulates axonal branching and dendritic elaboration (*150*). FMRP is also required during activity-dependent axon pruning, in which inadequate synaptic connections are removed through local lysosomal degeneration by glial cells and microtubule structure is disrupted in order to optimize neuronal networks and behavioural output (*151-153*). Sensory input enhances FMRP expression and function in synapses and drives changes in synaptic connections both during development of post-eclosion (*154, 155*). FMRP is strongly expressed in glia during development, following which time expression declines, suggesting that FMRP is required in glia for proper synaptic structure and maturation (*156*). Deficits in activity-dependent axon pruning have been associated with neurological disorders including ASD and FXS (*151, 157*).

FMRP regulates multiple aspects of neuronal architecture including dendritic elaboration, axonal branching, and synaptogenesis (*150*). Loss of FMRP results in enlarged and irregular synaptic boutons, and abnormal synaptic vesicle accumulation and altered neurotransmission due to pre- and post-synaptic defects (*150, 158*).

Regulation of Cytoskeleton

Neuronal morphology is linked to the cytoskeleton. FMRP functions in dendritic spine morphology by interacting with mRNAs involved in cytoskeleton regulation. The translation of key proteins regulating

cytoskeleton are repressed by FMRP, including MAP1b, Rac1, and Profilin (*76, 159-162*). MAP1b is negatively regulated by FMRP and is required for dendritic, axonal, and synaptic formation (*163, 164*). MAP1b is the first microtubule-associated protein to be expressed during neurogenesis and functions in growth cone motility and neurtie extension (*136, 162, 165, 166*). Profilin is required for the reorganization of cytoskeleton components involved in synaptic structure (*167*). Profilin is maintained at high levels throughout development and falls rapidly post-eclosion (*151*). In the absence of FMRP, Profilin up-regulated and levels are consistently high throughout development and persists following eclosion (*151*). Rac1 also functions in cytoskeleton remodeling and maturation and maintenance of synapses (*168, 169*). The overexpression of each protein mimics the synaptic abnormalities observed in FXS, indicating the importance of FMRP as a translational regulator in the normal developmental of synaptic structure (*76, 159, 160*).

Signalling Pathways

Metabotropic glutamate receptors (mGluR) Signalling

mGluR receptors are family G-protein-coupled receptors that regulate synaptic transmission and neuronal excitability through glutamatergic neurotransmission (*31, 170*). FMRP is regulated by neurotransmission from mGluR1 and mGluR5 receptors, together known as group 1 mGluRs (*31, 171*). Activation of group 1 mGluRs receptors initiates a signalling cascade that leads to the recruitment of transcription factors, translation factors, and FMRP and associated mRNA to synaptic sites, where FMRP regulates translation of target mRNAs (*17, 25, 31*). mGluR signalling has been implicated in synaptogenesis and protein synthesis-dependent plasticity (*33*).

Mammalian/Mechanistic Target of Rapamycin (mTOR) Signaling

mTOR is a protein kinase involved in the regulation of cell growth, proliferation, autophagy, and translation (*172*). Key components of the mTOR signalling pathway are present at synapses and control local protein synthesis through the initiation of cap-dependent translation (Figure 2-2) (*173*). mTOR is regulated by group 1 mGluRs (*174, 175*). mTOR signalling functions in neuronal growth, synaptic plasticity, and regulation of downstream signalling including PI3K, Akt, and cAMP (*176, 177*).

Cyclic Adenosine Monophosphate (cAMP) Signalling

cAMP is a secondary messenger molecule that functions in growth cone steering, synapse formation, neurotransmission, synaptic plasticity, and neuronal excitability (*178*). Processing of sensory information requires the initiation of secondary messenger signalling cascades, including cyclic-nucleotide cascades like cAMP (*179, 180*). Regulation of cAMP occurs through adenylate cyclase (AC) and phosophodiesterase (PDE), which are involved in the synthesis or degradation of cAMP respectively (*181, 182*). FMRP regulates AC production, which is required for cAMP synthesis and activation of downstream effectors (*22*). cAMP has a number of downstream effectors that also function in neuron growth, regulation, and signalling, including protein kinase A (PKA) and the transcription factor cAMP response element-binding protein (CREB) (*183*). CREB has been proposed to regulate FMRP, possibly through activation of group 1 mGluRs (*184, 185*).

2.1.2 Neuropathology of Fragile X Syndrome

FMRP is part of a ribonucleoprotein (mRNP) complex that is associated with RNA granules throughout the CNS, where FMRP functions in the transport, stability, and translation of target mRNAs involved in the establishment and maintenance of neuronal networks and regulation of key signalling pathways. Dysregulation of FMRP-dependent translation has been proposed to be the mechanism of pathology underlying abnormal neuronal development and function, ultimately altering network activity and function.

FMRP-deficient neurons exhibit changes in gene expression where 198 genes are down-regulated, many of which function in neuronal differentiation and development, axon pathfinding, and axonogenesis (*186*). In addition many miRNAs are also down-regulated in absence of FMRP (*186*).

The majority of polyribosomes are localized at the base of dendritic spines, where they function in the local translation of proteins that regulate synapse morphology through interactions with other proteins or signalling cascades (*187*). *Fmr1* and FMRP are found in both neural soma and dendritic processes (*25*). Activation of mGluR signalling stimulates the production of FMRP and the recruitment of FMRP-containing RNA granules to synaptic sites for translation (*188*). FMRP functions in repressing neuronal

growth by negatively regulation dendritic spine growth and elaboration (*76, 150*). A pathophysiological hallmark of FXS is the presence of immature dendritic spines, which display abnormally long, thin, and tortuous morphology, which has been associated with cognitive impairment and dysfunction within neuronal networks in FXS (*189, 190*). Immature neuronal morphology is thought to be a result of defects in axon pruning. There are two periods of FMRP-dependent axon pruning, axonogenesis and activity-dependent pruning (*151*). FMRP expression is highest during these two periods (*151*). In absence of FMRP, axon pruning does not occur resulting in increased axonal growth and over-branching (*150*). Structural changes results in alterations in synaptic functions (*158, 191*).

FMRP and its mRNA have been found in synaptoneurosomes, synapses containing the pre- and postsynaptic termini containing synaptic vesicles and translational machinery respectively, where it is thought to function in synaptogenesis and *de novo* protein synthesis required for synaptic plasticity (*29*). In absence of FMRP, there is a global increase in protein synthesis corresponding to loss of translational control of FMRP targets, and an increase in neuronal circuit excitability (*192*).

FMRP is required both pre- and post-synaptically (*158*). Loss of FMRP results in altered composition of receptors, ion channels, and neurotransmitters at synapses (*193*). Absence of FMRP results in molecular changes and disruptions in network activity of several signalling pathways, whose altered function have been implicated in the pathogenesis of FXS including PI3K, mTOR, mGluR, InS, Akt, and cAMP (*31, 33, 34, 78, 79, 194*).

2.1.3 Drosophila Stress Odourant (dSO) Avoidance Behaviour

Drosophila stress odour (dSO), an odourant emitted when flies are subjected to electrical or mechanical stress, elicits an innate avoidance behavioural response by Drosophila (*81*). A main component of dSO is $CO_{2(g)}$ (*81*). Flies exhibit innate avoidance behaviour to $CO_{2(g)}$ in a dose-dependent manner, suggesting that other repellent compounds comprise dSO (*81, 195*).

2.2 Materials and Methods

2.2.1 Drosophila Fly Stocks

Fly stocks were maintained at 22°C on standard cornmeal yeast media from Cold Spring Harbor Laboratory. Wild-type stocks were backcrossed to w^{1118}*isoCJ1* for 6 generations. $dfmr1^{B55}$ flies were obtained from Dr. Kendal Broadie (Vanderbilt University). To eliminate background effects all fly stocks used were outcrossed to the wild-type strain w^{1118}*isoCJ1*. $Gal80^{ts}$*Elav-Gal4,* $dfmr1^{3}$ flies, and $dfmr1^{3}$ flies containing a wild-type rescue transgene ($dfmr1^{3}$*WTR*) were obtained from Dr. Tom Jongens (University of Pennsylvania). *Elav-Gal4, OK107-Gal4, C747-Gal4, MB247-Gal4, Feb170-Gal4*, and $Gal80^{ts}$*C747-Gal4* flies were obtained from Dr. Tim Tully. *Repo-Gal4* flies were obtained from Dr. Sarah Hughes (University of Alberta). Dunce (6020) and Rutabaga (9404) flies were obtained from Bloomington Stock Centre. *UAS-dfmr1* flies were obtained from Dr. Kendal Broadie (Vanderbilt University). Transgenic dfmrRNAi lines were generated as previously described (*196*).

Note that in Drosophila *dfrm1* is not X-linked and is located on the third chromosome. The $dfmr1^{3}$ fly genome contains a larger deletion in the *dfmr1* gene than the genome of $dfmr1^{B55}$ flies (*197*).

2.2.2 The Gal4-UAS Binary Expression System

In order to manipulate gene expression, we utilized the Gal4-UAS binary expression system. The Gal4-UAS system utilizes two modules to manipulate gene expression, a driver and a drivee. The driver, or Gal4 transgene uses a promoter to direct gene expression (*65*). The drivee, or UAS-transgene contains the upstream activating sequence from a yeast gal promoter, which can be used to target genes of interest (*65*). The Gal4 driver binds to the UAS-transgene to promote expression of the gene of interest under the control of the Gal4 transcription factor (*65*).

When required to control both spatial and temporal expression of genes, we utilized the temperature sensitive transcriptional repressor, $Gal80^{ts}$. $Gal80^{ts}$ restricts Gal4 expression by binding to carboxyl terminal of Gal4 (*198*). At 18°C $Gal80^{ts}$ represses Gal4 activity and expression of UAS-transgene (*199*). At temperatures above 30°C $Gal80^{ts}$ cannot repress transcription of Gal4 and the UAS-transgene of interest is expressed (*199*).

2.2.3 Genetic Crosses

To determine the spatial requirement of FMRP in mediating dSO avoidance, we used RNAi against FMRP in order to knockdown/reduce expression of the FMRP protein. Using the Gal4-UAS system we generated crosses were by mating *Elav-Gal4*, *OK107-Gal4*, *Feb170-Gal4, and MB247* virgin females to *UAS-dfmr1RNAi*$^{1-7}$ males. The progeny of the generated crosses were utilized for avoidance testing.

Pan-neuronal knockdown of FMRP
Elav-Gal4 virgin females x *UAS-dfmr1RNAi*$^{1-7}$ males
WT virgin females *x WT* males

Mushroom body knockdown of FMRP
MB247 virgin females x *UAS-dfmr1RNAi*$^{1-7}$ males
OK107-Gal4 virgin females x *UAS-dfmr1RNAi*$^{1-7}$ males
C747-Gal4 virgin females x *UAS-dfmr1RNAi*$^{1-7}$ males
WT virgin females *x WT* males

Central complex knockdown of FMRP
Feb170-Gal4 virgin females x *UAS-dfmr1RNAi*$^{1-7}$ males
WT virgin females *x WT* males

Glia knockdown of FMRP
Repo-Gal4 virgin females x *UAS-dfmr1RNAi*$^{1-7}$ males
WT virgin females *x WT* males

To evaluate the possibility of genetic background effects, double FMRP mutant flies were generated by mating *dfmr1*^{B55} females with *dfmr1*^{3} males.

dfmr1^{B55} x *dfmr1*^{3}
dfmr1^{B55} virgin females x *WT* males
dfmr1^{B55} virgin females x *dfmr1*^{3} males
WT virgin females x *dfmr1*^{3} males
WT virgin females *x WT* males

FMRP knockdowns to evaluate the temporal requirement of FMRP were generated by mating *Gal80*^{ts}*Elav-Gal4*, and *Gal80*^{ts}*C747-Gal4* virgin females with *UAS-dfmr1RNAi*$^{1-7}$ males.

Temporal knockdown of FMRP pan-neuronally

Gal80^{ts}Elav-Gal4 virgin females x *UAS-dfmr1RNAi$^{1-7}$* males

WT virgin females *x WT* males

Temporal knockdown of FMRP in the mushroom body

Gal80^{ts}C747-Gal4 virgin females x *UAS-dfmr1RNAi$^{1-7}$* males

WT virgin females *x WT* males

FMRP was overexpressed, and spatially and temporally restricted in flies by mating *Elav-Gal4*, *OK107-Gal4,* and *Gal80^{ts}Elav-Gal4* females to *UAS-dfmr1* males.

Pan-neuronal overexpression of FMRP

Elav-Gal4 virgin females x *UAS-dfmr1* males

WT virgin females *x WT* males

Mushroom body overexpression of FMRP

OK107-Gal4 virgin females x *UAS-dfmr1* males

WT virgin females *x WT* males

Temporal overexpression of FMRP pan-neuronally

Gal80^{ts}Elav-Gal4 virgin females x *UAS-dfmr1* males

WT virgin females *x WT* males

Genetic crosses utilizing Gal80^{ts} temperature sensitive drivers were raised at 18°C and incubated at 30°C for 4 days prior to avoidance testing to activate RNAi expression. Flies were then tested at 30°C. Genetic control flies were raised at 18°C and subsequently tested at 18°C.

Flies were anesthetized using $CO_{2(g)}$ prior to sorting and collection. Crosses were maintained at 22°C on standard cornmeal yeast media.

2.2.4 Drosophila Stress Odourant (dSO) Avoidance Assay

The T-maze avoidance assay was conducted as previously described with modifications (*81*). All testing was performed in a temperature-controlled room maintained at 25°C and 70% humidity. To produce dSO a group of 50 flies (mixed gender) were vortexed (termed ‘emitter’, depicted in Figures 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-8, 2-9, 2-10, 2-11, and 2-12 to as ‘E’) for 1 minute (vortexed for 3 seconds followed by 5

seconds rest for 1 minute duration) in a 10mL Falcon tube sealed with Parafilm (Fisher Scientific 149598) at maximum speed (Fisher Vortex Mixer). Emitter flies were then removed from the Falcon tube and the dSO-containing Falcon tube was then placed into a T-maze. A new, unconditioned Falcon tube was placed opposite the dSO-containing tube. 50 naïve flies (termed 'responder'), depicted in Figures 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-8, 2-9, 2-10, 2-11, and 2-12 as 'R') were transferred into a new Falcon tube and loaded into the elevator of the T-maze. Responder flies were then given 1 minute to choose between the dSO-containing and the unconditioned Falcon tubes. Following the 1 minute testing period, flies were sequestered and avoidance response was scored.

Homologous-paired avoidance testing was conducted using emitter (E) and responder (R) flies from the same genotype. Heterologous-paired avoidance testing was conducted using different genotypes for both the emitter and responder flies.

For all dSO avoidance testing flies were 1-3 days old, with the exception of genetic crosses utilizing temperature-sensitive drivers. One day prior to avoidance testing, emitter and responder flies were aspirated into food vials; each pair of flies—one vial of emitter flies and one vial of responder flies—comprised an N of 1.

Genetic crosses utilizing temperature sensitive drivers were raised at 18°C and incubated at 30°C for 4 days prior to avoidance testing to activate RNAi expression. Flies were then tested at 30°C. Flies that were utilized as controls in which RNAi against FMRP was not expressed or flies in which FMRP was not over-expressed were raised at 18°C and subsequently tested at 18°C.

Avoidance was scored as Performance Index (PI), where PI was calculated by subtracting the number of flies in the dSO-containing Falcon tube from the number of flies in the unconditioned Falcon tube, and divided by the total number of flies tested.

2.2.5 Carbon Dioxide ($CO_{2(g)}$) Avoidance Assay

$CO_{2(g)}$ was utilized in place of emitter flies in $CO_{2(g)}$ avoidance testing. A flow-meter set at 0.5mL/min was used to administer $CO_{2(g)}$ into Falcon tubes, which were then momentarily sealed using Parafilm prior to being loaded into the T-maze. Responder flies were given 1 minute to choose between the $CO_{2(g)}$-

containing and the unconditioned Falcon tubes. Flies were then sequestered and avoidance response was scored.

For all $CO_{2(g)}$ avoidance testing flies were 1-3 days old. One day prior to avoidance testing, emitter and responder flies were aspirated into food vials; each pair of flies—one vial of emitter flies and one vial of responder flies—comprised an N of 1.

Avoidance was scored as Performance Index (PI), where PI was calculated by subtracting the number of flies in the dSO-containing Falcon tube from the number of flies in the unconditioned Falcon tube, and divided by the total number of flies tested.

2.2.6 Performance Index (PI) Calculation and Statistical Analysis

Performance Index was calculated in JMP® using the following formula:

Performance Index (PI) =

$$\frac{\#\text{ of flies in the unconditioned falcon tube (dSO/}CO_{2(g)}\text{-free tube) - \# of flies in dSO/}CO_{2(g)}\text{ falcon tube}}{\text{total \# of flies in trial}} \times 100\%$$

All statistical analysis was conducted using GraphPad Prism 6.

2.3 Results

2.3.1 Research Highlights/Summary

Drosophila exhibit robust avoidance behaviour to stress odourants emitted by other flies, termed Drosophila stress odourant (dSO), an innate behaviour resulting from pre-programmed neural circuits (*81*). Abnormal synaptic and dendritic formation may result in dysfunction that has been identified as an underlying cause of intellectual disability (ID) and autism spectrum disorders (ASD) in which information processing and behavioral functions are affected (*157, 200-202*). Fragile X Syndrome (FXS) is characterized by the loss of Fragile X mental retardation protein (FMRP), an mRNA-binding protein that functions in neuronal mRNA metabolism, namely in the translation of neuronal mRNAs involved in synaptic structure and function. FMRP plays a crucial role in neural circuit patterning/formation and the regulation of key signalling pathways (*107*). How this loss of FMRP affects avoidance behaviour remains unknown. Here we show that FXS flies exhibit a defect in dSO avoidance. Our results suggest that FMRP

is required developmentally, specifically in the mushroom body for higher-order processing, and in the establishment of neuronal networks.

2.3.2 The Fragile X Syndrome Mutants *FMR1*^{3} and *FMR*^{B55} Exhibit Decreased Drosophila Stress Odourant (dSO) Avoidance Behaviour

A neuropathological hallmark of Fragile X Syndrome is the formation of abnormal neuronal architecture that results in impaired function and thus neural circuitry and signaling dysfunction (*74, 76, 158, 191*). Loss of FMRP has been implicated in the improper establishment and function of neuronal networks underlying behavioural anomalies exhibited by FXS flies, including circadian rhythm, courtship behaviour, locomotive activities, social interactions, as well as in learning and memory deficits (*63, 75, 196, 197, 203*).

Given the requirement of FMRP in modulating other behaviours we sought to determine if this FMRP requirement also applied to dSO avoidance behaviour. It was predicted that much like the anomalies observed in other behaviours disrupted by loss of FMRP, we would also observe defects in dSO avoidance behaviour by Fragile X Syndrome (FXS) flies.

In order to determine the role of Fragile X Mental Retardation Protein (FMRP) in mediating dSO avoidance behaviour in Drosophila we utilized the two null alleles *FMR1*^{3} and *FMR*^{B55}. We found that in homologous-paired avoidance trials, *FMR*^{B55} and *FMR1*^{3} flies exhibited a decreased avoidance response as compared to wild-type (*WT)* flies (Figure 2-4 A). Expression of a promoter-driven genomic *dfrm1*^{3} transgene (*FMR1*^{3}*WTR*) rescued *FMR1*^{3} avoidance (Figure 2-4 A).

To discern whether the decreased avoidance response exhibited by the FXS mutants was due to a dSO processing or dSO emission deficiency, we conducted heterologous-paired avoidance trials in which wild-type flies were utilized as the 'emitter' or 'responder' and tested with the mutant flies of interest. *WT* flies exhibited normal avoidance in response to dSO emitted by *FMR*^{B55}, *FMR1*^{3}, and *FMR1*^{3}*WTR* flies (Figure 2-4 C). *FMR*^{B55} and *FMR1*^{3} flies exhibited decreased avoidance as compared to controls when *WT* flies were utilized as 'emitter' flies (Figure 2-4 B). These results suggest that *FMR*^{B55} and *FMR1*^{3} flies do not have a dSO emission defect, and that the diminished avoidance behaviour exhibited by the FXS mutants is the result of a dSO processing defect caused by the loss of FMRP.

Genetic background influences the penetrance and expressivity of mutations in Drosophila (*204*). In Drosophila the phenotypic effects of the *dfrm1* mutation are susceptible to genetic background effects, as shown by the variable penetrance of *dfmr1* in mushroom body (MB) resulting in a variety of architectural and functional defects (*205*).

To eliminate the possibility of background-dependence among the observed dSO avoidance behaviour between the two alleles, we crossed $FMR1^{3}$ and FMR^{B55} flies. The heteroallelic mutants, $FMR^{B55}/FMR1^{3}$, exhibited decreased avoidance behaviour compared wild-type flies (Figure 2-4 D). $FMR1^{3}/WT$ and FMR^{B55}/WT flies exhibit wild-type dSO avoidance behaviour (Figure 2-4 D). Collectively these results suggest that the observed phenotypic behaviour is not background-dependent.

2.3.3 Pan-Neuronal Knockdown of Fragile X Mental Retardation Protein (FMRP) Results in Decreased Drosophila Stress Odourant (dSO) Avoidance Behaviour

FMRP expression is ubiquitous (*206*). To determine the spatial requirement of FMRP in mediating avoidance behaviour we first used the pan-neuronal driver *Elav-Gal4* with *UAS-dfmr1RNAi* $^{1\text{-}7}$ to knockdown/reduce expression of FMRP. Pan-neuronal knockdown of FMRP resulted in a decreased avoidance response as compared to *WT* flies (Figure 2-5 A). To determine if the decreased avoidance behaviour caused by a dSO processing or dSO emission defect, we conducted heterologous-paired avoidance trials in which wild-type flies were utilized as the 'emitter' or 'responder' and tested with *Elav-Gal4:UAS-dfmr1RNAi* $^{1\text{-}7}$ flies. *WT* flies did not exhibit decreased avoidance behaviour to in response to dSO emission by *Elav-Gal4>UAS-dfmr1RNAi* $^{1\text{-}7}$ flies, suggesting that the decreased avoidance behaviour exhibited by *Elav-Gal4:UAS-dfmr1RNAi* $^{1\text{-}7}$ flies is a result of a dSO processing defect caused by knockdown of FMRP (Figure 2-5 B).

2.3.4 Knockdown of Fragile X Mental Retardation Protein (FMRP) in the Mushroom Body Results in Decreased Drosophila Stress Odourant (dSO) Avoidance Behaviour

Behavioural responses require integration and processing of sensory information in higher olfactory centres (*207, 208*). ORNs convey sensory information to the antennal lobe, which then transmits information to higher-order olfactory centres, the MB and the lateral horn.

In order to determine if FMRP was required in the mushroom body for dSO avoidance behaviour we utilized the MB-specific driver *OK107* to knockdown/reduce the expression of FMRP. Knockdown of FMRP in the MB resulted in a significantly decreased avoidance response by *OK107-Gal4>UAS-dfmr1RNAi*$^{1-7}$ flies compared to *WT* flies (Figure 2-6 A). To confirm that the decreased avoidance behaviour exhibited by *OK107-Gal4>UAS-dfmr1RNAi*$^{1-7}$ flies was due to sensory processing defect and not a dSO emission defect caused by the loss of FMRP in the MB, we tested *WT* flies against *OK107-Gal4:UAS-dfmr1RNAi*$^{1-7}$ dSO. *WT* flies did not exhibit any significant changes in avoidance behaviour when *OK107-Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies were utilized as dSO emitters (Figure 2-6 B). When *WT* flies were utilized as dSO emitters, *OK107-Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies exhibited decreased avoidance behaviour, suggesting that the decreased avoidance behaviour is a result of a dSO sensory processing defect caused by loss of FMRP in the MB (Figure 2-6).

Innate odour-associated behaviours are thought to exclusively rely on the lateral horn, and be MB-independent. Recent investigations have highlighted a new role for the MB in mediating innate odour driven behavioural responses, however these results suggest that the MB only functions in innate behaviours, like $CO_{2(g)}$ avoidance, in a starvation-dependent manner (*209*).

To confirm the role of the MB in mediating avoidance behaviour we utilized the MB-specific drivers *247* and *747* to knockdown/reduce FMRP. Use of the MB driver *274* to knockdown FMRP resulted in a significant defect in avoidance behaviour, where as use of the MB driver *747* did not (Figure 2-6 C, E). Absence of a dSO emission defect in *MB247Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies was confirmed by conducting heterologous-paired trials, in which no significant decrease in avoidance behaviour was exhibited by *WT* flies (Figure 2-6 C). Testing *C747Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies against *WT* dSO confirms the absence of a dSO emission defect as well as confirms lack of avoidance behaviour deficit (Figure 2-6 F).

Differences in dSO avoidance behaviour resulting from knockdown of FMRP in the MB is likely due to the regional specificity and the strength of expression of each individual driver within the MB. The driver *OK107-Gal4* targets and is strongly expression throughout the entire MB. The drivers *MB247-Gal4* and *C747-Gal4* both target the □, □, and □lobes of the MB, but the expression of 247 is greater in the □lobes, suggesting that this region may be more prominent in mediating avoidance behaviour (*210*). Furthermore

an extraneous target of the MB driver 247 are glia (*210*). Together these results suggest that FMRP is required in the MB for wild-type avoidance behaviour and in addition raises the possibility that glia may have an FMRP-dependent role in mediating avoidance behaviour.

2.3.5 Knockdown of Fragile X Mental Retardation Protein (FMRP) in the Glia Results in Decreased Drosophila Stress Odourant (dSO) Avoidance Behaviour

Neurons and glia comprise the central nervous system. Glia differ from neurons in that they lack axons and dendrites and therefore cannot participate in synaptic connections or electrical signal conveyance (*211*).

Glia instead have a crucial role in the developing CNS where they function in the establishment of neuronal circuitry by providing migratory cues for axon pathfinding and in meditating neuron survival, during which time FMRP expression is highest (*100, 152, 212*).

Using the *RepoGal4* driver, we reduced dfmr1 gene expression in glia. Knockdown of FMRP in glia resulted in decreased avoidance behaviour compared to *WT* flies (Figure 2-7 A).

Given the conflicting data about FMRP expression in glia, we wanted to determine if these flies also exhibited other phenotypic abnormalities characteristic of FXS (*74, 213, 214*). We accomplished this by knocking down FMRP in glia and then assayed for learning defects (See 4.2.2 for more information). *RepoGal4>UAS-dfmr1RNAi*$^{1\text{-}7}$ flies exhibit a learning deficit compared to *WT* flies (Figure 2-7 B). FMRP is expressed during development in neurons and glia, but is down-regulated in glia following development (*215-217*). These results suggest that FMRP is required in glia during development, the absence of which likely contributes to the Fragile X Syndrome phenotype.

2.3.6 Knockdown of Fragile X Mental Retardation Protein (FMRP) in the Central Complex Does Not Decrease Drosophila Stress Odourant (dSO) Avoidance Behaviour

The central complex is a higher-order processing centre in Drosophila, which serves as the integration site for sensory input with locomotive output and functions in the initiation and persistence of behaviours, as well as in learning and memory activities (*218*).

To determine if FMRP is required in the central complex for avoidance behaviour, we knocked down FMRP using the Feb170 driver. Knockdown of FMRP did not result in a significant defect in avoidance behaviour (Figure 2-8 A). *WT* flies did not exhibit any significant changes in avoidance behaviour when *Feb170-Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies were utilized as dSO emitters nor did *Feb170-Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies when *WT* flies were utilized as dSO emitters (Figure 2-7 B), suggesting that FMRP is not required in the central complex for wild-type avoidance behaviour.

2.3.7 Fragile X Mental Retardation Protein (FMRP) is Required for Carbon Dioxide ($CO_{2(g)}$) Avoidance in Drosophila

A significant portion of dSO is comprised of $CO_{2(g)}$ (*81*). To ascertain the contribution of FMRP in $CO_{2(g)}$ processing and subsequent modulation of avoidance behaviour we utilized the t-maze assay, where the responder flies were presented with $CO_{2(g)}$ instead of dSO. *FMR*^{B55} and *FMR1*^{3} flies exhibited decreased avoidance to $CO_{2(g)}$ compared to *WT* flies which exhibit normal avoidance to the presence of $CO_{2(g)}$ (Figure 2-9 A, B). The decreased avoidance to $CO_{2(g)}$ exhibited by FXS combined with the diminished dSO avoidance behaviour would suggest the presence of a global defect that impairs sensory processing in FXS flies.

2.3.8 Over-Expression of Fragile X Mental Retardation Protein (FMRP) in the Mushroom Body and Pan-Neuronally Results in Decreased Drosophila Stress Odourant (dSO) Avoidance Behaviour

FMRP is a negative regulator of neuronal architecture (*150*). Overexpression of FMRP results in a marked reduction in growth and branching of neurons, loss of synaptic differentiation, and severe MB structural defects (*74, 150*).

To determine if FMRP was required in a dose-dependent manner, we overexpressed FMRP pan-neuronally (*Elav-Gal4:UAS-dfmr1)* and in the MB. Overexpression of FMRP pan-neuronally and in the MB resulted in diminished avoidance behaviour (Figure 2-10 A, B). This suggests that FMRP is required in a dose-dependent manner.

2.3.9 Acute Pan-Neuronal Knockdown and Overexpression of Fragile X Mental Retardation Protein (FMRP) Does Not Result in Decreased Drosophila Stress Odourant (dSO) Avoidance Behaviour

FMRP expression is ubiquitous in the CNS, and as a result plays a central role in a number of key processes. During development FMRP functions in neurogenesis and activity-dependent axonal pruning (*219, 220*). In adults FMRP is required acutely for use-dependent axonal pruning, protein synthesis regulation, and regulation of neurotransmission (*221, 222*).

To determine if FMRP has an acute role in mediating avoidance behaviour we utilized the *Gal80^{ts}Elav-Gal4* driver to knockdown FMRP. Spatiotemporal reduction of FMRP gene expression pan-neuronally did not result in decreased avoidance behaviour compared to both *WT* flies and the control flies at 18°C in which RNAi expression is repressed (Figure 2-11 A).

We also utilized the *Gal80^{ts}Elav-Gal4* driver to acutely overexpress FMRP pan-neuronally. Similarly acute overexpression of FMRP did not result in decreased avoidance behaviour compared to both *WT* flies and the control flies at 18°C in which FMRP overexpression is repressed (Figure 2-11 B).

Taken together these results suggest that FMRP is not required acutely in mediating avoidance behaviour.

2.3.10 Acute Knockdown of Fragile X Mental Retardation Protein (FMRP) in the Mushroom Body Does Not Result in Decreased Drosophila Stress Odourant (dSO) Avoidance Behaviour

The MB is required in memory formation, where acute disruption of normal neurotransmission results in learning and memory deficits (*223-225*). Loss of FMRP in the MB results in memory defects (*196, 203*). Thus far our results demonstrate that FMRP plays a critical role in the MB during development in mediating dSO avoidance behaviour. We therefore asked whether FMRP was acutely required in the MB for dSO avoidance.

To determine if FMRP is acutely required in the MB for dSO avoidance behaviour, we utilized the *Gal80^{ts}C747-Gal4* driver to acutely disrupt FMRP expression in the MB. Acute spatiotemporal knockdown of FMRP in the MB did not result in decreased avoidance behaviour compared to both *WT* flies and the control flies at 18°C in which RNAi expression is repressed (Figure 2-12).

2.4 Discussion

2.4.1 Fragile X Mental Retardation Protein (FMRP) is Required for Drosophila Stress Odourant (dSO) Avoidance

Abnormal synaptic and dendritic formation may result in dysfunction that has been identified as an underlying cause of intellectual disability (ID) and autism spectrum disorders (ASD) in which information processing and behavioral functions are affected (*157, 200-202*). Fragile X Syndrome (FXS) is characterized by the loss of Fragile X mental retardation protein (FMRP), an mRNA-binding protein that functions in neuronal mRNA metabolism, namely in the translation of neuronal mRNAs involved in synaptic structure and function. FMRP regulates the translation of 5% of mRNA in the brain (*22*). FMRP binds to specific mRNAs to form mRNA-protein complexes that associate with polyribosomes to mediate mRNA translation. Loss of FMRP from the polyribosome mRNA-protein complexes results in translational profile shifts (*22*). Loss of translational regulation may lead to abnormalities in neuronal circuits and consequently modulation of behaviour observed in FXS (*226, 227*).

FMRP is ubiquitously expressed throughout the brain during embryonic development and found to have the highest expression in progenitors cells and newly differentiated neurons, identifying a new role for FMRP in the differentiation of stem cells (*206, 228-230*). FMRP functions in neurogenesis by regulating embryonic neural stem cell (eNSC) and adult neural stem cell proliferation (aNSC) (*100, 229*). In Drosophila, loss of FMRP results in a reduced number of quiescent neuroblasts, an over-proliferation of NSCs, and an increase in immature neuroblasts in adult brains due to altered cell cycle progression (*100*).

A reduction in neuronal and glial cell differentiation and an overabundance of stem cells are observed when FMRP is lost from aNSC populations in FXS mice while an increase in cells expressing neuronal cell linage markers is observed in eNSC populations (*230, 231*). Neurons that differentiate from aNSCs lacking FMRP display abnormal dendritic spines and altered calcium signalling, which is associated with changes in neuronal morphology and neurotransmission, and can be observed as early as during differentiation of NSCs suggesting that loss of the correct/proper differentiation of stem cells due to the loss of FMRP contributes to FXS neuropathology very early on during neurogenesis (*229, 232, 233*). The impaired differentiation and neurogenesis of stem cells results in learning deficits in FXS models (*231*).

Here we show that FXS flies exhibit a significant defect in dSO avoidance behaviour, independent of genetic background (Figure 2-4 D), which can be rescued through the expression of a promoter-driven genomic *dfrm1*^{3} transgene (*FMR1*^{3}*WTR*) (Figure 2-4 A-C).

Given that *WT* flies exhibit normal avoidance behaviour when FXS flies were utilized as the emitters, and that FXS flies still exhibited a significant defect in dSO avoidance when *WT* flies were utilized as the emitters, this would suggest that the diminished dSO avoidance response displayed by FXS flies is likely due to a sensory processing defect, and not an emission deficiency.

The diminished dSO avoidance exhibited by FXS flies coupled with the ability to rescue dSO avoidance through the expression of a *dfrm1*^{3} transgene suggests that FMRP is required for dSO processing and modulation of avoidance behaviour as FMRP plays a crucial role in neural circuit patterning/formation and the regulation of key signalling pathways (*107*).

2.4.2 Fragile X Mental Retardation Protein (FMRP) is Required Pan-Neuronally for Drosophila Stress Odourant (dSO) Avoidance

Development of neuronal connectivity underlying sensory processing within olfactory systems requires an intricate series of precisely coordinated events during development. FMRP is required during neurogenesis, axon pathfinding, synaptogenesis, activity-dependent synaptic pruning, and use-dependent circuit remodeling for the establishment of neural circuitry (*192, 234*).

Drosophila stress odorant (dSO) processing occurs through the olfactory system, as demonstrated by the surgical removal of antennae which abolished avoidance response (*81*). Pan-neuronal knockdown of FMRP allowed us to localize the requirement of FMRP in the processing of olfactory information to neurons. While others have shown the antenna to be the most important in olfactory processing, our results suggest an important role for higher olfactory centres in processing in mediating dSO avoidance (Figure 2-5 A-D). As we did not knockdown FMRP expression in the antenna, we show that despite detection by antenna, the conveyance and processing of olfactory information is likely inhibited due to loss of FMRP. This is exemplified the by significant decrease in dSO avoidance behaviour exhibited by *Elav-Gal4>UAS-dfmr1RNAi* $^{1\text{-}7}$ flies (Figure 2-5 A, B).

The mechanism of dysfunction underlying decreased dSO avoidance may occur through translational regulation of FMRP at pre- and post-synaptic sites, as well as FMRP regulation of neurotransmitter release. FMRP localizes in axons of pre-synaptic neurons, where it functions in the translation of pre-synaptic proteins (*18, 22, 235, 236*). Loss of FMRP results in altered expression of both pre- and post-synaptic protein targets as well as changes in the number of synaptic vesicles and kinetics of synaptic vesicle recycling resulting in altered neurotransmission (*237-239*). Neurotransmission is dependent on synapse morphology and generation of action potentials (*240*). FMRP regulates neurotransmission in a translation-independent manner by modulating the sensitivity of voltage-sensitive calcium channels to calcium and the generation of action potentials (*222*). FMRP-dependent loss of calcium sensitivity in voltage-sensitive calcium channels results in a broadening of action potentials, short-term plasticity deficits, and impaired transmission of information due to a decrease in the release of secretory vesicles (*222*). Short-term plasticity is required for rapid information processing and has been implicated in other cognitive impairment disorders such as Rett Syndrome and autism (*241-243*).

2.4.3 Fragile X Mental Retardation Protein (FMRP) is Required in the Mushroom Body for Drosophila Stress Odourant (dSO) Avoidance

The MB functions in complex adaptive behaviours such as choice behaviours, sleep, memory, and visual context generalization (*244-246*). Innate olfactory-driven behaviours are thought to be exclusively mediated by the lateral horn given the extensive characterization of the MB in olfactory-based adaptive responses (*247*). The exact role of the MB in mediating olfactory-driven avoidance behaviours still requires investigation.

The group that first reported the phenomena of dSO avoidance behaviour found that ablation of the MB using hydroxyurea and inhibition of neurotransmitter release in the MB did not result in diminished dSO avoidance behaviour and therefore not required for modulating this behaviour (*81*). Here we identify a novel role for the MB in mediating olfactory driven dSO avoidance behaviour. Pan-neuronal knockdown of FMRP, which targets MB Kenyon cells, as well as knockdown of FMRP in the MB using two MB-specific driver lines resulted in a significant decreased dSO avoidance behaviour (Figure 2-5 A, B; Figure 2-6 A-

D). Our results suggest that contrary to expectations, the MB functions in dSO avoidance behaviour, likely in an FMRP-dependent manner where FMRP is required for processing of olfactory information.

Flies use olfactory neural circuitry for immediate sensory processing and modulation of innate behaviours (*248*). Avoidance behaviour is hardwired by the same olfactory circuitry that mediates $CO_{2(g)}$ avoidance as artificial stimulation of neurons (ab1c) that elicits dSO/$CO_{2(g)}$ avoidance also results in an avoidance response (*249*). Presence of an odour evokes an increase in Ca^{2+} in the MB, indicating an increase in Kenyon cell activity suggesting that the MB functions in immediate sensory integration and could mediate innate behaviour (*248, 250*). Furthermore analysis of aborization patterns in the MB and lateral horn revealed that third-order neurons in the MB can receive input from a variety of second-order projection neurons, while third-order neurons within the lateral horn only form synaptic connections with a subset of second order projection neurons (*208*). This suggests that the MB functions in the processing of a broad range of sensory input and subsequent modulation of behavioural responses.

It is possible that ablation of the MB did not affect dSO avoidance behaviour as other higher olfactory centers, such as the lateral horn, may also function simultaneously in olfactory processing required for dSO avoidance behaviour. Blocking neurotransmission in the MB results in a decrease in odour-driven attraction but not in odour-driven avoidance, suggesting that the MB functions in odour-driven attraction but not odour-driven avoidance, which is mediated by the lateral horn (*251*). Inhibiting neurotransmission in both the lateral horn and MB results in a decrease in both odour-driven attraction and odour-driven avoidance (*251*). Olfactory-driven behaviours therefore may be a combination of MB and lateral horn output (*251*).

Given the role of FMRP in neural circuit patterning/formation and the regulation of key signalling pathways, it is possible that loss of FMRP results in signalling dysfunction that can be attributed to the decreased avoidance behaviour not observed by simple ablation of the MB (*107*). Improper establishment of and signaling within neural circuitry meditating dSO avoidance behaviour may inhibit conveyance of sensory input to higher olfactory centres for processing. This reinforces the idea that innate olfactory-driven behaviours may be modulated by a complex output from more than one higher olfactory centre, as

signalling and processing of sensory input still occurred, albeit to an unknown extent, when the MB functioning was altered and *dfmr1* was not transcriptionally silenced.

FMRP regulates the neuronal elaboration and synaptic function in all MB Kenyon cells (*150*). Loss of FMRP from MB Kenyon cells results in aberrant dendritic elaboration and axonal branching which display incorrect axonal trajectories into incorrect brain regions, and abnormal synaptic vesicle cycling (*150*). MB neurons convey sensory information to other brain regions that modulate motor output (*252*). It is possible that loss of FMRP results in the improper integration of sensory information in the MB in order to modulate motor response associated with avoidance behaviour (*225*).

It has also been suggested that the MB functions in innate-olfactory driven avoidance in a context dependent manner. Inhibition of signalling output in all MB Kenyon cells results in decreased $CO_{2(g)}$ avoidance behaviour only when flies had been in a starvation state for 24 hours, suggesting that the MB functions in $CO_{2(g)}$ avoidance, but only in a starvation-dependent manner (*209*). Our results did not support this conclusion, however consistent with our results, use of the driver *OK107-Gal4* to block MB output in all Kenyon cells resulted in a significant decrease in avoidance response to $CO_{2(g)}$ regardless of starvation state (*209*).

Here we show a novel, context-independent role for the MB in mediating dSO avoidance behaviour (Figure 2-6 A-F). The exact role of the MB in modulating avoidance behaviour still requires elucidation. It would be worth investigating whether the loss of FMRP in other olfactory centres, specifically the ORNs, antennal lobe, and lateral horn also results affects olfactory processing and modulation of dSO avoidance behaviour.

2.4.4 Fragile X Mental Retardation Protein (FMRP) is Required in the Mushroom Body □ Lobe for Drosophila Stress Odourant (dSO) Avoidance

Kenyon cells in the MB are classified into three subtypes, □/□, □□/□□, or □, based on their morphology and trajectories within the MB (*253, 254*). □/□ and □□/□□ Kenyon cells bifurcate to form vertical □ and □□ lobes, and horizontal □ and □□ lobes. □ Kenyon cells bifurcate to form horizontal □ lobes (*254-256*).

□Kenyon cells are the first to form, likely in the embryo as elaborate dendrites and axonal projections are established in third instar larva (*256*). □/□ Kenyon cells appear in later larval stages and persist into adult life, maintaining connections with input and output neurons (*256*). □Kenyon cells serve as pioneer neurons in larva for □/□ Kenyon cells and during metamorphosis □Kenyon cells undergo dramatic rearrangement (*256*). Functional studies in learning and memory suggest that each Kenyon cell subtype has distinct functions (*257-259*).

Unlike the significant decrease in dSO avoidance that resulted from using the *OK107-Gal4* and *MB247-Gal4* driver lines to knockdown FMRP in the MB, use of the C*747-Gal4* driver line did not result in a significant decrease in dSO avoidance (Figure 2-6 A-F). These differences are likely due to regional specificity and strength of expression of each individual driver within the MB. The *OK107-Gal4* and *MB247-Gal4* driver lines strongly target expression in □, □, and □Kenyon cells, while *C747-Gal4* expression is weaker in □Kenyon cells (*210*).

In response to an odour, Ca^{2+} influx occurs primarily in □Kenyon cells (*260*). Expression of the dopaminergic signaling MB-specific D1-like dopamine receptor, DopR, in □Kenyon cells is necessary to rescue all forms of olfactory-associated memory, while expression in □/□ and □□/□□Kenyon cells is not (*257*). Olfactory processing therefore may occur first in □Kenyon cells and then subsequently in □/□ and □□/□□Kenyon cells.

Inactivation of neuronal output □□/□□cells and a in a defined region of □/□ Kenyon cells only resulted in decrease avoidance to $CO_{2(g)}$ in a starvation-dependent manner, while inactivation on neuronal output in □/□ Kenyon cells did not result in diminished $CO_{2(g)}$ avoidance regardless of starvation state (*209*).

It is possible that given the role of □Kenyon cells in providing guidance routes for □/□ Kenyon cells during development, that abnormal functioning and neural connectivity caused by the loss of FMRP in this region alters correct connectivity of □/□ Kenyon cells which persist into adulthood. Furthermore as □Kenyon cells form synaptic connections twice during development, it is possible that improper development and signalling during synaptogenesis during the embryonic stages has a cumulative effect on/contributes to the malformation of □Kenyon cell remodeling during metamorphosis.

This suggests that individual populations of Kenyon cells may have differential roles in the modulation of dSO avoidance, and that Kenyon cells may have a primary role in mediating processing and avoidance.

2.4.5 Fragile X Mental Retardation Protein (FMRP) is Required in the Glia for Drosophila Stress Odourant (dSO) Avoidance

NSCs differentiate into intermediate progenitors (IPCs) and then to neurons and glia (*261*). Loss of FMRP results in decreased differentiation of IPCs into neuronal and glial cells due to the misexpression of stem cell specific genes and the loss of cell cycle regulation (*100, 103, 231, 262*). Cyclin-dependent kinase 4 (CDK4) and cyclin D1 cell cycle regulating proteins were both identified as mRNA targets specific to FMRP in NSCs (*103*). Loss of FMRP results in altered CDK4 expression at synapses suggesting an interaction in mediating proliferation and that FMRP is required for glia cell development (*156, 263*).

Glia are required for the establishment of neuronal circuitry and function in all essential steps of neural development (*152*). Glia provide guidance cues for growth cones, facilitate bundling, fasciculation and ensheathment of axons, insulate neurons, modulate synaptic transmission, and maintain neurotransmitter homeostasis in synapses (*152*).

In early development glial cells regulate neuron proliferation during larval neurogenesis (*264*). Reciprocal interactions between glia and neurons guide glia to their correct destination and regulate glial cell numbers (*265*). Glia are required to convey nutrient-dependent fat body signalling to neuroblasts that have entered quiescence (*102, 266*). Conveyance of nutrient-dependent information by glia triggers neuroblasts re-entry into the cell cycle to give rise to mature neurons (*102*).

Glia function in axon pathfinding by providing guidance cues to growth cones, specialized sensorimotor structures at the end of axons that allows axons to migrate following a specific trajectory by interpreting external guidance cues (*141*). Glia also function in spatial patterning of neuronal circuits by forming boundaries in order to restrict axon and dendrite growth within targeted areas (*267, 268*).

Glia are required for the patterning of antennal lobe and protoglomeruli formation (*269*). Glial cells and sensory neurons project into the antennal lobe simultaneously likely to regulate sensory neurons arboritization and glomeruli establishment (*82, 270*). During neurogenesis axonal projections from ORNs contact “sorting zone” glia which halts pathfinding in order to organize ORN axons into fascicles that make up specific glomeruli (*271-273*).

Glial cells are required for synaptogenesis (*274, 275*). Neurons innervate targets prior to forming synapses, which coincides with astrocyte/glia cell generation (*276*). When cultured in the absence of astrocytes, neurons exhibit a significant reduction in synaptic activity, a reduction in the number of synapse formation, and decreased synapse stability marked by the rapid disappearance of synapses when astrocytes where removed from culture (*275-277*). It is thought that glia/astrocytes release diffusible factors that promote synapse formation (*278, 279*). Furthermore astrocytes stabilize and promote the maturation of newly formed synapses (*280*).

Glia also participate in the regulation of neurotransmission at synapses by maintaining ion and neurotransmission homeostasis by removing excess ions and neurotransmitters as well as secreting neuromodulators (*152, 281*). Release of neurotransmitters from a pre-synaptic terminal activates mGluR receptors on astrocytes/glial cells triggering an influx of Ca^{2+} in the astrocyte and a release of gliotransmitters, which then modulate both the pre- and post-synaptic neurons (*282*). Moreover glia can regulate GABAergic transmission by altering ion gradients in developing neurons and conversion of excitatory and inhibitory signalling input (*283*).

Glia also function in refinement of neuronal circuitry. Neuronal circuitry refinement occurs through a process known as axon pruning in which extraneous synaptic connections are eliminated by glia through lysosomal degradation (*284-287*). Remodeling of the □lobe of the mushroom body at the end of larval development utilizes axon pruning, in which inadequate synaptic connections are removed through local lysosomal degeneration by glia cells (*287*). Glia function in the localized pruning of dendrites and terminal axon branches of projection neurons, which relays sensory input from olfactory neurons to the mushroom body (*152, 153*).

FMRP is expressed in glial cells early in development, where it has been shown to be required for a number of key steps during neural development, following which FMRP is down-regulated in mature glial cells (*288*). FMRP is required in glial cells to control the proliferation of neural stem cells and to mediate the balance between glia and neurons during neurogenesis (*100, 103, 215*). Exit of neuroblasts from quiescence requires insulin signalling via glial cells (*102*). Insulin-like peptides secreted by glial cells bind to insulin receptors that triggers the activation of the PI3K/Akt signalling pathway which leads to the subsequent proliferation of neuroblasts (*102, 289*). Glia regulate their own insulin-like peptides production through FMRP (*215*).

Glia modulate neuronal architecture by regulating dendrite morphology. When neurons were cultured in the presence of FMRP-deficient astrocytes, abnormal dendrite morphology was observed (*290*). When grown in the presence of wild-type astrocytes, FMRP-deficient neurons exhibited normal dendrite morphology (*290*). Impaired synaptic protein expression is also observed in neurons in the presence of FMRP-deficient astrocytes, which is not observed when FMRP-deficient neurons were cultured in the presence of wild-type astrocytes (*290*). FMRP also regulates activity dependent axon pruning modulated by glial cells (*151*). Loss of FMRP from glia prevents correct synaptic pruning and neural circuitry establishment that likely contributes to the signalling dysfunction in FXS.

MB Kenyon cells are derived from four neuroblasts in the embryonic brain that also give rise to a set of indistinguishable glia cells (*291*). Sequential derivation of glial cells and MB Kenyons cells from the same set of neuroblasts would indicate that these cells would have similar FMRP requirements during development for proper formation and function. Our results show that FMRP is required during development in both MB Kenyon cells and glial cells for dSO avoidance. Glial cell dysfunction has been linked to a number of neurodevelopmental disorders in which abnormal synaptic function contributes to neuropathology such as Rett Syndrome, Down Syndrome, Fragile X Syndrome, and autism (*213, 290, 292*). Here we show that knockdown of FMRP in glia resulted in a significant decrease in dSO avoidance behaviour (Figure 2-7 A, B). This further establishes a requirement for FMRP is establishing neuronal circuitry that mediates avoidance behaviour.

2.4.6. Fragile X Mental Retardation Protein (FMRP) is Not Required in the Central Complex for Drosophila Stress Odourant (dSO) Avoidance

The central complex is a multisensory neutrophil that functions in visual, mechanosensory, and olfactory information processing (*293*). Loss of FMRP has been implicated in abnormal behaviours regulated by the central complex such as memory, spatial orientation, and locomotive activities (*293*).

Contrary to expectations, FMRP is not required in the central complex for dSO avoidance behaviour. Our results suggest that the MB has the most prominent role in mediating dSO avoidance (Figure 2-8 A, B). Synapse formation does not occur in the central complex until late in development and direct connections between the central complex and mushroom body are absent, rather the central complex is indirectly connected to sensory brain structures and the ventral nerve cord (*293, 294*). Furthermore the central complex is comprised of a protocerebral bridge, a fan-shaped body, and an ellipsoid body, where connections between each neutrophil is limited, suggesting that each neutrophil may function independently as the ellipsoid body specifically has been shown to be required for specific behaviours such as learning (*218*). Despite that anatomical conservation of central complex across insects, knowledge about functional roles is based on a few comparative studies where functions vary among species (*293*).

The central complex has been shown to be required for novelty choice learning, associative pattern learning, and visual orientation learning in Drosophila, all which rely on sensory input into the central complex from the visual system (*295*).

The lateral horn and the MB are considered to be higher olfactory centres in Drosophila, and less is known about olfactory information processing beyond these two regions (*293*). It is likely that avoidance hierarchical sensory processing in the fly brain utilizes the well-known antennal lobe to mushroom body pathway (*296*). Here we show that FMRP is not required in the central complex for dSO avoidance, however this does eliminate the possibility that the central complex may function in dSO avoidance at all, rather it may function in an FMRP-independent manner.

Future research should examine the expression of FMRP in the central complex throughout and following development to characterize the role of FMRP in the central complex. Moreover blocking

neurotransmission in the central complex neurophils would provide evidence as to whether the central complex functions in dSO avoidance.

2.4.7 Fragile X Mental Retardation Protein (FMRP) is Required for Carbon Dioxide ($CO_{2(g)}$) Avoidance in Drosophila

Drosophila can detect $CO_{2(g)}$ in concentrations as little as 0.02% (*195*). Elucidation of the conveyance of $CO_{2(g)}$ sensory information from olfactory receptors revealed that a single population of antennal olfactory receptor neurons (ORNs)—Gr21a/Gr63a receptors—synapse with projection neurons (PNs) in the antennal lobe (*81, 195, 297, 298*). ORNs converge onto the V-glomerulus, where four projections convey information to higher processing centres, two of which exclusively connect to the lateral horn, and the remaining an atypical bilateral projection neuron—bilateral ventral projection neuron—that send projections from the V-glomerulus to both the lateral horn and the MB (*209, 299, 300*).
To further complicate matters, $CO_{2(g)}$ avoidance behaviour can be modified in a context-dependent manner in which the presence of an attractant odour inhibits the sensory processing that mediates avoidance behaviour (*301*) . Three projection neurons that innervate the V-glomerulus—PN_v-1, PN_v-2, and PN_v-3—mediate $CO_{2(g)}$ avoidance in concentration dependent manner (*302*). Following detection of $CO_{2(g)}$ one of two pathways is activated for processing, a low-$CO_{2(g)}$ pathway (0.5% $CO_{2(g)}$) that is mediated by the projection neuron PN_v-1 and a high-$CO_{2(g)}$ pathway ($CO_{2(g)}$ 5%) mediated by the projection neuron PN_v-2 (*302*). The low-$CO_{2(g)}$ pathway but not the high-$CO_{2(g)}$ pathway, is subject to regulation and can be inhibited by PN_v-3, which is activated in response to food odours and high concentrations of $CO_{2(g)}$ (*302*).

Drosophila also exhibit innate avoidance to $CO_{2(g)}$, a main component of dSO (*81*). Here we show that developmental loss of FMRP results in decreased avoidance behaviour to $CO_{2(g)}$ as demonstrated by the significant decrease in PI scores of both *FMR1*^{3} and *FMR*^{B55} flies as compared to *WT* flies and *FMR1*^{3}*WTR* (Figure 2-9 A, B). Similarly to dSO avoidance, $CO_{2(g)}$ avoidance behaviour is rescued in *FMR1*^{3} flies through the addition of promoter driven *dfrm1*^{3} transgene (Figure 2-9 A, B). This genetic rescue suggests that FMRP functions in development in the establishment of neuronal networks that

mediate $CO_{2(g)}$ processing and modulation of avoidance behaviour. The exact role of FMRP in establishing the neural circuitry that mediates $CO_{2(g)}$ avoidance remains unknown. Further research will be required to provide a mechanism through which FMRP functions in the establishment of complex neural circuitry underlying the processing of sensory information.

2.4.8 Over-Expression of Fragile X Mental Retardation Protein (FMRP) Results in Decreased Drosophila Stress Odourant (dSO) Avoidance

FMRP is a negative regulator of neuronal architecture, where the overexpression of FMRP results in a marked reduction in growth and branching of neurons, loss of synaptic differentiation, and severe MB structural defects (*74, 150*). Overexpression of FMRP also results in the loss of the dendritic 'claws' in the MB, which form synapses with PNs (*150, 256*). Each Kenyon cell 'claw' contacts a single PN bouton which can contribute sensory input into a single Kenyon cell claw at 3-4 presynaptic sites (*96, 303*). Thus loss of dendritic 'claws' would likely result in impaired information transmission to the MB for processing.

Here we show that overexpression of FMRP pan-neuronally and overexpression of FMRP in the MB both results in decreased dSO avoidance behaviour suggesting that FMRP is required in a dose-dependent manner (Figure 2-10 A, B). Loss of translational repression by FMRP may result in overexpression of dose sensitive genes. Within neuronal networks dosage effects are crucial, where network function and homeostasis relies on excitatory and inhibitory input and the expression of many genes involved directly or indirectly in neurotransmission, including receptors, neurotransmitter synthesis and degradation, signaling and effector proteins, and regulators of transcription and translation (*304*).

2.4.9 Fragile X Mental Retardation Protein (FMRP) is Not Acutely Required for Drosophila Stress Odourant (dSO) Avoidance

Time-dependent requirements of FMRP during development have been identified in a number of neuronal networks for proper structure and functioning. Small ventrolateral neurons (sLNvs), a key subset of clock neurons, exhibit a time-dependent requirement for FMRP during a transient window at a late stage of development when synaptic pruning occurs and is required for proper neuronal architecture and circuitry, where the reintroduction of the mature protein cannot rescue architecture in this subset (*305*).

Reintroduction of FMRP in *dfmr1* null larva partially rescues neuromuscular junction (NMJ) structural defects but not functional defects as synaptic vesicle cycling remains abnormal (*158*).

Acute knockdown of FMRP pan-neuronally (Figure 2-11) or in the MB (Figure 2-12) did not result in decreased avoidance behaviour, nor did acute over-expression of FMRP pan-neuronally.
Despite the prominent role of FMRP in the MB in mediating dSO avoidance, acute over-expression and knockdown did not result in decreased dSO avoidance. This may be a result of the driver line used. We used the *Gal80^{ts}C747-Gal4* driver line to over express and knock down FMRP. When we utilized the C747-*Gal4* driver line to knockdown FMRP in the MB throughout development, we did not observe any significant decrease in dSO avoidance, suggesting that specific regions within the MB are more prominent in mediating avoidance or that the driver line had a functional defect. However, pan-neuronal disruption did not result in decreased avoidance and therefore it is likely that FMRP is not acutely required in the MB.

Thus far our results have established a developmental role for FMRP in mediating dSO avoidance behaviour. We show that FXS flies exhibit decreased avoidance response to $CO_{2(g)}$ This confirms not only the requirement of FMRP for dSO avoidance behaviour, but also identifies the FMRP requirement for wild-type $CO_{2(g)}$ avoidance. Acute disruption of FMRP in the MB of adult flies does not result in diminished dSO avoidance where as knockdown of FMRP throughout development results in decreased avoidance behaviour, suggesting that FMRP is required developmentally in the MB, where it likely functions in the establishment of the neural circuitry that mediates dSO avoidance, and by extension $CO_{2(g)}$ avoidance.

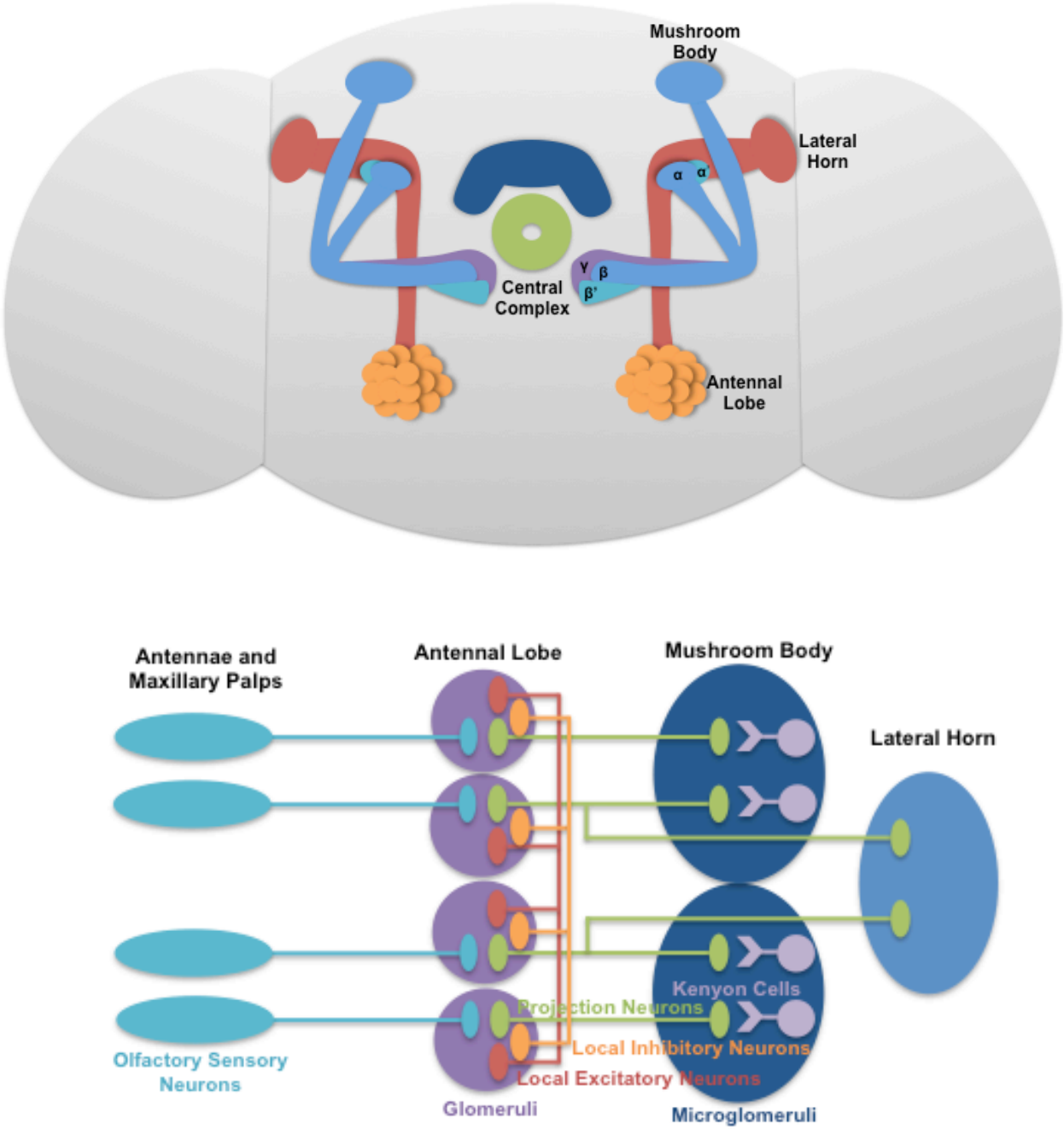

Figure 2-1. Drosophila olfactory system.
Olfactory sensory neurons (OSN) transmit information to the antennal lobe, where OSNs synapse with projection neurons (PN) in discrete structures called glomeruli. Each glomerulus also contains input from local neurons (LNs). PNs then transmit information to one of two higher processing centres, the lateral horn (LH) or the mushroom body (MB). In the MB, Kenyon cells synapse with projection neurons from the AL.

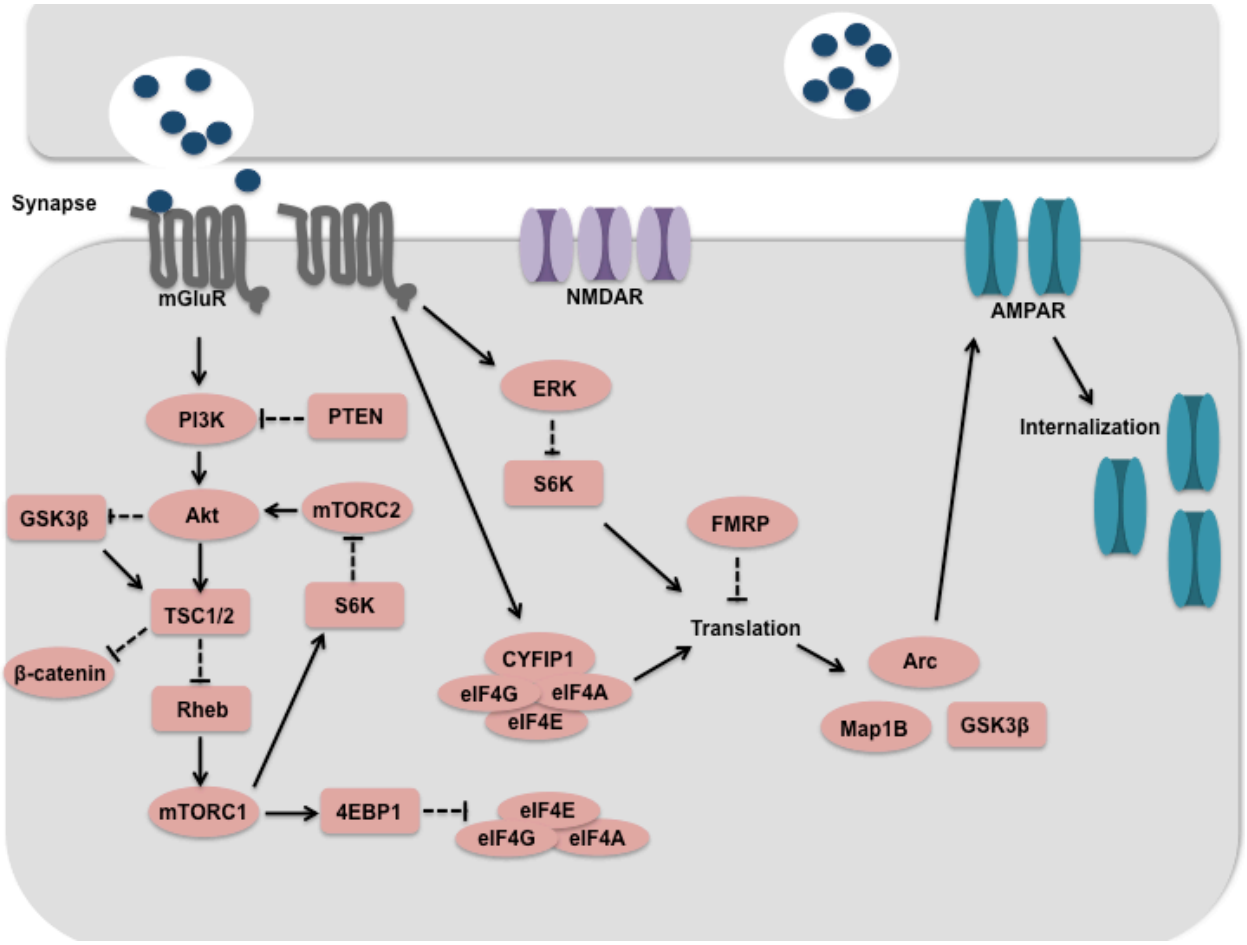

Figure 2.2 mGluR and mTOR signalling pathways implicated in Fragile X Syndrome.
Activation of mGluR receptors initiates an intracellular signalling cascade involving PI3K, mTOR, and Akt that regulates translation. In absence of FMRP, mGluR signalling is exaggerated and translational regulation of key target mRNAs is lost resulting in an increase in the internalization of AMPAR receptors.

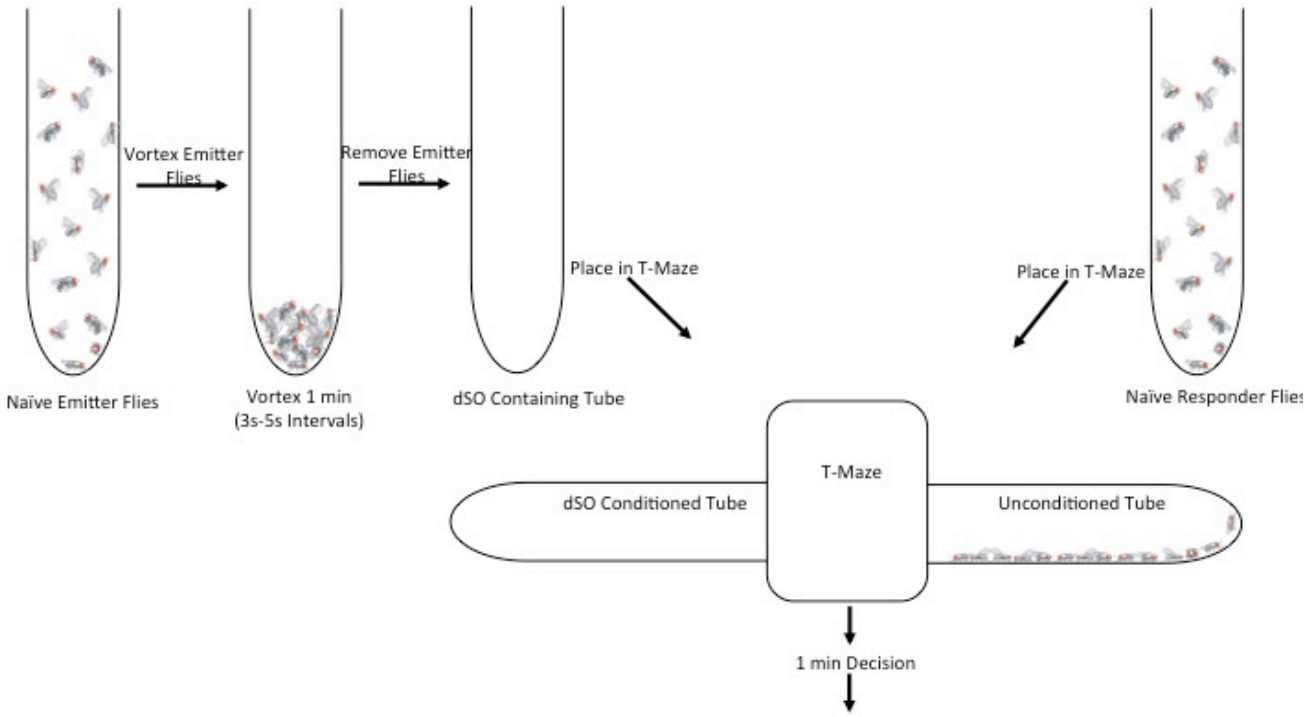

Figure 2-3. Drosophila stress odourant (dSO) avoidance assay.
The T-maze avoidance assay was conducted as previously described with modifications. All testing was performed in a temperature-controlled room maintained at 25°C and 70% humidity. To produce dSO a group of 50 flies (mixed gender) were vortexed (termed 'emitter', depicted in graphical representations as 'E') for 1 minute (vortexed for 3 seconds followed by 5 seconds rest for 1 minute duration) in a 10mL Falcon tube sealed with Parafilm (Fisher Scientific 149598) at maximum speed (Fisher Vortex Mixer). Emitter flies were then removed from the Falcon tube and the dSO-containing Falcon tube was then placed into a T-maze. A new, unconditioned Falcon tube was placed opposite the dSO-containing tube. 50 naïve flies (termed 'responder'), depicted in graphical representations as 'R') were transferred into a new Falcon tube and loaded into the elevator of the T-maze. Responder flies were then given 1 minute to choose between the dSO-containing and the unconditioned Falcon tubes. Following the 1 minute testing period, flies were sequestered and avoidance response was scored.
Homologous-paired avoidance testing was conducted using emitter (E) and responder (R) flies from the same genotype. Heterologous-paired avoidance testing was conducted using different genotypes for both the emitter and responder flies. For all avoidance testing, with the exception of drug treatment testing, flies were 1-3 days old. One day prior to avoidance testing, emitter and responder flies were aspirated into food vials; each pair of flies—one vial of emitter flies and one vial of responder flies—comprised an N of 1.
Avoidance was scored as Performance Index (PI), where PI was calculated by subtracting the number of flies in the dSO-containing Falcon tube from the number of flies in the unconditioned Falcon tube, and divided by the total number of flies tested.

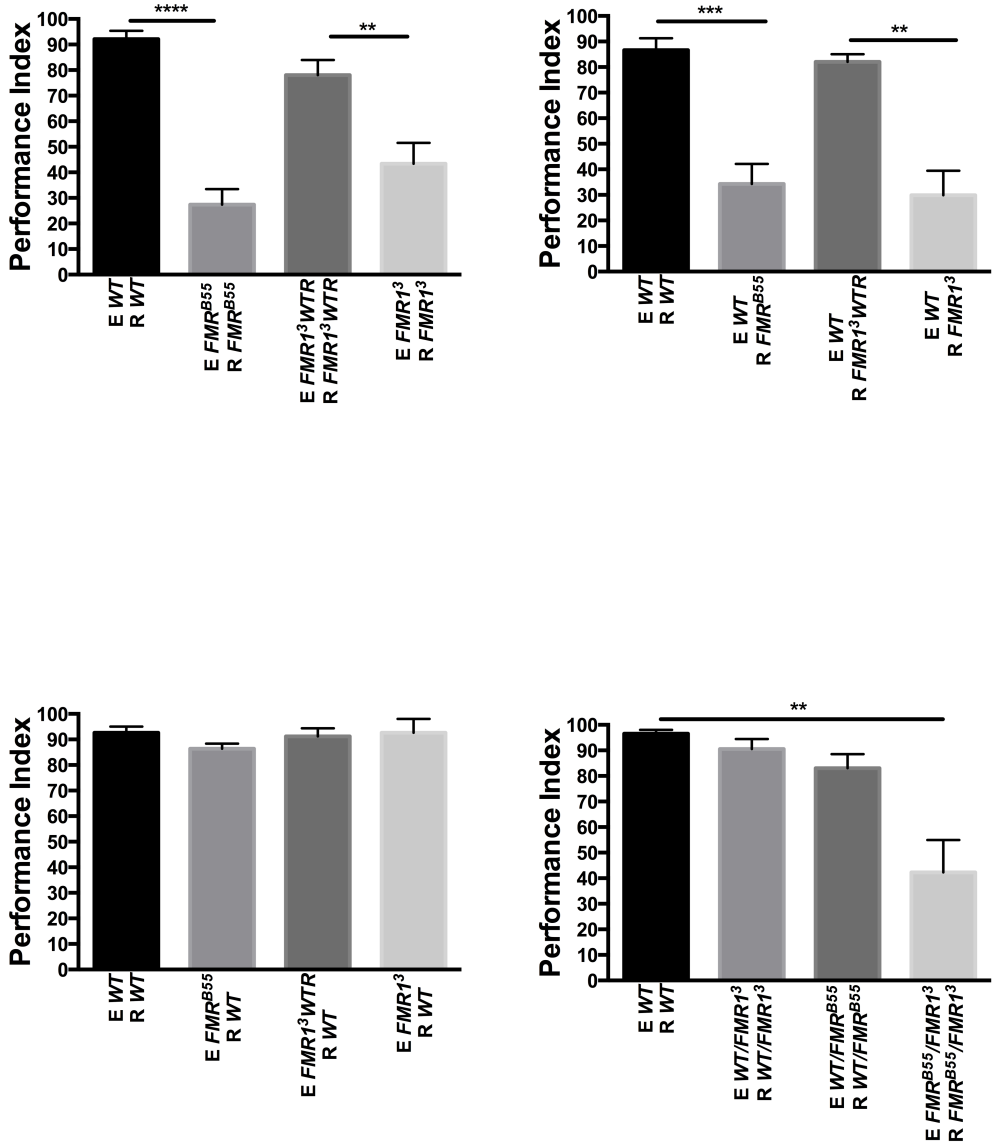

Figure 2-4. Fragile X Mental Retardation Protein (FMRP) is required for avoidance of Drosophila melanogaster stress odourant (dSO).

(A) *FMR*^{B55} mutants exhibit a significantly lower avoidance in response to dSO compared to *WT* flies (T-test P<0.0001; N=8); avoidance is quantified as Performance Index (PI). *FMR1*^{3} exhibit decreased avoidance compared to *FMR1*^{3}*WTR* flies the avoidance of which is rescued genetically through the addition of a genomic *dfmr1*^{3} fragment (T-test P=0.0049; N=8).
'E' denotes flies that have undergone 1 minute of vortexing to produce dSO, these flies are the 'emitters'. 'R' denotes naïve flies that are being tested for avoidance response to dSO, these flies are the 'responders'. Dso avoidance behaviour is scored as Performance Index (PI).
(B) *FMR*^{B55} flies exhibit decreased avoidance behaviour to *WT* dSO (T-test P=0.0001; N=12). *FMR1*^{3} also flies exhibit diminished avoidance behaviour to *WT* dSO as compared to *FMR1*^{3}*WTR* flies (T-test P=0.0018; N=12).
(C) *WT* flies did not exhibit decreased avoidance behaviour to dSO emitted by *FMR*^{B55}, (T-test P=0.0988; N=5), *FMR1*^{3} (T-test P=0.9897; N=5), and *FMR1*^{3}*WTR* flies (T-test P=0.7153; N=5).
(D) *FMR*^{B55}*/FMR1*^{3} flies exhibit decreased avoidance compared to *WT* flies (ANOVA P=0.0001; N=7). Avoidance behaviour is genetically rescued in *FMR*^{B55}*/WT* (ANOVA P=0.9348; N=7) and *FMR1*^{3}*/WT* (ANOVA P=0.5638; N=7) flies. *FMR*^{B55}*/FMR1*^{3} flies exhibit decrease avoidance behavior compared to *FMR1*^{3}*/WT (*ANOVA P=0.0004; N=7) and *FMR*^{B55}*/WT* (ANOVA P=0.0028; N=7) flies. All graphs depict mean ± S.E.M..

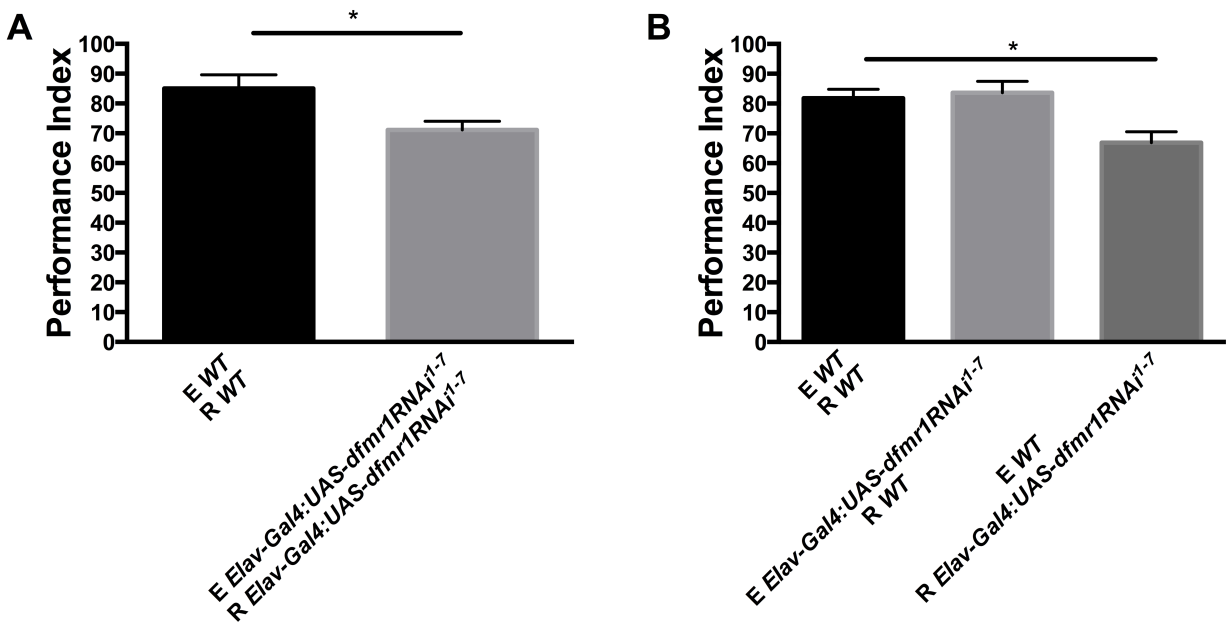

Figure 2-5. Pan-Neuronal knockdown of Fragile X Mental Retardation Protein (FMRP) results in decreased Drosophila stress odourant (dSO) avoidance behaviour.
(A) Pan-neuronal knockdown of FMRP, *Elav-Gal4:UAS-dfmr1RNAi* $^{1-7}$*,* results in decreased avoidance to dSO compared to *WT* flies (T-test P=0.0409; N=20).
(B) *WT* flies did not exhibit any significant decrease in avoidance behavior to dSO emitted by *Elav-Gal4:UAS-dfmr1RNAi* $^{1-7}$ flies (T-test P=0.00285;N=12). *Elav-Gal4:UAS-dfmr1RNAi* $^{1-7}$ flies exhibit decreased avoidance behaviour to *WT* dSO as compared to *WT* flies (T-test P=0.7653;N=10). All graphs depict mean ± S.E.M.

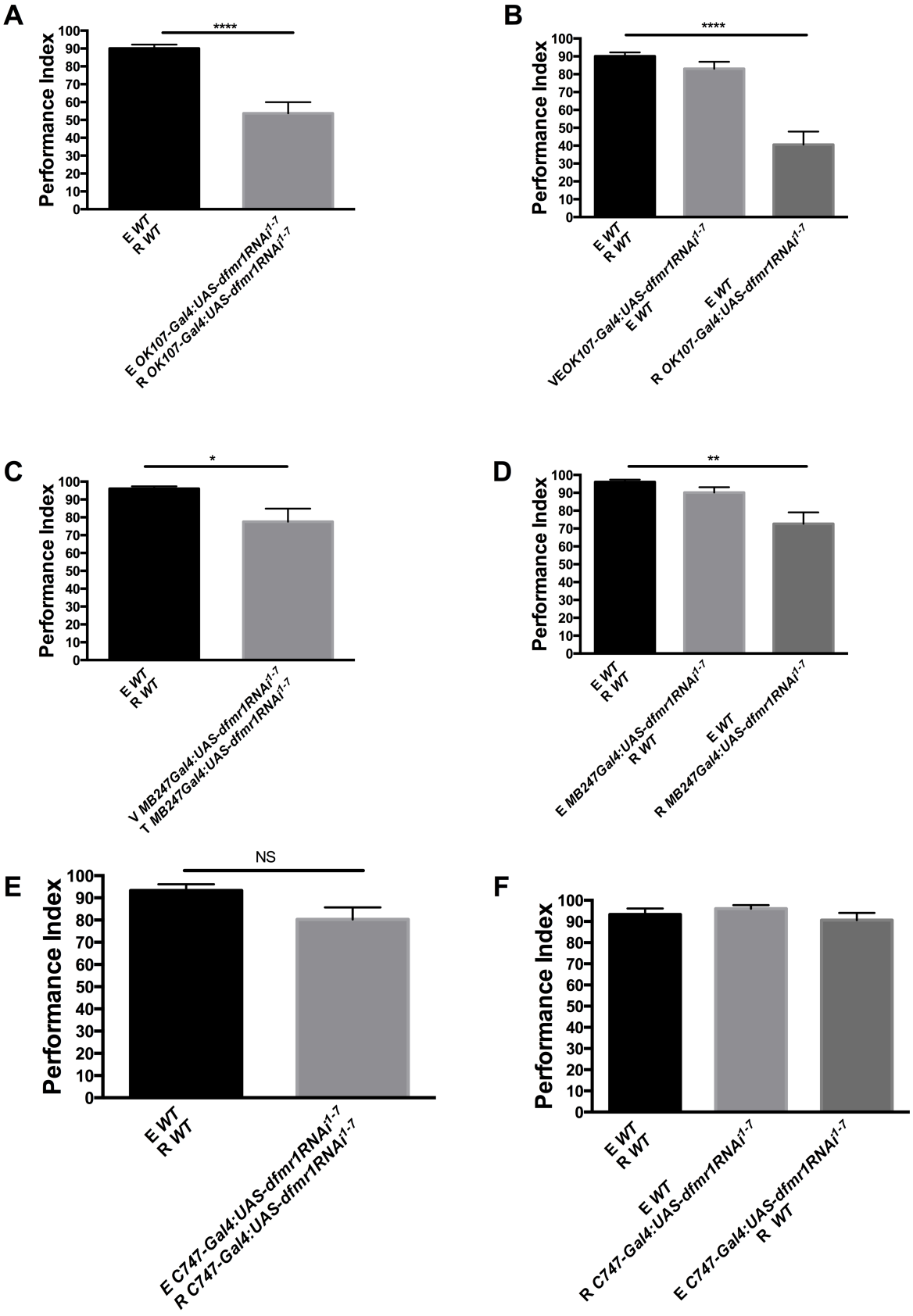
A
Performance Index
****
E WT
R WT
E OK107-Gal4:UAS-dfmr1RNAi1-7
R OK107-Gal4:UAS-dfmr1RNAi1-7
B
Performance Index
****
E WT
R WT
VEOK107-Gal4:UAS-dfmr1RNAi1-7
E WT
E WT
R OK107-Gal4:UAS-dfmr1RNAi1-7
C
Performance Index
*
E WT
R WT
V MB247Gal4:UAS-dfmr1RNAi1-7
T MB247Gal4:UAS-dfmr1RNAi1-7
D
Performance Index
**
E WT
R WT
E MB247Gal4:UAS-dfmr1RNAi1-7
R WT
E WT
R MB247Gal4:UAS-dfmr1RNAi1-7
E
Performance Index
NS
E WT
R WT
E C747-Gal4:UAS-dfmr1RNAi1-7
R C747-Gal4:UAS-dfmr1RNAi1-7
F
Performance Index
E WT
R WT
E WT
R C747-Gal4:UAS-dfmr1RNAi1-7
E C747-Gal4:UAS-dfmr1RNAi1-7
R WT

Figure 2-6. Knockdown of Fragile X Mental Retardation Protein (FMRP) in the mushroom body results in decreased Drosophila stress odourant (dSO) avoidance behaviour.
(A) *OK107-Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies exhibit significantly decreased avoidance to dSO compared to *WT* flies (T-test P<0.0001; N=12).
(B) *OK107-Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies exhibit a significantly decreased avoidance response when tested against *WT* dSO (T-test P<0.0001; N=8). *WT* flies exhibited normal avoidance behaviour when tested against dSO produced by *OK107>FmrRNAi(1-7)* flies (T-test P=0.1240; N=8).
(C) *MB247Gal4;UAS-dfmr1RNAi*$^{1-7}$ flies exhibited diminished avoidance behaviour as compared to *WT* flies (T-test P=0.0239; N=10).
(D) *MB247Gal4;UAS-dfmr1RNAi*$^{1-7}$ flies exhibit a significantly decreased avoidance response when tested against *WT* dSO (T-test P=0.0016; N=8). *WT* flies exhibited normal avoidance behaviour when tested against dSO produced by *MB247Gal4;UAS-dfmr1RNAi*$^{1-7}$ flies (T-test P=0.0707; N=8).
(E) *C747-Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies did not exhibit any significant decrease in dSO avoidance behaviour (T-test P=0.0858; N=16).
(F) *WT flies did not exhibit any significant decrease in dSO avoidance behaviour in response to C747-Gal4:UAS-dfmr1RNAi*$^{1-7}$ *dSO* (T-test P=0.5653; N=6). *C747-Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies did not exhibit any significantly defect in dSO avoidance when *WT* flies were utilized as the emitters (T-test P=0.5325; N=6). All graphs depict mean ± S.E.M.

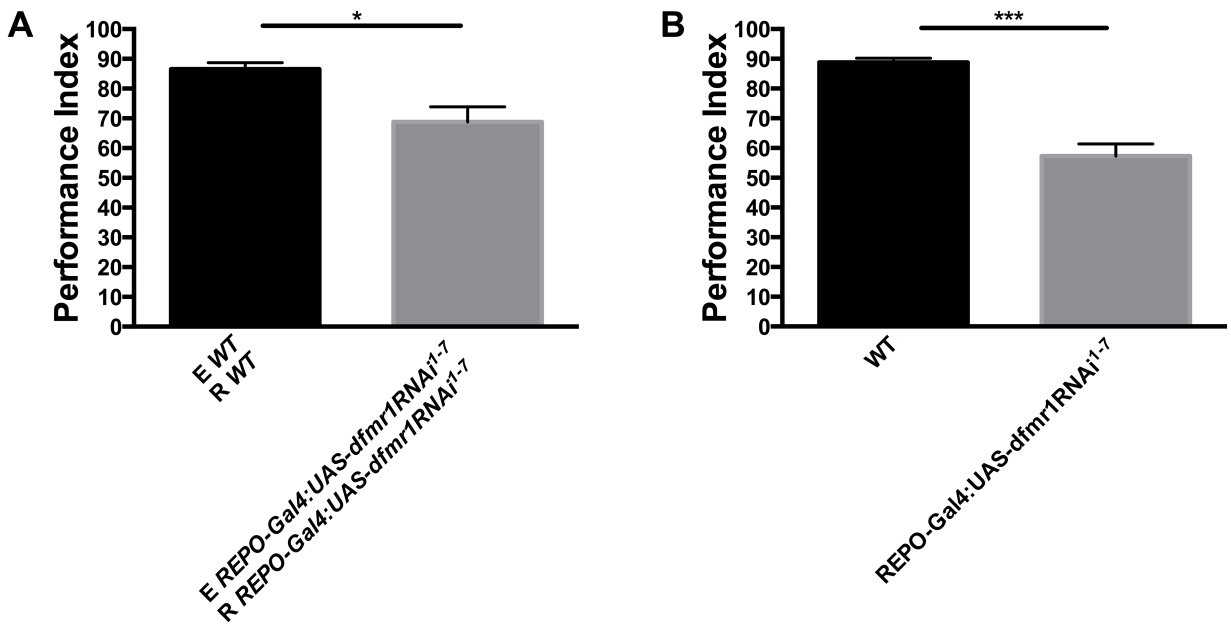

Figure 2-7. Fragile X Mental Retardation Protein (FMRP) is required in the Glia for Drosophila stress odourant (dSO) avoidance behaviour and learning.
(A) *REPO-Gal4:UAS-dfmr1RNAi*$^{1\text{-}7}$ flies exhibited diminished avoidance behaviour as compared to *WT* flies (T-test P=0.0345; N=13).
(B) *REPO-Gal4:UAS-dfmr1RNAi*$^{1\text{-}7}$ flies exhibited significantly lower learning compared to *WT* flies (T-test P=0.004; N=4). All graphs depict mean ± S.E.M.

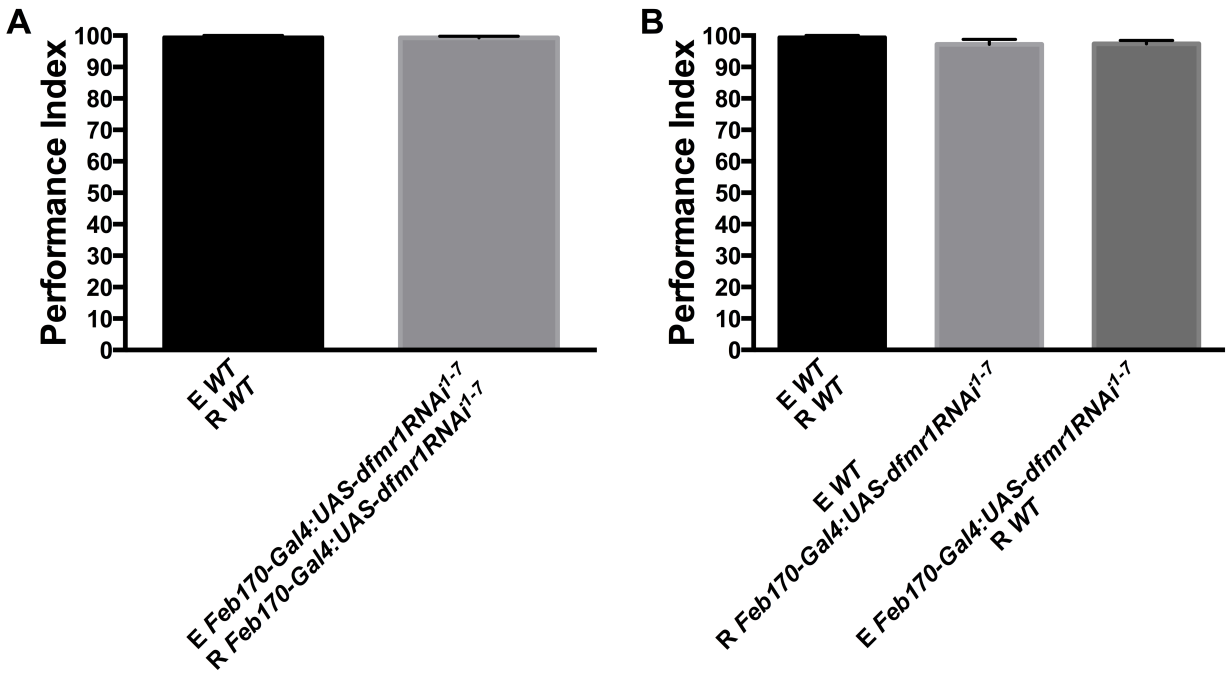

Figure 2-8. Fragile X Mental Retardation Protein (FMRP) is not required in the central complex for Drosophila stress odourant (dSO) avoidance behaviour.
(A) *Feb170-Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies did not exhibit any defect in avoidance response compared to *WT* flies (T-test P=0.8973; N=10).
(B) *Feb170-Gal4:UAS-dfmr1RNAi*$^{1-7}$ flies did not exhibit any defect in avoidance when tested against *WT* dSO (T-test P=0.2119; N=10). All graphs depict mean ± S.E.M.

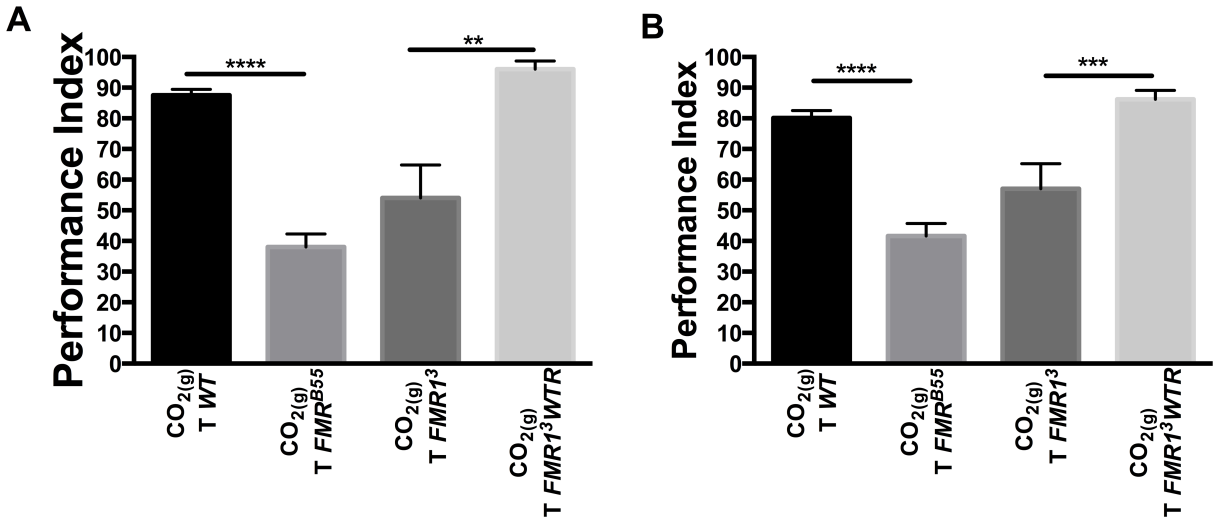

Figure 2-9. Fragile X Mental Retardation Protein (FMRP) is required for carbon dioxide ($CO_{2(g)}$) avoidance in Drosophila.
(A) *FMR^{B55}* (T-test P<0.0001; N=6) and *$FMR1^{3}$* (T-test P=0.013; N=6) flies exhibited significantly decreased avoidance to $CO_{2(g)}$ at a concentration of 0.2 mL/min compared to *WT* flies.
(B) *FMR^{B55}* (T-test P<0.0001; N=10) and *$FMR1^{3}$* (T-test P=0.009; N=13) flies exhibited significantly decreased avoidance to $CO_{2(g)}$ at a concentration of 0.5 mL/min compared to *WT* flies. All graphs depict mean ± S.E.M.

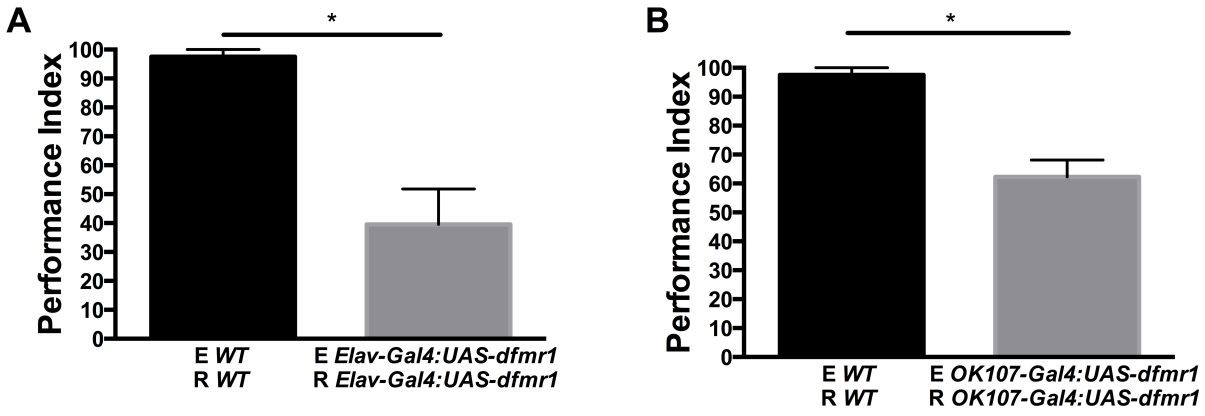

Figure 2-10. Overexpression of Fragile X Mental Retardation Protein (FMRP) in the mushroom body and pan-neuronally results in decreased Drosophila stress odourant (dSO) avoidance behaviour.

(A) *Elav-Gal4:UAS-dfmr1* flies exhibited decreased avoidance as compared to *WT* flies (T-test P=0.0016; N=8).

(B) *OK107-Gal4:UAS-dfmr1* exhibited a decrease in avoidance response as compared to *WT* flies (T-test P=0.00418; N=6). All graphs depict mean ± S.E.M.

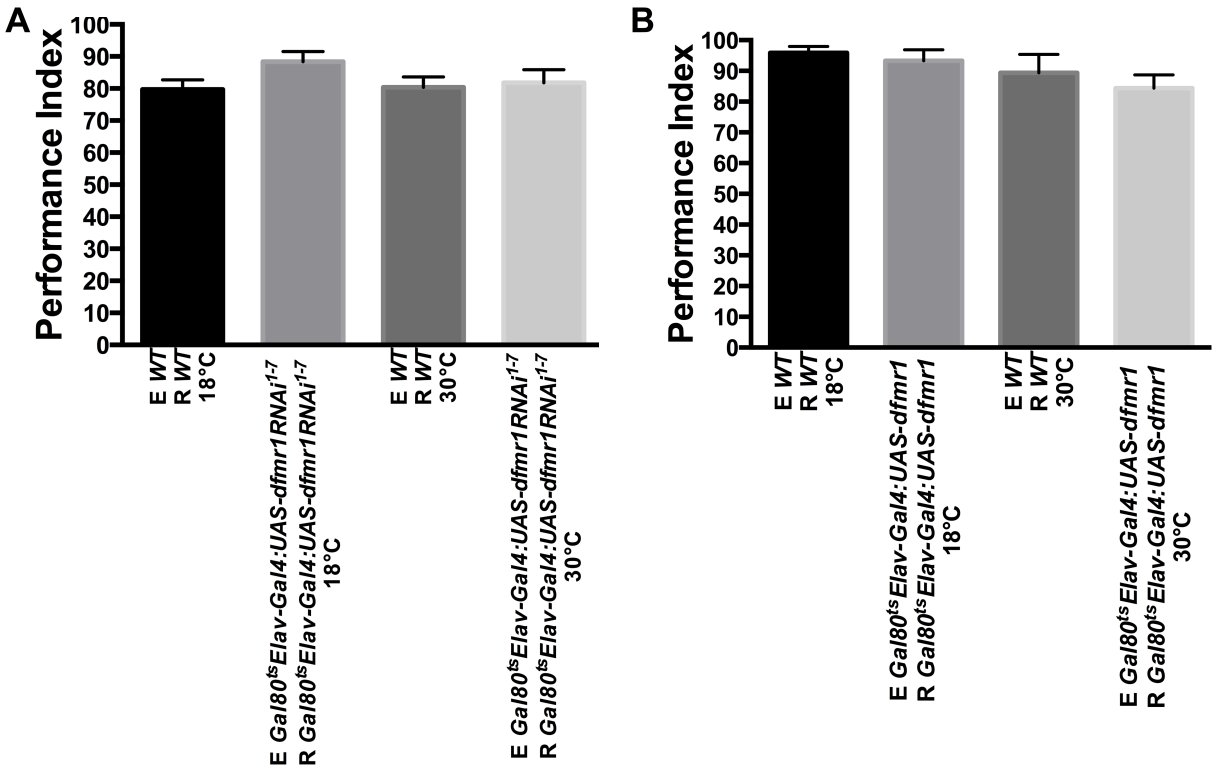

Figure 2-11. Acute pan-neuronal disruption of Fragile X Mental Retardation Protein (FMRP) does not result in decreased Drosophila stress odourant (dSO) avoidance behaviour.

(A) At 18°C RNAi is inactive; *Gal80^{ts}Elav-Gal4:UAS-dfmr1RNAi$^{1-7}$* did not exhibit an significant decrease in avoidance behaviour as compared to *WT* flies (ANOVA P=0.2924; N=8). At 30°C RNAi is no longer repressed; *Gal80^{ts}Elav-Gal4:UAS-dfmr1RNAi$^{1-7}$* flies did not exhibit any significant decrease in avoidance compared to *WT* flies (ANOVA P=0.9929; N=8) or RNAi inactive *Gal80^{ts}Elav-Gal4:UAS-dfmr1RNAi$^{1-7}$* flies at 18°C (ANOVA P=0.6121; N=8). *WT* flies exhibited no significant difference in avoidance behaviour at 18°C or 30°C (ANOVA P=0.9983; N=8).

(B) FMRP overexpression is repressed at 18°C; *Gal80^{ts}Elav-Gal4:UAS-dfmr1* flies did not exhibit did any significant difference in avoidance behaviour as compared to *WT* flies (ANOVA P=0.9704; N=7). Acute overexpression of FMRP at 30°C did not results in any significant difference in avoidance behaviour by *Gal80^{ts}Elav-Gal4:UAS-dfmr1* flies as compared to both *WT* flies (ANOVA P=0.1803; N=7) and control flies at 18°C (ANOVA P=0.3789; N=7). *WT* flies did not exhibit any significant difference in avoidance behaviour at 18°C or 30°C (ANOVA P=0.7424; N=7). All graphs depict mean ± S.E.M.s

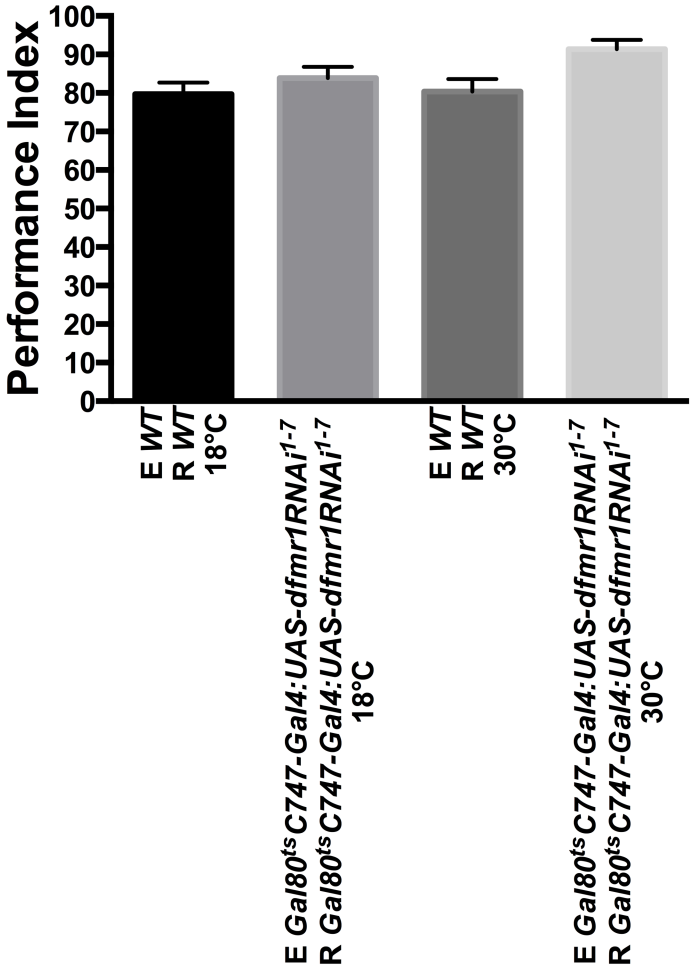

Figure 2-12. Acute knockdown of Fragile X Mental Retardation Protein (FMRP) in the mushroom body does not result in decreased Drosophila stress odourant (dSO) avoidance behaviour.
When RNAi expression is repressed at 18°C, *Gal80^{ts}C747-Gal4:UAS-dfmr1RNAi$^{1-7}$* did not exhibit an significant decrease in avoidance behaviour as compared to *WT* flies (ANOVA P=0.7439; N=17). At 30°C when RNAi expression is no longer repressed *Gal80^{ts}C747-Gal4:UAS-dfmr1RNAi$^{1-7}$* flies did not exhibit any significant decrease in avoidance compared to *WT* flies (ANOVA P=0.0.0593; N=17) or RNAi inactive *Gal80^{ts}C747-Gal4:UAS-dfmr1RNAi$^{1-7}$* flies at 18°C (ANOVA P=0.8475; N=17). *WT* flies exhibited no significant difference in avoidance behaviour at 18°C or 30°C (ANOVA P=0.9985; N=17). Graphs depicts mean ± S.E.M.

CHAPTER 3- The Cyclic Adenosine Monophosphate (cAMP) Signalling Pathway Interacts with Fragile X Mental Retardation Protein (FMRP) to Mediate Avoidance Behaviour

3.1 Introduction

3.1.1 The Cyclic Adenosine Monophosphate (cAMP) Signalling Pathway

Cyclic adenosine 3′,5′-monophosphate (cAMP) is an intracellular secondary messenger molecule that functions in signal transduction in the CNS. The cAMP cascade functions in mediating neuronal growth and differentiation, organization and regulation of cytoskeleton structure, neurotransmitter synthesis and transmission, and receptor sensitivity (*306*).

The cAMP signalling pathway is activated by the binding of a ligand to a G protein-coupled receptor (GPCR), which activates the production of cAMP (*307*). cAMP levels are regulated by the action of two enzymes, adenylyl cyclase (AC) and phosphodiesterase (PDE), which synthesize and degrade cAMP respectively (*181, 182, 308-310*). AC catalyzes the conversion of ATP to cAMP while PDE degrades cAMP molecules by hydrolyzing the phosphodiester bond (Figure 3-1) (*308, 309*).
A primary downstream effector of cAMP is protein kinase A (PKA), which modulates cAMP signalling by phosphorylating targets within the pathway as well as ACs and PDEs (*311*). PKA is a serine/threonine protein kinase that modulates gene expression and cellular responses with the help of A kinase anchoring proteins (AKAPs) that target PKA to specific substrates (*312*). AKAPs provide specificity in cAMP signal transduction. In addition to PKA, the cAMP cascade has other downstream targets including cyclic nucleotide-gated (CNG) ion channels that increase electrical activity in cells and guanine-nucleotide-exchange factors (GEF) EPACs, which function in modulating cell morphology and secretory vesicle dynamics (*313*).

During axon guidance, growth cones are directed by guidance factors that are either attractive or repulsive (*140*). Growth cone response to guidance cues may depend on the cytosolic levels of cAMP. Increases in cAMP levels increase attraction towards a guidance factor and decreases in cAMP levels results in repulsion (*145, 146*). Thus the same guidance cue can be both attractive and repulsive based on cAMP activity. Cytosolic cAMP levels may be regulated by guidance cues themselves or by cell-cell

interactions in which contact with 'guidepost' cells may trigger a change in cAMP levels resulting in repulsion, permitting the axon to continue towards the next target (*314*). Furthermore down stream targets of cAMP include proteins that mediate cytoskeleton morphology and function in growth cone turning (*315*).

cAMP regulates neurotransmission. A number of neurotransmitters signal via the cAMP cascade through specific GPCRs including serotonin, adrenaline, dopamine, and GABA (*316*). The cAMP pathway has been shown to enhance neuronal excitability and increase vesicle neurotransmitter release (*317, 318*). Odour detection is mediated by the cAMP signalling cascade. The cAMP signalling cascade is initiated in ORNs in response to the binding of an odour to a G-protein couple receptor, triggering the synthesis of cAMP by AC which results in the opening of CNG ion channels (*319-321*). ORNs also express PDEs, which degrades cAMP and negatively regulates signal transduction (*321*).
Changes in signal transduction results in altered sensory processing and behavioural responses (*322, 323*).

3.1.2 Fragile X Mental Retardation Protein (FMRP) Regulation of the Cyclic Adenosine Monophosphate (cAMP) Signalling Pathway

The secondary messenger molecule cAMP plays a crucial role in signal transduction and modulation of physiological processes within neurons, specifically cAMP is critical for neural development. The cAMP signalling cascade has a broad range of targets and as such has a wide spread effects. Dysregulation of the cAMP signalling cascade has been proposed to be one of the mechanisms underlying FXS neuropathology (*79*).

cAMP levels are regulated by AC and PDE. In the absence of FMRP, cAMP levels are decreased suggesting that FMPR regulates the production of cAMP (*78*). Although the exact mechanism by which FMRP regulates cAMP still requires elucidation, one of FMRP's 500 cargo mRNAs is adenylate cyclase (AC) (*12, 13, 22*). Loss of FMRP from RNP complexes also corresponds with a decrease in AC mRNA

levels, suggesting that FMRP regulates cAMP production through its enzymatic conversion from ATP by AC (*77-79*).

In absence of AC, a decrease in the number of synaptic boutons, axon branches, and docked vesicles is observed; in absence of PDE function an increased number of docked vesicles and poorly defined synaptic specialization is observed (*181, 324-326*). AC and PDE are important for cAMP homeostasis. Impairment in the functioning of either enzymes results in defects in neuron development and function.

3.2 Methods and Materials

3.2.1 Drosophila Fly Stocks

See 2.2.1 for full description.

3.2.2 The Gal4-UAS Binary Expression System

See 2.2.2 for full description.

3.2.3 Genetic Crosses

To determine the genetic interaction between cAMP and FMRP in mediating dSO avoidance behaviour, heteroallelic flies were generated by mating rut^1 and dnc^1 virgin females with $dfmr1^3$ males to produce the following crosses (Note that in Drosophila *dfrm1* is not X-linked and is located on the third chromosome.):

$\underline{rut^1 x dfmr1^3}$

rut^1 virgin females x $dfmr1^3$ males
rut^1 virgin females x *WT* males
WT virgin females x $dfmr1^3$ males
WT virgin females x *WT* males

$\underline{dnc^1 x dfmr1^3}$

dnc^1 virgin females x $dfmr1^3$ males
dnc^1 virgin females x *WT* males
WT virgin females x $dfmr1^3$ males
WT virgin females x *WT* males

The progeny of the crosses were utilized for avoidance testing. Flies were anesthetized using $CO_{2(g)}$ prior to sorting and collection. Crosses were maintained at 22°C on standard cornmeal yeast media.

3.2.4 Drosophila Stress Odourant (dSO) Avoidance Assay

See 2.2.4 for full description.

3.2.5 Performance Index (PI) Calculation and Statistical Analysis

See 2.2.6 for full description.

3.2.6 Drosophila Brain Immunohistochemistry

Dso Exposure. To produce dSO a group of 50 emitter flies (mixed gender) were vortexed for 1 minute (flies were vortexed for 3 seconds followed by 5 seconds rest for a 1 minute duration) in a 10mL Falcon tube sealed with Parafilm (Fisher Scientific 149598) at maximum speed (Fisher Vortex Mixer). Emitter flies were removed from Falcon tube and a group of 50 1-3 day old wild-type naïve responder flies (mixed gender) were transferred into the dSO-containing Falcon tube that was sealed with Parafilm for 1 minute. After 1 minute exposure to dSO, naïve responder flies were placed on ice for 2 minutes and heads of female responder flies were removed and placed in cold 1X PBS for dissection. Unexposed flies were placed in an unconditioned 10mL Falcon tube sealed with Parafilm for 1 minute. Flies were subsequently placed on ice for 2 minutes and heads of female flies were removed and placed in cold 1X PBS for dissection.

Dissection and Processing. Fly heads were dissected in 1X PBS to remove brains. Following removal, brains were transferred to 4% PFA to fix for 10 minutes at room temperature. Following the 10minute fixation period, brains were transferred into a penetration/fixation buffer (0.25% Triton 4% PFA) and placed under vacuum for 1.5 hours. Brains were then incubated in a penetration/blocking buffer (2%PBST, 10% NGS, 0.02% Sodium Azide) on rocker for 2 hours at 4^{o}C and following completion, transferred to primary antibody solution (1:1000 □-PKA Abcam ab118531 and 1% PBST with 0.25% NGS) and incubated overnight at 4^{o}C. Following overnight incubation, brains were washed 3 times in 1% PBST for 20 minutes. Brains were subsequently incubated with secondary antibody solution (1:200 Cy3 □-Rabbit Jackson ImmunoResearch 111-165-003 and 1% PBST with 0.25% NGS) overnight at 4^{o}C. Following incubation with secondary antibody, brains were washed 3 times with 1% PBST and mounted

using FocusClear (Cedarlane FC-101). Imaging was completed using Zeiss LSM 700 confocal microscope and images were quantified using ImageJ.

3.2.7 Pharmacological Intervention

LiCl. Lithium Chloride (LiCl) (Sigma L9650) was added directly to the standard cornmeal yeast medium for drug administration. Flies were set up in food bottles containing 10mM LiCl or the vehicle for 4 days and transferred to food vials containing 10mM LiCl or the vehicle the day prior to testing. Emitter flies were not treated with LiCl. Flies were maintained at 22°C throughout treatment duration.

IBMX. 3-isobutyl-1-methylxanthine (IBMX) (Sigma I7018) was added to standard cornmeal yeast media for drug administration. Flies were placed in food bottles containing 0.05 mg/mL IBMX or the vehicle for 4 days and transferred to food vials containing 0.05mg/mL IMBX or the vehicle the day prior to testing. Emitter flies were not treated with IBMX. Flies were maintained at 22°C throughout treatment duration.

8-CPT. 8-(4-Chlorophenylthio)adenosine 3'-,5'-cyclic monophosphate sodium salt (8-CPT) (Sigma C3912) was administered to flies on 2.1cm Whatman filter paper (Fisher WHT1540321). Flies were placed in vials containing 240µL of 8-CPT with 5% sucrose or vehicle (5% sucrose) and treated for 5 days prior to testing. Flies were transferred to new vials containing fresh 8-CPT or 5% sucrose daily. Emitter flies were not treated with 8-CPT. Flies were maintained at 22°C throughout treatment duration.

3.3 Results

3.3.1 Research Highlights/Summary

FMRP plays a crucial role in neural circuit patterning/formation and the regulation of key signalling pathways (*107*). Cyclic adenosine monophosphate (cAMP) is the most abundant secondary messenger in the CNS and modulates many critical neural processes including neuronal development and function. In Fragile X Syndrome cAMP signalling is misregulated due to the loss of FMRP (*306, 327*). Deficiencies in the cAMP signalling pathway is one of the mechanisms underlying Fragile X Syndrome pathology (*77-79*). Here we show that cAMP is required for avoidance and identify the cAMP cascade as a key signalling pathway underlying avoidance behaviour dysfunction in FXS. Through pharmacological

intervention targeting the misregulated cAMP pathway we show that avoidance behaviour can be rescued in FXS flies, demonstrating the ability to ameliorate a developmental abnormality.

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3.3.2 The Cyclic Adenosine Monophosphate (cAMP) Mutants *Dnc*^{1} and *Rut*^{1} Exhibit Decreased Drosophila Stress Odourant (dSO) Avoidance Behaviour

The cAMP signalling pathway is the most prevalent major secondary messenger system in the CNS and functions in the development of neuronal networks, signal transduction, and axon pathfinding. In FXS cAMP metabolism is altered due to a loss of FMRP and as a result neurotransmitters and receptors signaling through the cAMP cascade are deficient (*77, 327, 328*).

To determine the requirement of cAMP in mediating avoidance behaviour we used the cAMP mutants dunce (*dnc*) and rutabaga (*rut*), which encodes a cAMP-specific phosphodiesterase (PDE) and a Ca^{2+}/calmodulin-activated adenylyl cyclase (AC) respectively (*181, 324, 325*). These mutations in the cAMP signalling pathway results in synapse dysmorphologies. Decrease in the number of synaptic boutons, axon branches, and docked vesicles compared to wild-type flies are characteristic of *rut* mutants, while *dnc* mutants possess an increased number of docked vesicles but poorly defined synaptic specialization (*326, 329*). Both *dnc* and *rut* flies exhibited decreased avoidance as compared to *WT* flies (Figure 3-2 A). *Rut and dnc* flies exhibited decreased avoidance behaviour when *WT* flies were utilized as dSO emitters (Figure 3-2 B). *WT* flies did not exhibit any significant changes in avoidance behaviour when *rut and dnc* flies were utilized as dSO emitters (Figure 3-2 C). Absence of a dSO emission defect suggests that cAMP homeostasis is required for dSO avoidance behaviour.

3.3.3 Protein Kinase A (PKA) Levels Increased in Response to Drosophila Stress Odourant (dSO) Exposure

PKA is a direct target of cAMP metabolism. The inactive form of PKA consists of 2 regulatory subunits and 2 catalytic subunits. PKA is activated in response to elevated cAMP levels, in which cAMP binds to regulatory subunits of PKA, resulting in the disassociation of catalytic subunits (*330*). To confirm the predicted requirement of cAMP in dSO avoidance behaviour we examined the relative PKA levels in *WT* fly brains in response to dSO exposure by utilizing a catalytic subunit-specific PKA antibody. PKA levels

were significantly elevated in *WT* brains following dSO exposure compared to naïve, unexposed *WT* flies (Figure 3-3 A, B). This result suggests that cAMP signalling mediates/is activated and participates in dSO avoidance behavior.

3.3.4 Cyclic Adenosine Monophosphate (cAMP) Interacts with Fragile X Mental Retardation Protein (FMRP) to Mediate Drosophila Stress Odourant (dSO) Avoidance Behaviour

Rut and *dnc* are preferentially expressed at elevated levels in the MB, where both FMRP and cAMP activity are both required for axon pruning (*286, 331-333*). Given that FMRP is a regulator of the cAMP signalling pathway, we sought to determine the genetic interaction between FMRP and cAMP in mediating avoidance behaviour. We tested this by examining the genetic interaction of the $dfmr1^3$ allele with both *rut* and *dnc.*

Rut^1/WT, $rut^1/FMR1^3$, and $dnc^1/FMR1^3$ flies exhibited significant decreased dSO avoidance behaviour compared to *WT* flies (Figure 3-4 A, D). There was no significant decrease in dSO avoidance response exhibited by $FMR1^3/WT$ and dnc^1/WT flies, suggesting genetic rescue (Figure 3-4 A, D).

To confirm that the decreased avoidance behaviour exhibited by flies was due to sensory processing defect and not a dSO emission defect caused by the interaction of $dfmr1^3$ with *rut* and *dnc*, we tested *WT* flies against the cAMP-FMRP mutants dSO. *WT* flies did not exhibit any significant changes in avoidance behaviour when $FMR1^3/$WT, rut^1/WT, $rut^1/FMR1^3$, dnc^1/WT, and $dnc^1/FMR1^3$ flies were utilized as dSO emitters (Figure 3-4 C,F). When *WT* flies were utilized as dSO emitters, rut^1/WT, $rut^1/FMR1^3$, and $dnc^1/FMR1^3$ flies exhibited decreased dSO avoidance behaviour (Figure 3-4 B.F). $FMR1^3/WT$ and dnc^1/WT flies exhibited no significant decrease in avoidance behaviour when *WT* flies were utilized as emitters (Figure 3-4 B, F). Taken together these results suggest that FMRP and cAMP interact to mediate wild-type avoidance behaviour.

3.3.5 5-Day Lithium Treatment Increases Drosophila Stress Odourant (dSO) Avoidance Behaviour in the Fragile X Syndrome FMR^{B55} Mutants

Given the role of cAMP in mediating avoidance behaviour, we wanted to determine if avoidance behaviour could be rescued through pharmacological intervention targeting the cAMP signalling pathway. We asked if lithium, which increases cAMP levels by enhancing basal levels of AC, could rescue

avoidance behaviour (*334*). Following 5-day treatment with 10mM lithium *FMR*^{B55} flies exhibited significant increase in avoidance as compared to *FMR*^{B55} flies treated with vehicle only (Figure 3-5).

3.3.6 5-Day IBMX Treatment Increases Drosophila Stress Odourant (dSO) Avoidance Behaviour in the Fragile X Syndrome Mutants *FMR1*^{3} and *FMR*^{B55}

Next we asked whether IBMX, a non-specific PDE inhibitor, could rescue avoidance behaviour in FXS flies. 5-day IBMX administration resulted in a significant increase in avoidance behaviour exhibited by *FMR*^{B55} and *FMR1*^{3} flies as compared to *FMR*^{B55} and *FMR1*^{3} flies treated with vehicle (Figure 3-6 A, B).

3.3.7 5-Day 8-CPT Treatment Rescues Drosophila Stress Odourant (dSO) Avoidance Behaviour in the Fragile X Syndrome *FMR*^{B55} Mutants

To confirm specificity of cAMP action in rescuing dSO avoidance response, we asked if use of a cAMP analog could rescue avoidance behaviour. 5-day 8-CPT administration resulted in a significant rescue of dSO avoidance in *FMR*^{B55} and *FMR1*^{3} flies (Figure 3-7 A, B). Collectively these results suggest that the FMRP-dependent loss and misregulation of cAMP signalling results in defects in avoidance behaviour as exhibited by FXS flies.

3.4 Discussion

3.4.1 Cyclic Adenosine Monophosphate (cAMP) interacts with Fragile X Mental Retardation Protein (FMRP) to Mediate Drosophila Stress Odourant (dSO) Avoidance Behaviour

cAMP plays a critical role in neural functioning, in both the regulating neuronal excitability and establishment of neural circuitry (*317, 335, 336*). Disruptions in cAMP metabolism results in abnormalities in synaptic transmission, activity-dependent facilitation and potentiation of neurotransmitter release in the NMJ—specifically kinetics and output from growth cones and presynaptic terminals— growth cone motility, aborization, MB structure, altered K^+ channel regulation, and action potential generation (*317, 335-341*). Mutations in genes regulating cAMP metabolism have been implicated behaviour abnormalities, including altered jump-and-flight escape response and learning and memory (*182, 309, 324, 342*).

Here we show that cAMP is required for dSO avoidance behaviour. Our results demonstrate that cAMP is activated in response to dSO exposure in *WT* flies and that mutations in cAMP regulatory genes, *rut* and *dnc,* result in decreased dSO avoidance behaviour (Figure 3-2 A-C).

Neuronal morphology is crucial for neuronal function. Abnormal neuronal morphology is a neuropathological hallmark of neurodevelopmental disorders (*343*). Structural differences in neuronal morphology caused by these mutations results in differences in synaptic neurotransmitter release. *Rut* mutants have less docked vesicles, and while *dnc* mutants posses a greater number of docked vesicles; and they display poorly defined synaptic structure (*326, 329*). Collectively these results indicate the importance of cAMP both developmentally for the establishment of neuronal circuitry governing dSO processing, as well as acutely for proper signalling within these networks as both mutants display instability in synaptic output and firing patterns, which likely contributes to perturbations in information processing resulting in decreased dSO avoidance behaviour (*344*).

Loss of FMRP results in altered signalling/function in several key signalling pathways involved in neuronal development including PI3K, mGluR, mTOR, GSK3, cAMP, and insulin signalling (InS) (*33, 77, 194, 345-349*). FMRP regulates the translation of 5% of mRNA in the brain (*22*). FMRP binds to specific mRNAs to form mRNA-protein complexes that associate with polyribosomes to mediate mRNA translation. Loss of FMRP from the polyribosome mRNA-protein complexes results in translational profile shifts (*22*). FMRP regulates cAMP production through its enzymatic conversion from ATP by AC (*77-79*).

Decreased AC mRNAs levels have been observed in FXS tissues (*22, 263, 350*). This decrease in AC mRNAs may account for the FMRP-dependent decrease in cAMP levels observed in FXS models, including human, mouse, and Drosophila neural cells (*327*). Loss of translational regulation may lead to abnormalities in neuronal circuits and consequently modulation of behaviour observed in FXS (*226, 227*).

FMRP can alter protein expression through cAMP signal transduction (*351*). cAMP can induce changes in neuronal functioning through the activation of the cAMP-dependent enzyme, PKA. Activation of PKA results in the phosphorylation of other kinases, transcription factors, and ion channels. Like *rut* and *dnc*, PKA (*DC0*) is also preferentially expressed in the MB (*352*). A downstream target of cAMP is the

transcription factor CREB (cAMP response element binding protein). CREB phosphorylation promotes transcription of genes containing the cAMP-response elements, which results in long-term physiological changes in the CNS and regulates synaptic structure in the larval NMJ (*353, 354*).

Calcium signalling is required in a number of numbers of neural processes involving FMRP including synaptogenesis, synaptic pruning, and plasticity (*355-357*). FMRP regulates the expression of key calcium-binding proteins and the Ca^{2+}-dependent depolarization of neurons (*358*). *Dfrm1* mutants exhibit altered expression of mRNA of key calcium-binding proteins and defects in the release of Ca^{2+} from internal stores (*358*). Ca^{2+} influx occurs through NMDA-type glutamate receptors (NMDARs), which are regulated by PKA (*359*). FMRP regulates Ca^{2+} signalling in excitatory and inhibitory neurons in the MB during development (*360*). cAMP and FMRP may both exist in a feedback loop as when an increase in cAMP levels in *dfmr1*^{3}/+ mutants was observed, an increase in FMRP was also seen, suggesting the FMRP acutely responds to and is regulated by cAMP levels (*185*).

Phenotypes of *rut* and *dnc* are complex as cAMP has many downstream targets, including channel permeability, signal transduction, and gene expression (*344, 361*). Glutamatergic and cholinergic synapses, which utilizes □-aminobutyric acid as a neurotransmitter, are regulated by cAMP signalling (*318, 341*). GABA has a critical role in the modulation of coordinated behaviors, such as learning and memory (*362*). Binding of an odourant to an odourant receptor and subsequent activation of an ORN initiates the cAMP signalling cascade (*363, 364*). Initiation of cAMP signalling cascade and opening of cyclic nucleotide-gated channels is dependent on synthesis of cAMP by AC (*364*). GABAergic transmission is altered in cAMP mutants, *rut* and *dnc,* suggesting that cAMP modulates GABAergic transmission (*318*).

The MB is extensively innervated by GABAergic neurons (*95*). Our results demonstrate that the MB has a key role in mediating dSO avoidance. During development GABAergic signalling regulates key processes such as cell proliferation, differentiation, migration, and synapse maturation (*365*). GABAergic transmission is regulated by the CAMP signalling pathway (*318*). PKA mediates GABA receptor sensitivity and GABAergic transmission (*318, 366*). Local neurons are also GABAergic and involved in

olfactory information processing by mediating odour response (*88, 90*). Furthermore loss of FMRP impairs expression of GABAergic receptors (*263, 367*).

Failure to initiate a proper cAMP signalling cascade in the MB of *rut* and *dmr1*^{3} mutants may account for inability to rescue dSO avoidance in *rut*^{1}/*FMR1*^{3} mutants. Furthermore failure to rescue dSO avoidance behaviour within wild-type range in *rut*^{1}/*WT* flies coupled with the lack of significant difference in avoidance behaviour between *rut*^{1}/*WT* and *rut*^{1}/*FMR1*^{3} flies suggests that AC is required in a dose-dependent manner as AC is the limiting factor in cAMP synthesis (*368*).

Taken together our results demonstrate that cAMP and FMRP interact to mediate dSO avoidance behaviour. *Rut*, *dnc*, and *PKA* are preferentially expressed in the MB, indicating their importance in mediating signalling and development within the MB, which we have identified to have a key role in modulating avoidance behaviour. FMRP is required to regulate development. Loss of FMRP results in improperly established neural circuits and dysfunction of signalling cascades that have been associated with behavioural abnormalities (*306*).

3.4.2 Pharmacological Intervention Targeting Cyclic Adenosine Monophosphate (cAMP) Rescues Drosophila Stress Odourant (dSO) Avoidance Behaviour in Fragile X Syndrome Flies

The transmission and processing of sensory information occurs in genetically pre-determined neuronal networks established during development. Abnormal gene expression within complex gene networks results in abnormalities in information processing due to perturbations in neural connectivity and ultimately dysfunction within complex neuronal networks (*304*). Here we show that cyclic adenosine monophosphate (cAMP) is required for avoidance and identify the cAMP cascade as a key signalling pathway underlying avoidance behaviour dysfunction in FXS (Figure 3-5; 3-6; 3-7).

By targeting the cAMP-signalling pathway using three pharmacological approaches, an AC-activator, a PDE-inhibitor, and a cAMP analog, we were able to confirm that cAMP signalling is required for dSO avoidance, and that loss of FMRP results in signalling dysfunction within this pathway.

Collectively our results suggest that FMRP is required developmentally to regulate cAMP signalling in the establishment of neuronal networks that mediate dSO sensory processing and avoidance behaviour,

specifically in the MB for higher-order processing, where loss of *dfmr1*^{3} results in neuronal structural and functional defects (*205*).

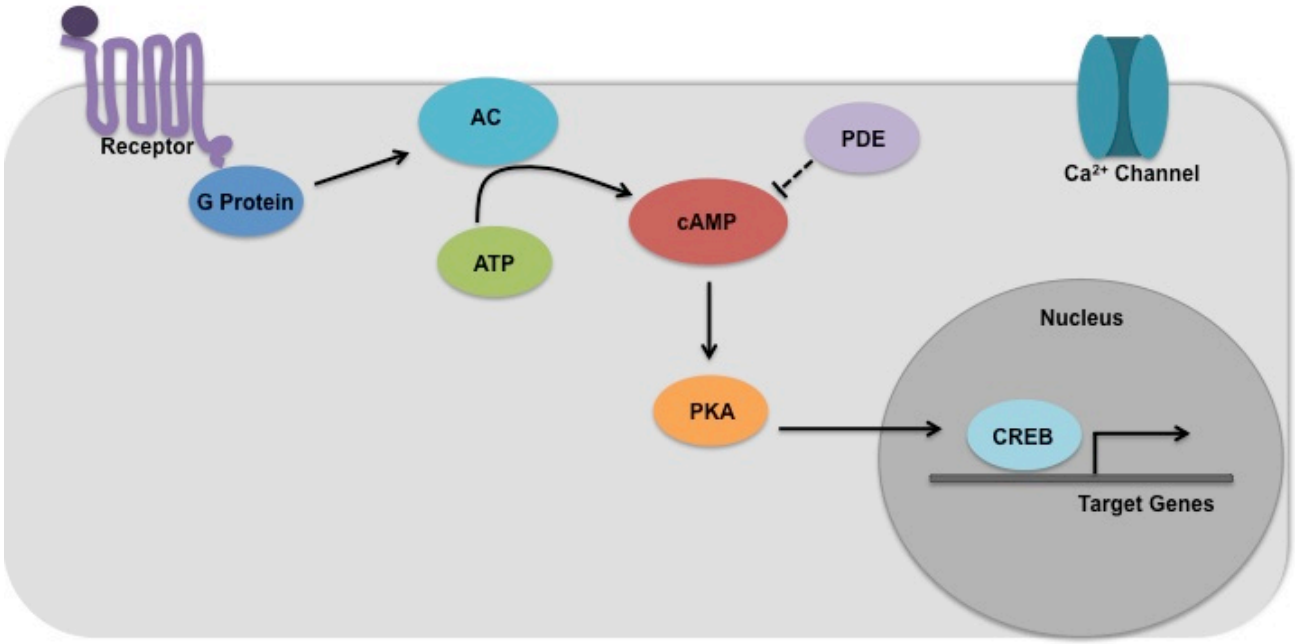

Figure 3-1. cAMP signalling pathway.
Activation of G-protein couple receptors initiates the catalytic conversion of ATP to cAMP by AC. cAMP activates PKA, a main downstream effector of cAMP signalling and transcription of Wnt targeted genes. cAMP levels are regulated through the activity of PDE.

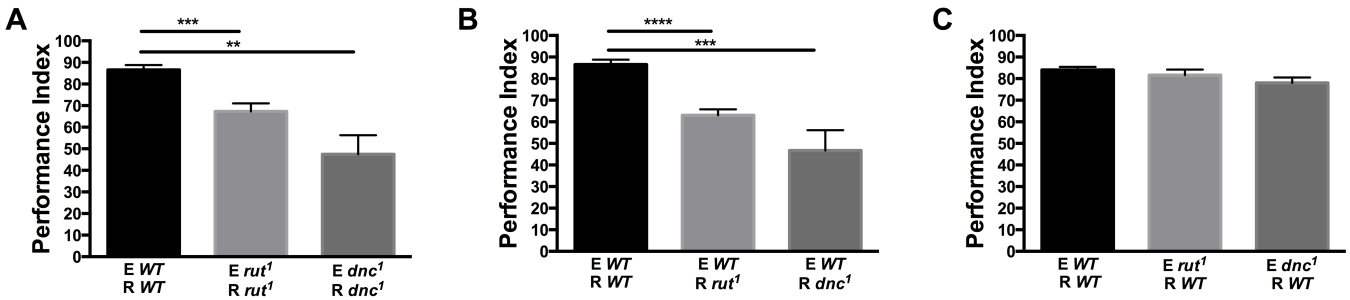

Figure 3-2. The cyclic adenosine monophosphate (cAMP) mutants dnc1 and rut1 exhibit decreased Drosophila stress odourant (dSO) avoidance behaviour.

(A) The cAMP phosphodiesterase mutant dunce (dnc1) shows a defect in avoidance behaviour compared to WT flies (T-test P=0.0011; N=6). Similarly the cAMP adenylyl cyclase mutant rutabaga (rut1) shows significantly decreased avoidance compared to WT flies (T-test P=0.0009; N=6).
(B) Rut (T-test P<0.0001; N=6) and dnc flies (T-test P=0.0007; N=6) exhibited decreased avoidance when tested against dSO produced by WT flies.
(C) WT flies exhibit normal avoidance to dSO emitted by the cAMP mutants dnc (T-test P=0.1170; N=6) and rut (T-test P=0.5180; N=6). All graphs depict mean ± S.E.M.

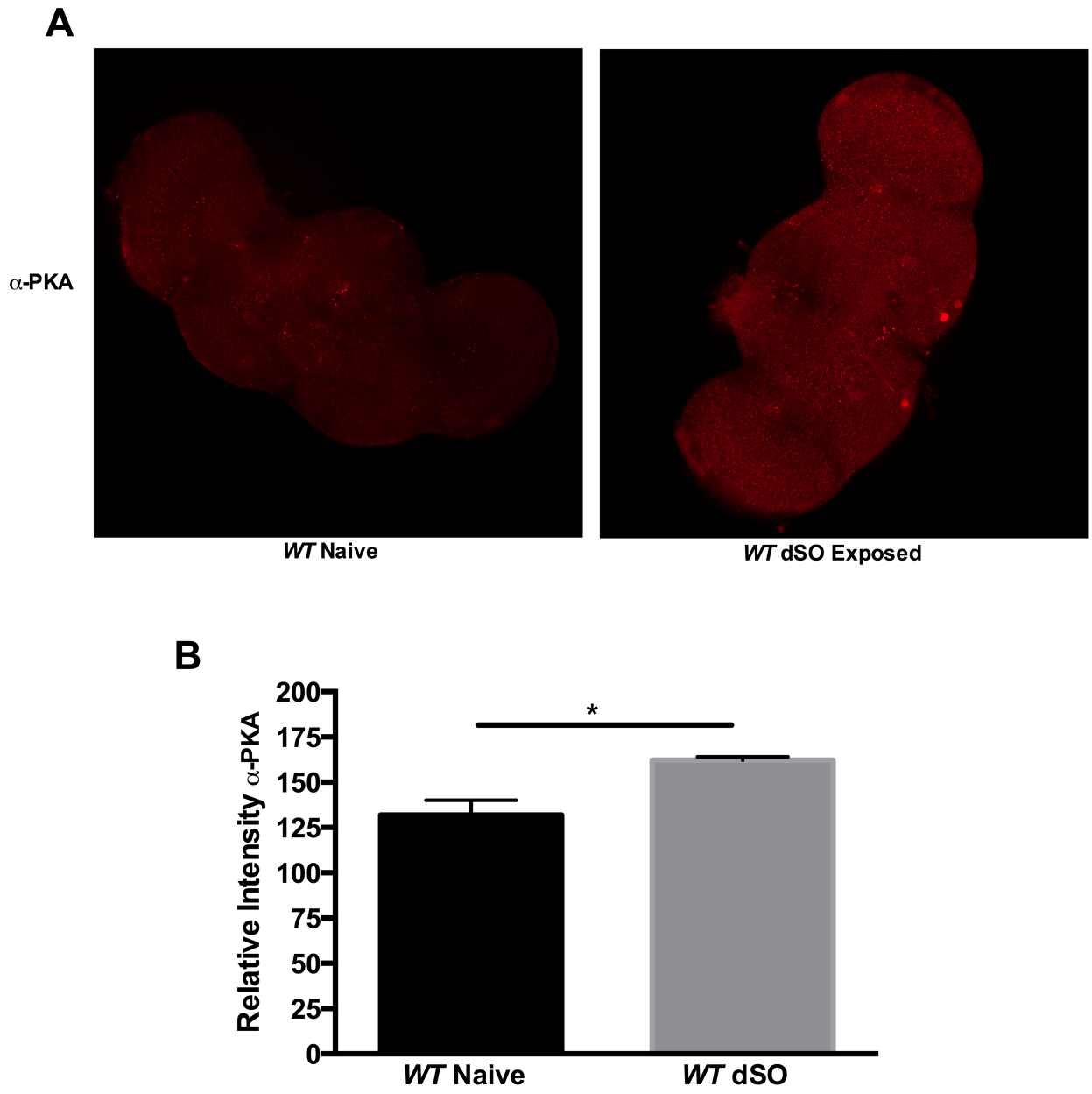

Figure 3-3. Protein kinase A (PKA) levels increased following exposure to Drosophila stress odourant (dSO).
(A) PKA levels in dSO exposed and unexposed *WT* fly brains.
(B) dSO exposure results in a significant increase in PKA levels in *WT* brains compared to unexposed control (T-test P=0.00226; N=3). Graph depicts mean ± S.E.M.

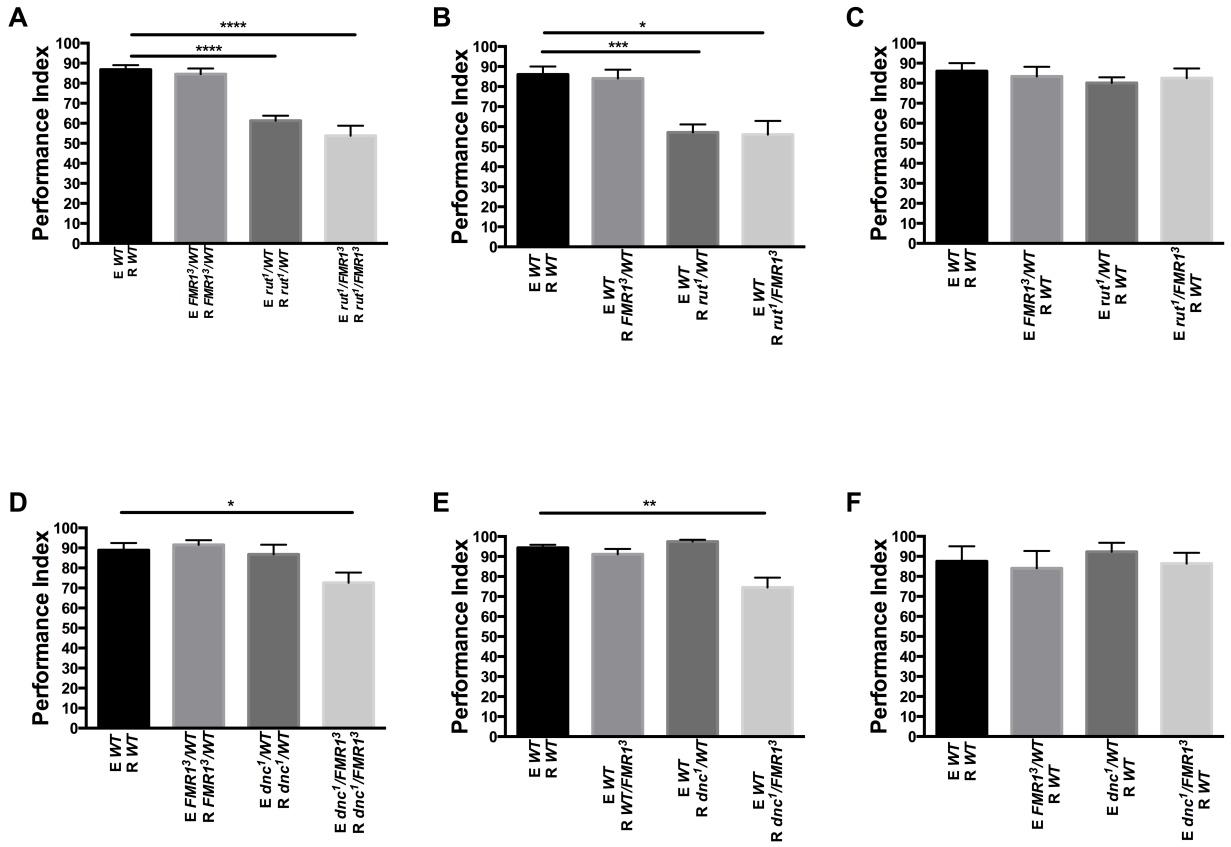

Figure 3-4. Cyclic adenosine monophosphate (cAMP) Interacts with Fragile X Mental Retardation Protein (FMRP) to mediate Drosophila stress odourant (dSO) avoidance behaviour.

(A) *rut*^{1}*/WT* flies (T-test P<0.0001; N=16) and *rut*^{1}*/ FMR1*^{3} flies (T-test P<0.0001; N=16) exhibit decreased avoidance behaviour compared to WT flies. *FMR1*^{3}*/WT* flies exhibit wild-type avoidance to dSO (T-test P=0.5272; N=16). No significant difference in avoidance between *rut*^{1}*/ FMR1*^{3} and *rut*^{1}*/WT* (T-test P=0.2374; N=16).

(B) *rut*^{1}*/WT* flies (T-test P=0.0005; N=9) and *rut*^{1}*/FMR1*^{3} flies (T-test P=0.0102; N=9) exhibit decreased avoidance to dSO produced by WT flies. *FMR1*^{3}*/WT* flies exhibit wild-type avoidance to dSO emitted by *WT* flies (T-test P=0.7725; N=9).

(C) *WT* flies exhibit normal avoidance when tested against dSO produced by FMR1^{3}/WT flies (T-test P=0.7188; N=9), *rut*^{1}*/WT* flies (T-test P=0.2459; N=9), and *rut*^{1}*/FMR1*^{3} flies (T-test P=0.6422; N=9).

(D) Wild-type avoidance is exhibited by *dnc*^{1}*/WT* flies (T-test P=0.7330, N=14) and *FMR1*^{3}*/WT* flies (T-test P=0.5330; N=14). *dnc*^{1}*/FMR1*^{3} flies exhibit decreased avoidance as compared to *WT* flies (T-test P=0.0149; N=14).

(E) Decreased avoidance to *WT* dSO is exhibited by *dnc*^{1}*/FMR1*^{3} flies as compared to *WT* flies (T-test P=0.0011, N=10). *dnc*^{1}*/WT* flies (T-test P=0.1025, N=10) and *FMR1*^{3}*/WT* flies (T-test P=0.2919, N=10) exhibited wild-type avoidance.

(F) *WT* flies do not exhibit any defect in avoidance when tested against dSO emitted by *dnc*^{1}*/FMR1*^{3} flies (T-test P=0.9116; N=4), *dnc*^{1}*/WT* flies (T-test P=0.6070, N=4), and *FMR1*^{3}*/WT* flies (T-test P=0.7729; N=4). All graphs depict mean ± S.E.M.

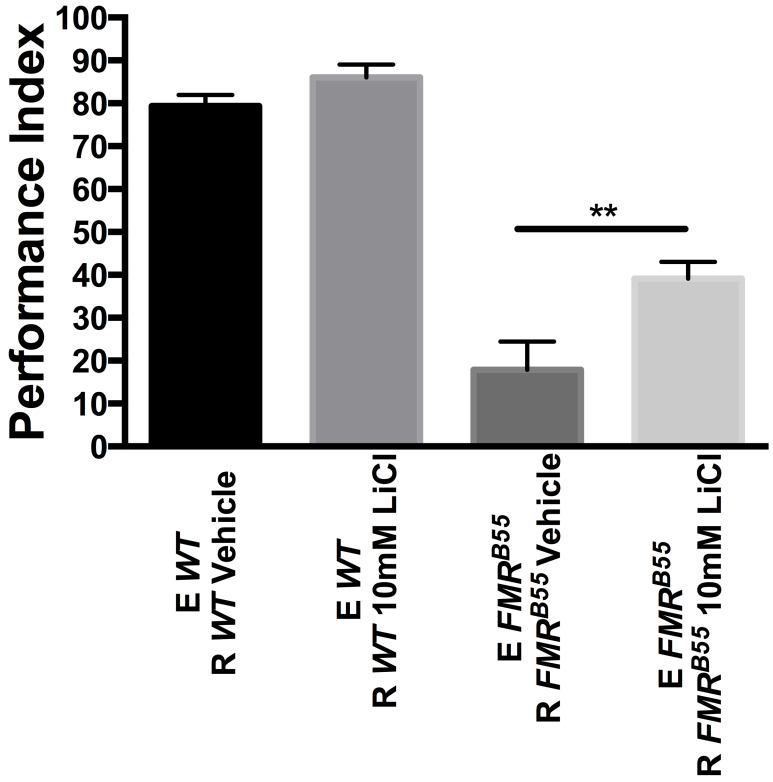

Figure 3-5. 5-Day lithium treatment increases Drosophila stress odourant (dSO) avoidance behaviour in the Fragile X Syndrome FMR^{B55} mutant.
5-day treatment of *FMR*^{B55} flies with 10mM lithium results in significantly increased avoidance compared to *FMR*^{B55} on vehicle (T-test P=0.0094; N=15). No significant difference in avoidance behaviour observed in *WT* flies following 5-day treatment with 10mM lithium as compared to vehicle (T-test P=0.0999; N=15). Graph depicts mean ± S.E.M.

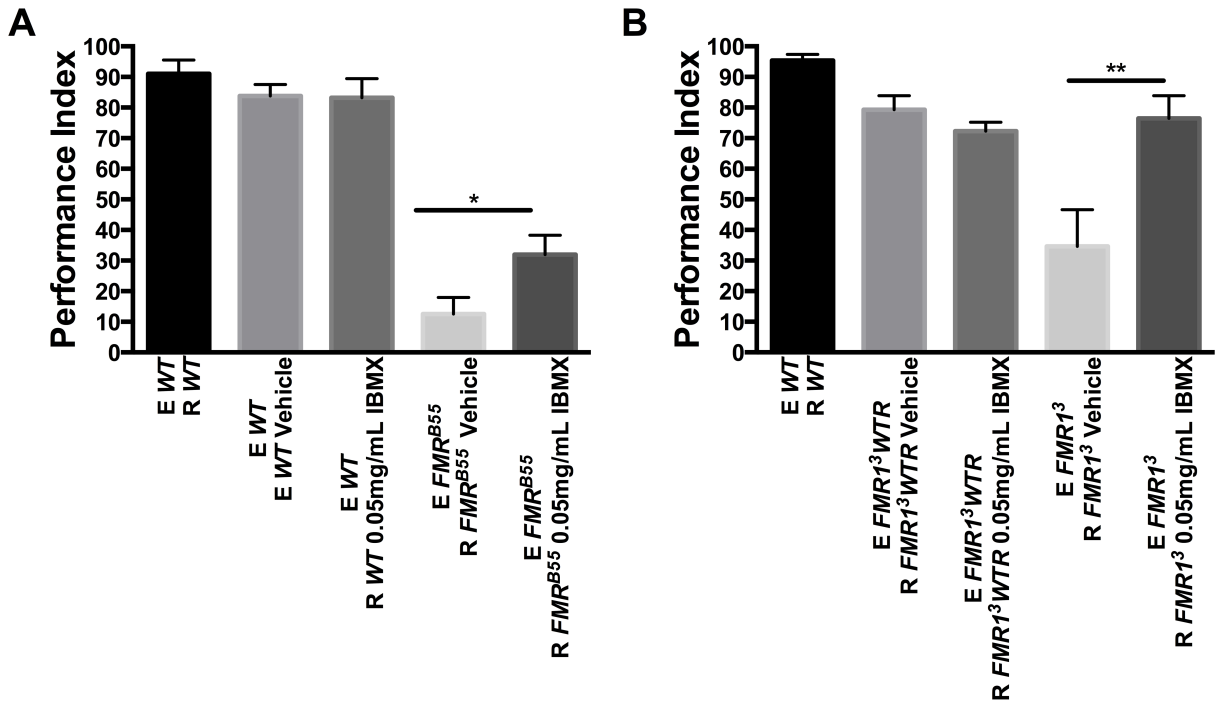

Figure 3-6. 5-Day IBMX treatment increases Drosophila stress odourant (dSO) avoidance behaviour in the Fragile X Syndrome Mutants FMR1^{3} and FMR^{B55}.
(A) 5-day treatment of *FMR^{B55}* flies with 0.05mg/mL IBMX results in significantly increased avoidance compared to *FMR^{B55}* on vehicle (T-test P=0.0282; N=14). No significant difference in avoidance behaviour observed in *WT* flies following 5-day treatment with 0.05mg/mL IBMX as compared to vehicle (T-test P=0.9379; N=14).
(B) 5-day treatment of *FMR1^{3}* flies with 0.05mg/mL IBMX resulted in a significantly increase in avoidance compared to *FMR^{B55}* on vehicle (T-test P=0.0068; N=13). No significant difference in avoidance behaviour observed in *FMR1^{3}WTR* flies following 5-day treatment with 0.05mg/mL IBMX as compared to vehicle (T-test P=0.02077; N=13). All graphs depict mean ± S.E.M.

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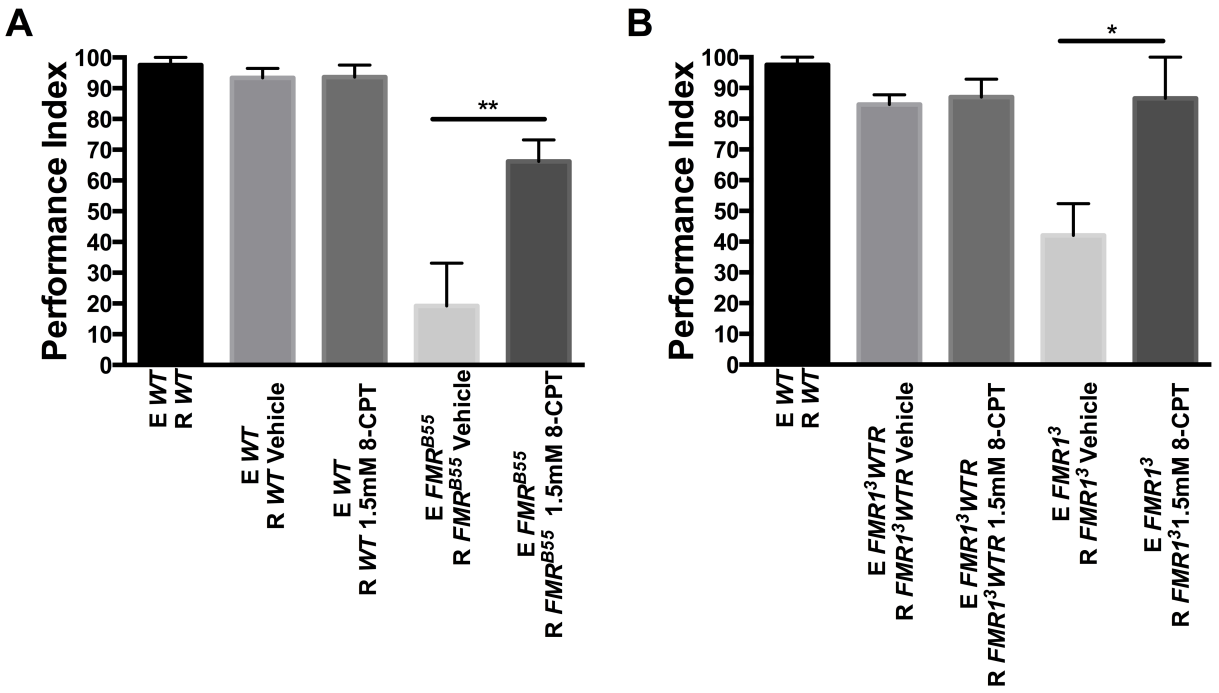

Figure 3-7. 5-Day 8-CPT treatment increases Drosophila stress odourant (dSO) avoidance behaviour in the Fragile X Syndrome mutants FMR1^{3} and FMR^{B55}.
(A) *FMR^{B55}* flies treated for 5-days with 1.5mM 8-CPT exhibited significantly increased avoidance behaviour as compared to vehicle (T-test P=0.0073; N=5). 5-day treatment of *WT* flies with 1.5mM 8-CPT did not result in any significant difference in avoidance behaviour as compared to vehicle (T-test P=0.09688; N=5).
(B) *FMR1^{3}* flies treated for 5-days with 1.5mM 8-CPT exhibited significantly increased avoidance behaviour as compared to vehicle (T-test P=0.0252; N=6). 5-day treatment of FMR1^{3}*WTR* flies with 1.5mM 8-CPT did not result in any significant difference in avoidance behaviour as compared to vehicle (T-test P=0.07334; N=6). All graphs depict mean ± S.E.M.

CHAPTER 4- Characterization of Armadillo in Fragile X Syndrome and Assaying for Learning Reversal and Long-Term Memory Reversal Defects in Fragile X Syndrome

4.1 Introduction

4.1.1 Learning and Long-Term Memory in Drosophila

Drosophi*la* provides an excellent model for genetic based disease research. Drosophila have a low level of genetic redundancy, high degree of conservation of human genes and signalling pathways, and variety of genetic tools allows for genetic manipulation to elucidate the molecular mechanism underlying disease pathology (*55, 58*). This is particularly true for neurological and cognitive abnormalities/disorders, for which 87% of genes implicated in cognitive impairment are conserved in Drosophila (*369*). As such, the use of Drosophila has emerged as a powerful tool in identifying genes influencing cognitive/neurological dysfunction and testing candidate molecules for amelioration of said dysfunction.

The classical Pavlovian olfactory associative conditioning paradigm has been used extensively to study learning and memory in Drosophila. Utilizing this paradigm 'teaches/trains' flies to associate an odour paired with a shock and subsequently allows for testing of memory performance. During the training paradigm, two odours are presented to the flies sequentially, one of which is paired with an electrical foot-shock while the other is not (*324*). Following the training period, flies are then presented with the two odours simultaneously, without the presence of a foot-shock, and memory performance is evaluated based on the flies' ability to remember to avoid the odour that was paired with a foot-shock during the training period (*203, 324*). This paradigm can be used to test the consolidation of memory at different time points following training, including short-term (or 'learning'), intermediate (or middle-term), and long-term memory (*203, 370*). Each of these memory phases have been associated with specific genes and pathways that, when disrupted, impairs memory consolidation (*371*).

Memory is a dynamic process involving the coding, storage, and retrieval of information. Short-term memory (or 'learning') describes the immediate recall of information, is highly labile, and only persists for up to two hours following a training session (*203*). Intermediate (or middle-term) memory is slightly more stable and can persist for up to several hours following training (*203*). In addition both short-term memory

and intermediate memory are also present after massed training, which consists of flies undergoing a series of repeated training sessions without a rest period (*203*). Long-term memory results after flies undergo spaced training in which flies receive a series of repeated training sessions with a 15-minute rest period between each training session (*203*). Spaced training produces a stable protein-synthesis dependent form of long-term memory that can persist for up to a week following training, while massed training produces a less stable, protein-synthesis independent form of memory, not long-term memory, that decays much quicker than the protein-synthesis dependent form of memory (*203*).

Memory formation resulting from the classical Pavlovian olfactory associative conditioning paradigm requires information processing by the olfactory system. Volatile odourants bind to one or more of the 1300 olfactory receptor neurons (ORNs) located on the antennae and maxillary palps of Drosophila *(86, 321, 372)*. Each ORN expresses one type of odorant receptor (OR) protein, along with the chaperon receptor Or83b that interacts directly with the OR and functions in the integration of OR proteins within ORNs (*373-375*). Binding of an odourant to a receptor initiates a signalling cascade that results in the generation of a specific sequence of action potentials based on the properties of the odourant (*376*). ORNs axons project to the antennal lobe (AL), where they synapse with projection neurons (PNs) or local interneurons (LNs) within discrete regions called glomeruli, all axons of ORNs expressing the same OR project to the same glomerulus (*377, 378*). Each odour is capable of evoking activity within a discrete set of glomeruli (*89, 379*). PNs send axonal projections to both the mushroom body (MB) calyx and the lateral horn (LH). MB neurons, or Kenyon cells project their dendrites into the MB calyx to form synapses with PN axons arranged in discrete regions called microglomeruli (*93, 94*). Each microglomerulus consists of a bouton formed from the synaptic connection between the PN and Kenyon cells and several local interneurons (*95*).

Olfactory memory traces can be identified within the olfactory circuit. Cellular memory traces are known as ‘engrams’, which refers to the molecular, physiological, or structural changes that occurs in neurons in response to learning (*380*). Short-term memory traces have been identified in the PNs of the AL (*381*).

Intermediate memory traces are observed in DPM neurons (*382*). Long-term memory traces are localized to the MB (*253*).

The MB has a crucial role in mediating learning and long-term memory formation learning and memory formation requires distinct populations of Kenyon cells. MB Kenyon cells are classified into three subtypes, /, , or , based on their morphology and trajectories within the MB (*253, 254*). / and Kenyon cells bifurcate to form vertical and lobes and horizontal and lobes, which are proposed to function in long-term memory, and Kenyon cells bifurcate to form horizontal lobes, which have been implicated in short-term memory formation (*254-256, 258, 383*).

Key genes involved in learning and memory formation are preferentially expressed at elevated levels in the MB, including those of key signalling pathways such as the cyclic adenosine monophosphate signalling (cAMP) pathway *rutabaga, dunce, protein kinase A*, and *CREB* (*331, 332, 352*). The cAMP signalling pathway is required for both learning and memory. *Rutabaga* (*rut*) encodes a Ca^{2+}/calmodulin-dependent adenylyl cyclase (AC) that catalyzed the conversion of ATP into cAMP, and *dunce* (*dnc*) encodes a phosphodiesterase that degrades cAMP (*181, 308*). Loss of function of either gene results in learning and memory defects (*309, 325*). Protein kinase A (PKA) is a main downstream effector of the cAMP signalling cascade that acts in the modulation of cAMP signalling by phosphorylating targets including those involved in the regulation of ion channels, spontaneous neurotransmitter release, neuron firing patterns, and synaptic strength (*384-388*). Loss of PKA also results in learning and memory defects (*389*). A main target of PKA is the transcription factor CREB (cAMP response element-binding protein) which is required protein synthesis-dependent long-term memory (*390*).
cAMP also functions in the modulation of other signalling pathways implicated in learning and memory. Mammalian/mechanistic target of rapamycin (mTOR) is a protein kinase that functions in translational regulation and is required for synaptic plasticity and long-term memory (*391*). mTOR is part of the PI3K/Akt/mTOR signalling pathway where mTOR functions as the site of integration for PI3K and Akt signalling (*176, 392*).

mTOR exists as two functionally and biochemically distinct complexes, mTORC1 and mTORC2. mTORC1 regulates two main proteins required for initiation of the translation, p70 ribosomal S6 kinase (S6K) and the eIF4E-binding proteins (4E-BPs) (*393, 394*). mTORC1 integrates nutrient availability and growth factors to regulate key cellular processes. Tuberous sclerosis 1 (TSC1) and tuberous sclerosis 2 (TSC2) are upstream regulators of mTORC1. Phosphorylation of TSC1/TSC2 by effector kinases of upstream signalling pathways, Akt, PI3K, and S6K, activates mTORC1 (*395-398*). mTORC2 is less well characterized, however mTORC2 activity is enhanced by TSC1/TSC2 and has been shown to up-regulate Akt signalling through direct phosphorylation (*399, 400*). Both mTORC1 and mTORC2 are regulated by cAMP, where cAMP inhibits the activation and promotes the disassociation of mTORC1 and mTORC2 via PKA (*401*). Key components of the mTOR signalling pathway are present in synapses and influence protein synthesis in response to stimulation by NMDA receptors, AMPA receptors, and mGluRs (*177*). Dysregulation of mTOR signalling has been associated with neurological disorders, including autism and Fragile X Syndrome (*177*).

4.1.2 The Akt Signalling Pathway Misregulation in Fragile X Syndrome

Fragile X Syndrome is caused by the loss of Fragile X Mental Retardation Protein (FMRP), which acts as a negative regulator of translation at synapses. In absence of FMRP a number of signalling pathways are misregulated. Exaggerated signalling through group 1 mGluRs (metabotropic glutamate receptors) has been proposed to be the underlying mechanism behind FXS pathology (*31*). mTOR is activated in synapses in response to stimulation by group 1 mGluRs (*174*). Loss of FMRP results in increased synaptic activity of PI3K, and aberrant over activation of the PI3K/Akt/mTOR signalling pathway (*34, 194*). Activation of PI3K results in the phosphorylation and activation of Akt (protein kinase B) (*402*). Akt is a serine/threonine protein kinase that integrates a myriad of signaling regulating glucose metabolism, cell proliferation and migration, and apoptosis (*403*). In the CNS, Akt has a crucial role in mediating neuronal growth and survival, neuronal morphology, receptor localization and function, synapse formation, and synaptic plasticity (*404-411*). In FXS, there is an increase in phosphorylated Akt levels (*33*).

4.1.3 The Wnt Signalling Pathway

Wnts are secreted glycoproteins that are essential to neurogenesis, neuronal migration, axon guidance, dendrite development, and synapse formation (*412, 413*). Wnts can signal through a number of receptors including frizzled (Fz) receptors, tyrosine kinase-like receptor (Ryk), the orphan receptor tyrosine kinase (Ror2), and insulin-like growth factor 1 receptor (IGF-1r) (*414, 415*). Binding of Wnts to receptors can initiate a number of intracellular signalling cascades the canonical Wnt pathway, the divergent Wnt canonical pathway, the planar cell polarity pathway, and the calcium Wnt pathway. The canonical Wnt pathway is the best characterized in which the binding of Wnt ligands activates the scaffolding protein, Dishevelled (Dvl), that inhibits the function of a destruction complex formed by axin, adenomatous polyposis coli (APC), and the serine/threonine kinase glycogen synthase kinase-3β (GSK-3β) (Figure 4-1) (*416*). The destruction complex marks -catenin for degradation via phosphorylation by GSK-3 (*417*). Inhibition of the destruction complex by Wnt signalling results in the accumulation of -catenin in the cytoplasm and its subsequent translocation to the nucleus, where -catenin interacts with Tcf/Lef transcription factors to modulate Wnt targeted gene expression (Figure 4-1) (*418*).

The divergent Wnt pathway regulates the stability of microtubule networks by modulating the phosphorylation of microtubule associated proteins (*419*). In the planar cell polarity pathway, activation of Dvl results in the activation of the small Rho-GTPases and the c-Jun-N-terminal kinase (JNK), which leads to reorganization of the actin and microtubule cytoskeleton (*416, 420*). The calcium Wnt pathway activates protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and the nuclear translocation of the transcription factor nuclear factor of activated T cells (NFACT) through the Wnt-dependent increase in intracellular Ca^{2+} levels (*421*). In absence of FMRP, altered Wnt signalling occurs (*422*).

4.1.4 Synaptic Plasticity and Clinical Observations of the Cognitive Defects

A neuropathological hallmark of FXS is the abnormal synaptic structure in which there is a hyper-abundance of long, thin, immature dendritic spines (*423*). Wnt proteins regulate structural components of synapses, including density, maturity, and arborization of dendritic spines (*424*). Specifically □-catenin has been proposed to be a critical mediator of dendritic morphology and is required for proper localization of synaptic vesicles in axons (*425, 426*). Overexpression □-catenin results in increased dendritic arborization through its interaction with the cell adhesion molecule, N-cadherin (*425*). N-cadherin is predominately expressed in the CNS and functions during development in neurite outgrowth, dendritic arborization, and synaptogenesis (*425, 427, 428*). Post-development, N-cadherin and □-catenin are localized to synapses where they modulate synaptic function and plasticity (*429-432*). Together □-catenin and N-cadherin form a complex that links the cytoskeleton to the extracellular environment and forms synaptic junctions (*433*).

A well-established clinical feature of FXS is the presence of cognitive defects, specifically impairment of learning and memory. FXS is caused by the transcriptional silencing of the *FMR1* gene and subsequent loss of its gene product, FMRP. FMRP binds to and controls the translation of target mRNAs at synapses. In absence of FMRP, the translational regulation of key proteins is lost and a global increase in protein synthesis is observed (*217*). Loss of FMRP results in dysfunction in a number of key signalling pathways required for learning and memory.

Wnt ligands modulate signalling at both excitatory and inhibitory synapses. Wnt proteins induce expression and maintenance of GABA receptors and enhance GABA signalling through receptor recycling (*434*). Wnts also induce insertion of glutamate receptors in post-synaptic regions and functions in the modulation of *N*-methyl-D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) signalling (*435, 436*). NMDA and AMPA receptors function in long-term potentiation (LTP), an activity-dependent long-term change in synaptic efficacy (*437*). Induction of LTP results in modifications of dendritic spines and synaptic structure (*438*). Synaptic efficacy is altered by the redistribution of AMPA receptors to potentiate or weaken synaptic connections (*437*). LTP is the

mechanism underlying synaptic plasticity and required for learning and memory (*439*). Wnt signalling facilitates LTP by increasing neurotransmitter release and recycling of synaptic vesicles (*440-442*).

-Catenin and N-cadherin also function in LTP. Cell adhesion molecules are thought to mediate activity-dependent changes at synapses (*443*). -Catenin associates with N-cadherin, which mediates interactions with cytoskeleton components (*433*). Phosphorylation of -catenin reduces affinity for N-cadherin, which down regulates cell adhesion (*444*). Depolarization of neurons modifies the arrangement of N-cadherin at synapses to enhance and maintain synaptic connections that occur during LTP (*430*). Depolarization also results in the recruitment of -catenin to the activated synapse, where N-cadherin cytoplasmic tails can couple to cytoskeleton components through -catenin, which has been proposed to strengthen connections (*445, 446*). N-cadherin can activate Rho family GTPases, which also function in synaptic structure and plasticity (*447, 448*). Furthermore a protein synthesis dependent increase of N-cadherin is crucial for the induction of LTP (*429*).

Wnt signalling has been implicated in post-developmental neurogenesis, a process in which adult progenitors cells undergo fate specification, proliferation, differentiation, and integration into pre-existing neural networks (*449*). During development Wnt proteins regulate stem cell differentiation (*450*). Activation of -catenin modulates proliferation and expansion of neural progenitors cell populations (*450*). Following development Wnts promote differentiation and maturation of progenitors cells into mature neurons (*451, 452*). Adult neurogenesis is crucial for learning and memory (*449*).

4.1.5 -Catenin/Armadillo is a Target of Akt

The Wnt and PI3K/Akt signalling pathways have common signalling intermediates, suggesting a molecular intersection between the two pathways and a means of cross-regulating a variety of physiological events (*453, 454*).

Wnt activates mTOR by inhibiting GSK-3β, which negatively regulates mTOR through TSC2 activation (*455*). Wnt signalling activates Akt, which associates with Dvl to enhance the phosphorylation of GSK3 in the destruction complex resulting in its inactivation and the stabilization and nuclear accumulation of -

catenin (*454*). Akt can also activate β-catenin through direct phosphorylation, which results in its disassociation from adhesion connections and increases its transcriptional activity (*456*). N-cadherin also regulates β-catenin through Akt, in which a decrease in N-cadherin levels results in decreased Akt activation and decreased phosphorylation of GSK3 and β-catenin (*457*). N-cadherin can activate Akt through PI3K, which can inturn stimulate β-catenin signalling (*458*).

4.2 Methods and Materials

4.2.1 Drosophila Fly Stocks

Fly stocks were maintained at 22°C on standard cornmeal yeast media from Cold Spring Harbor Laboratory. Wild-type stocks were backcrossed to w^{1118}*isoCJ1* for 6 generations. $Dfmr1^{B55}$ flies were obtained from Dr. Kendal Broadie (Vanderbilt University). To eliminate background effects all fly stocks were outcrossed to the wild-type strain w^{1118}*isoCJ1*. $Dfmr1^{3}$ flies and $dfmr1^{3}$ flies containing a wild-type rescue transgene ($dfmr1^{3}$*WTR*) were obtained from Dr. Tom Jongens (University of Pennsylvania). Wild-type Elav-Gal4 flies were obtained from Dr. Tim Tully. Armadillo/β-catenin (8370) flies were obtained from Bloomington Stock Centre.

4.2.2 Classical Pavlovian Olfactory Learning and Memory Formation

Learning

To examine the role of armadillo/β-catenin in learning, we utilized the classical olfactory conditioning paradigm. Approximately 100 flies were aspirated into a training chamber containing an electrifiable cooper grid, and the tube was inserted into a T-maze. During training, flies were first allowed to acclimate to an odoulress airflow for 1.5 minutes. Following acclimation, flies were then exposed to the conditioned stimulus (CS+), either 3-octanol (OCT) or 3-methylcyclohexanol (MCH), paired with a foot-shock (unconditioned stimulus; US) for one minute (12x 1.25s pulses of 60V DC at 5s interpulse intervals). Odours were delivered at equally aversive concentrations in an airflow of 750ml/min. Following CS presentation and pulsed shock conditioning, the training chamber was cleared with blank air for 45 seconds. A second unconditioned stimulus (CS-), either OCT or MCH; opposite of CS+ odour, was then introduced to the flies for 1 minute and chamber was cleared using blank air for 45 seconds. Flies were

then tapped into the elevator of the T-maze and lowered to a choice point where they were given two minutes to choose between the CS+ and CS- odours delivered through opposite collection tubes. Following two minute choice point, the elevator was raised and flies were sequestered and counted (Figure 4-2). Performance Index (PI) was used to evaluate learning capabilities.

All flies were 1-3 days old. One day prior to completing the classical olfactory conditioning paradigm, flies were set up in bottles containing yeast-media and filter paper.

Long-Term Memory

To evaluate the role of armadillo/□-catenin in long-term memory, we utilized massed training and spaced training, which are also based on the classical olfactory conditioning paradigm. During spaced training two groups of flies were exposed to 10 training sessions, in which each group received different odours as the CS+. Flies were given a 15 minute rest period between training sessions, allowing for protein synthesis to occur, resulting in a more stable longer lasting form of memory. Following training flies were kept at 18°C overnight and memory was evaluated following day by placing trained flies into T-maze, and like learning, flies were given two minutes to choose between the CS+ and CS- odours delivered through opposite collection tubes.

Massed training in similar to spaced training in which two groups of flies receive 10 training session with separate CS+ odours, however there is no rest period between training blocks resulting in a less stable form of memory that is protein synthesis independent (Figure 4-3).

Performance Index (PI) was used to evaluate memory.

All flies were 1-3 days old. One day prior to completing the classical olfactory conditioning paradigm, flies were set up in bottles containing yeast-media and filter paper.

4.2.3 Adapted Olfactory Learning and Memory Formation to Assay for Learning Reversal Defects

Learning Reversal

To evaluate the ability of flies to ‘re-learn’, we employed a modified version of classical olfactory conditioning paradigm. This paradigm in similar to the classical olfactory conditioning paradigm learning paradigm in that flies are presented with CS+ and CS-, but instead of immediately assessing learning

ability, flies are ‘re-trained’ where the CS- odour becomes the CS+ odour, and the CS+ odour becomes the CS-.

Following the two training sessions flies are presented with both odours simultaneously and given two minutes choose between the CS+ and CS- (Figure 4-2). Performance Index (PI) was calculated based on the last odour used as CS+ to evaluate ability to ‘re-learn’.

Long-Term Memory Reversal

In order to evaluate synaptic plasticity the ability of flies to ‘re-learn’ and form new long-term memories based on new information, we exposed flies to two rounds of spaced or massed training. Similar to normal spaced and massed training, two groups of flies were exposed to 10 training sessions, in which each group received different odours as the CS+, however following completion of 10 training sessions flies were trained again, but receive the opposite odour as the CS+.

Following the long-term memory training and long-term memory reversal training, flies were kept at 18°C overnight and memory was evaluated following day by placing trained flies into T-maze, where flies were given two minutes to choose between the CS+ and CS- odours delivered through opposite collection tubes (Figure 4-3). Performance Index (PI) was calculated based on the last odour used as CS+ to evaluate synaptic plasticity and ability to form new memories.

4.2.4 Task-Relevant Sensory and Motor Controls

Sensory acuity tests are required in olfactory-based assays to eliminate the possibility that observed results are not due to genotype specific sensory deficits in odour discrimination, shock reactivity, or mobility. To test naïve odour avoidance, flies are placed in T-maze and given two minutes to choose between air and an odour (OCT or MCH). As both OCT and MCH are naturally aversive to flies, the flies should avoid the odour and position themselves on the side containing air. Naïve odour response is scored as Performance Index (PI).

To test electric shock reactivity, flies are given two minutes to choose between two electrifiable cooper grids, one of which is connected to an electrical current while the other remains unpowered. Flies should avoid electrified cooper grid. Shock reactivity is scored at Performance index (PI).

4.2.5 Performance Index (PI) Calculation and Statistical Analysis

One complete experiment consists of training and testing two groups of flies. The CS+ is OCT and the CS- is MCH for one group; the CS+ is MCH and the CS- is OCT for the second group. The performance index (PI) is calculated as the average of the number of flies that avoid the shock-associated odor minus the number of flies avoiding the control odor for each group of flies trained in one experiment..

Performance Index was calculated in JMP® using the following formula:

Performance Index (PI) =

$$PI = \left(\frac{\#\text{ CS- flies} - \#\text{ CS+ flies}}{\text{total \# of flies}} \right) /2 + \left(\frac{\#\text{ CS- flies} - \#\text{ CS+ flies}}{\text{total \# of flies}} \right) /2 \times 100\%$$

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.

All statistical analysis was conducted using GraphPad Prism 6.

4.2.6 The Gal4-UAS Binary Expression System

In order to manipulate gene expression, we utilized the Gal4-UAS binary expression system. The Gal4-UAS system utilizes two modules to manipulate gene expression, a driver and a drivee. The driver, or Gal4transgene uses a promoter to direct gene expression (*65*). The drivee, or UAS-transgene contains the upstream activating sequence from a yeast gal promoter, which can be used to target genes of interest (*65*). The Gal4 driver binds to the UAS-transgene to promote expression of the gene of interest under the control of the Gal4 transcription factor (*65*).

4.2.7 Genetic Crosses

In order to determine the effect of the over-expression of armadillo/β-catenin in learning and long-term memory formation, we used the pan-neuronal driver *Elav-Gal4* to over-expression armadillo/β-catenin.

Pan-neuronal overexpression of armadillo/□-catenin

Elav-Gal4 females x *P(UAS-arm.Exel)3* males

Elav-Gal4 females x *WT* males

WT females x *P(UAS-arm.Exel)3* males

WT females x *WT* males

Progeny of the crosses was used for learning and long-term memory assays. Flies were anesthetized using $CO_{2(g)}$ prior to sorting and collection. Crosses were maintained at 22°C on standard cornmeal yeast media.

4.3 Results

4.3.1 Research Highlights/Summary

Wnt signaling is involved in regulating CNS development, and following development functions in cognition and behavior by regulating synaptic plasticity (*440, 459*). Depolarization of neurons results in the release of Wnts that activate NMDA receptors promoting dendritic spine growth and activity-dependent synaptic structure modifications; this synaptic plasticity is pivotal to learning and memory formation (*446, 460*). Armadillo/β-catenin is the main effector of the Wnt signalling pathway and mediates structural changes required for learning and memory (*460*). Armadillo/β-catenin is regulated by Akt signalling, which is up-regulated in FXS (*34*). Armadillo/β-catenin levels are also elevated in absence of FMRP. Here we show that over-expression of Armadillo/β-catenin results in learning and long-term memory defects and likely contributes to FXS pathology. Furthermore we demonstrate that FXS flies exhibit a learning reversal and long-term memory reversal defect, which may be a result of abnormal Armadillo/β-catenin expression resulting in synaptic function and remodeling defects.

4.3.2 Pan-Neuronal Overexpression of Armadillo results in Learning and Long-Term Memory Defects

Synaptic plasticity involves the rapid rearrangement of cytoskeleton structure at synapses and the formation of new synaptic connections (*438*). These changes rely on cell adhesion molecules to strengthen or weaken connections between pre- and post-synaptic neurons (*461*). Alterations in synaptic

structure and connectivity are mediated by cadherin-Armadillo/β-catenin complexes that are localized to synaptic junctions (*462*). Disruption of cadherin-Armadillo/β-catenin complexes, specifically Armadillo/β-catenin can alter synapse assembly and plasticity (*426*).

In FXS the PI3K/mTOR/Akt signalling pathway is misregulated and Akt levels are elevated (*33, 177*). Given that Akt can activate Armadillo/β-catenin, and that Akt levels are evaluated in FXS, it is anticipated that Armadillo/β-catenin is overactive. We therefore wanted to determine if the hyperactivity of Armadillo/β-catenin contributes to FXS pathology.

In order to determine how the overexpression of Armadillo/β-catenin contributes to learning and memory defects characteristic of FXS, we utilized the pan-neuronal driver *Elav-Gal4* to drive overexpression of Armadillo/β-catenin. Pan-neuronal overexpression of Armadillo/β-catenin results in a significant decrease in learning compared to *WT* flies (Figure 4-4 A). Similarly, the pan-neuronal overexpression of Armadillo/β-catenin resulted in a significant defect in spaced training compared to *WT* and control flies (Figure 4-4 B).

4.3.3 The Fragile X Syndrome Mutant *FMR^{B55}* Exhibit Impaired Learning Reversal

NMDAR and mGluR activation triggers a signalling cascade that mediates downstream effectors of neuronal development, and synaptic plasticity. NMDAR mediated neural activity modifies interactions within the cadherin-Armadillo/β-catenin complex, resulting in the accumulation of cadherin in the plasma membrane, which blocks NMDAR mediated synaptic plasticity (*463*). In absence of FMRP, signalling through both NMDAR and mGluR receptors is aberrant (*31*).

Utilizing a modified learning paradigm, we found that *FMR^{B55}* flies exhibited a significant defect in learning reversal as compared to *WT* flies (Figure 4-5 A,B).

4.3.4 The Fragile X Syndrome Mutant *FMR^{B55}* Exhibit Long-Term Memory Reversal Defects

FMR^{B55} flies exhibited a significant defect in spaced training long-term memory reversal as compared to *WT* flies (Figure 4-5 A,B). No significant defect in massed training long-term memory reversal was observed in *FMR^{B55}* flies as compared to *WT* flies (Figure 4-6 C,D).

4.4 Discussion

4.4.1 The Hyperactivity of Armadillo Contributes to Learning and Long-Term Memory Defects Observed In Fragile X Syndrome

mGluR signalling activates the mTOR/PI3K/Akt pathway, which results in the phosphorylation and inactivation of GSK-3β. GSK3 regulates Armadillo/β-catenin via the destruction complex, where it phosphorylates and marks Armadillo/β-catenin for degradation. However lower levels of the phosphorylated GSK3 protein are observed in FXS, while the total GSK3 protein levels remain the same, indicating GSK3 is hyperactive (*345*). This is counterintuitive given that Akt activity is elevated, which would result in the inactivation of GSK3 and an accumulation of Armadillo/β-catenin.

Given that both Akt and GSK3 are hyperactive in absence of FMRP, it is possible that GSK3 is inadequately regulated as multiple signalling pathways converge on GSK3, many of which are dysregulated in FXS. Exaggerated mGluR signalling and the hyperactivity of GSK3 in FXS can be rescued with treatment of the mGluR inhibitor MPEP, which decreases exaggerated mGluR signalling and results in an increase in the levels of the phosphorylated GSK-3β protein suggesting misregulation of signalling between mGluR and GSK3 (*345*). Furthermore hyperactivity of GSK3 can be ameliorated with lithium, which also modulates the PI3K signalling pathway, implicating another mechanism by which GSK3 is misregulated (*464, 465*). FMRP regulates translation of GSK3, which in turn regulates a number of transcription factors (*18*). Loss of transcriptional control by GSK3 may exacerbate the loss of translational control in absence of FMRP (*466*).

Despite increased GSK3 activity, an increase in Armadillo/β-catenin levels is likely mediated through Akt. Akt can activate armadillo/β-catenin through direct phosphorylation, which results in its disassociation from adhesion connections and increases its transcriptional activity (*456*). As previously mentioned, Akt activity is elevated in FXS, suggesting that Akt increases Armadillo/β-catenin levels through direct interaction rather than by mediating its stability through phosphorylation of GSK3 and de-activation of the destruction complex.

Synaptic plasticity requires architectural modification of the cytoskeleton in dendrites (*438*). Most excitatory synapses terminate in dendritic spines (*467, 468*). Following a learning event, dendritic spines exhibit changes in morphology and changes in expression of Armadillo/β-catenin mRNA (*469*). Cadherin-Armadillo/β-catenin complexes exist in dendritic spines. Depolarization results in the rapid redistribution of Armadillo/β-catenin. Following learning, Armadillo/β-catenin is phosphorylated which decreases its interaction with cadherin resulting in its dissociation from cadherin-Armadillo/β-catenin complexes (*460*). This may serve to weaken synaptic connections to allow for synaptic remodeling as following a period of destabilization of cadherin-Armadillo/β-catenin complexes, Armadillo/β-catenin re-localizes to the complexes to presumably strengthen new connections (*460*).

The initial labile phase of memory formation, such as learning and short-term memory, does not require Armadillo/β-catenin and may not be affected by its dysregulation, but conversion of short-term memory into long-term memory requires Armadillo/β-catenin (*470*). This may explain why we observed mild learning defects when Armadillo/β-catenin was over-expressed compared to the significant defect in long-term memory compared to *WT* flies when Armadillo/β-catenin was over-expressed (Figure 4-4). Overexpression of Armadillo/β-catenin results in a neuronal phenotype similar to that of FXS, where overexpression results in increased arborization (*425, 471*).

4.4.2 Fragile X Syndrome Mutants *FMR1*^{3} and *FMR*^{B55} Exhibit Long-Term Memory Reversal Defects

Synaptic plasticity is the process in which activity-dependent long-term modifications within existing neural circuits results in alterations in the efficacy of synaptic transmission (*472*). Long-term modifications in synaptic efficacy result from long-term potentiation (LTP) and long-term depression (LTD), which increases or decreases synaptic efficacy respectively (*31*). Synaptic plasticity is mediated by the coupling of mGluRs and NMDARs to the mTOR/PI3K/Akt signalling pathways to regulate translation (*473*). FMRP regulates approximately 5% of mRNAs, including both pre- and post-synaptic proteins (*18, 22*). FMRP modulates approximately 30% of both pre- and post-synaptic proteins including NMDAR, mGluR, and mTOR (*18, 235*). Loss of FMRP also results in the loss of key regulators of these signalling pathways, including TSC2, PI3K, and GSK3 (*18*). In absence of FMRP mGluR signalling is exaggerated as well as

the signalling pathways coupled to it, resulting in an increase in mGluR mediated protein synthesis (*31, 33*).

FXS is characterized by defects in synaptic plasticity. mGluR activation results in the synthesis of FMRP which functions in synaptic plasticity by modulating translation of target mRNAs (*30, 69, 474*). FMRP is regulated by post-translational modifications, in which the phosphorylation of the protein inhibits translation, while translation is up-regulated when FMRP is not phosphorylated (*17, 475*). Maintenance of LTD requires rapid protein synthesis immediately following induction by mGluRs (*171*). In absence of FMRP, proteins required for LTD are over-expressed in dendrites, resulting in a protein-synthesis independent form of plasticity (*31*). Not only does FMRP regulate proteins required for LTD maintenance, it also regulates 62% of the mRNA coding for proteins in the mGluR complex, 31% in the NMDAR, and 33% in the AMPAR complex, all of which are required for the induction of LTD (*18*). Furthermore FMRP also regulates the translation of potassium channels, loss of which may alter excitability and threshold for LTP induction (*476, 477*).

Loss of FMRP results in excessive AMPA receptor internalization in response to mGluR signalling (*478*). Stabilization of Armadillo/β-catenin results in the stabilization of cadherins in synaptic membranes impairing AMPA receptor endocytosis (*479*). Disruption of AMPA receptor endocytosis abolishes LTD (*479*). Disruption of LTD impairs learning reversal (*480, 481*). Stabilization of cadherin-Armadillo/β-catenin complexes abolishes LTD, disrupting synaptic plasticity and results in significant defects in learning reversal (*479*). LTP is achieved through the removal of AMPA receptors from synapses, and does not appear to be affected by stabilization of Armadillo/β-catenin (*479*).

Here we show that FMR^{B55} flies exhibit a significant defect in learning reversal and long-term memory reversal compared to *WT* counterparts (Figure 4-4 A, B). Given that LTD involves the weakening of synaptic connections by altering stability and distribution of cadherin-Armadillo/β-catenin complexes, it is possible that the necessary weakening of existing synaptic connections is greatly impaired during the initial long-term memory training, and that minimal changes in synaptic connectivity is achieved through

the initial training and is also impaired during long-term memory reversal training. Furthermore Armadillo/β-catenin is required specifically in the MB for the formation of long-term memory traces (*470*).

Because FMRP functions in the translational regulation of a significant number of neuronal mRNAs, it is possible that defects in learning reversal and long-term memory reversal may be mediated through a combination of mechanisms.

Neuronal excitability is regulated by γ-amino butyric acid (GABA), the primary inhibitory neurotransmitter in the CNS (*482*). Olfactory neural circuits that mediate learning and memory formation are highly innervated by GABAergic interneurons and thus subject to regulation by GABA. Mushroom bodies (MB) have been identified as a critical component of the olfactory neural circuit required for learning and memory in Drosophila and has recently been implicated in learning reversal (*254, 370, 483-485*). The MB is also highly innervated by GABAergic interneurons which have been shown to function in olfactory memory formation (*95, 486*). In the MB, anterior paired lateral neurons (APL) provide the source of GABAergic modulation (*96, 487, 488*). Intracellular calcium levels increase in APL neurons in response to delivery of odours and electrical shocks, resulting in neurotransmitter release (*489*). Inhibition of GABA release enhances classical learning and memory formation, but impairs learning reversal (*483, 485-487, 490*). Reducing GABA in APL neurons is a physiological and not development effect that impairs the flies' ability to suppress initial memory in reversal training (*483, 485*).

GABA receptor subunits are targets of FMRP translational regulation (*367, 491, 492*). In absence of FMRP the GABA signalling is down-regulated. In FXS models there is a significant reduction in the number of GABA receptor subunits, proteins required for GABA transport and GABA catabolism, GABA synthesis, as well as a significant decrease of GABAergic inputs into multiple brain regions (*493*).

Changes in the cytoskeleton of dendritic spines are mediated by the small GTPase, Rac1 (*169, 494*). Rac1 functions in the structural plasticity of dendritic spines by regulating actin polymerization in response to activation by glutamatergic activity required for LTP (*169, 495, 496*). Rac1 and FMRP are both located in the dendritic spines, where FMRP has been suggested to regulate spine morphology through Rac1 (*497*). In FXS, Rac1 is up-regulated (*498*). Rac1 is required for learning reversal but not initial learning (*499*). Elevated Rac1 in the MB results in accelerated memory decay (*500*).

Here we demonstrate the FXS mutant, *FMR*^{B55} exhibit a significant defect in learning reversal and long-term memory reversal compared to *WT* counterparts (Figure 4-5 B; 4-6 B, D). Given that GABA is down regulated in FXS, and inhibition of GABA transmission has been associated with learning reversal defects, it is likely that the down-regulation of GABA in FXS is responsible for the observed learning and long-term memory reversal defects. Furthermore Rac1 levels are elevated in absence of FMRP, suggesting that FMRP is required to maintain optimal protein levels required for remodeling of the cytoskeleton during activity-dependent plasticity (*500*).

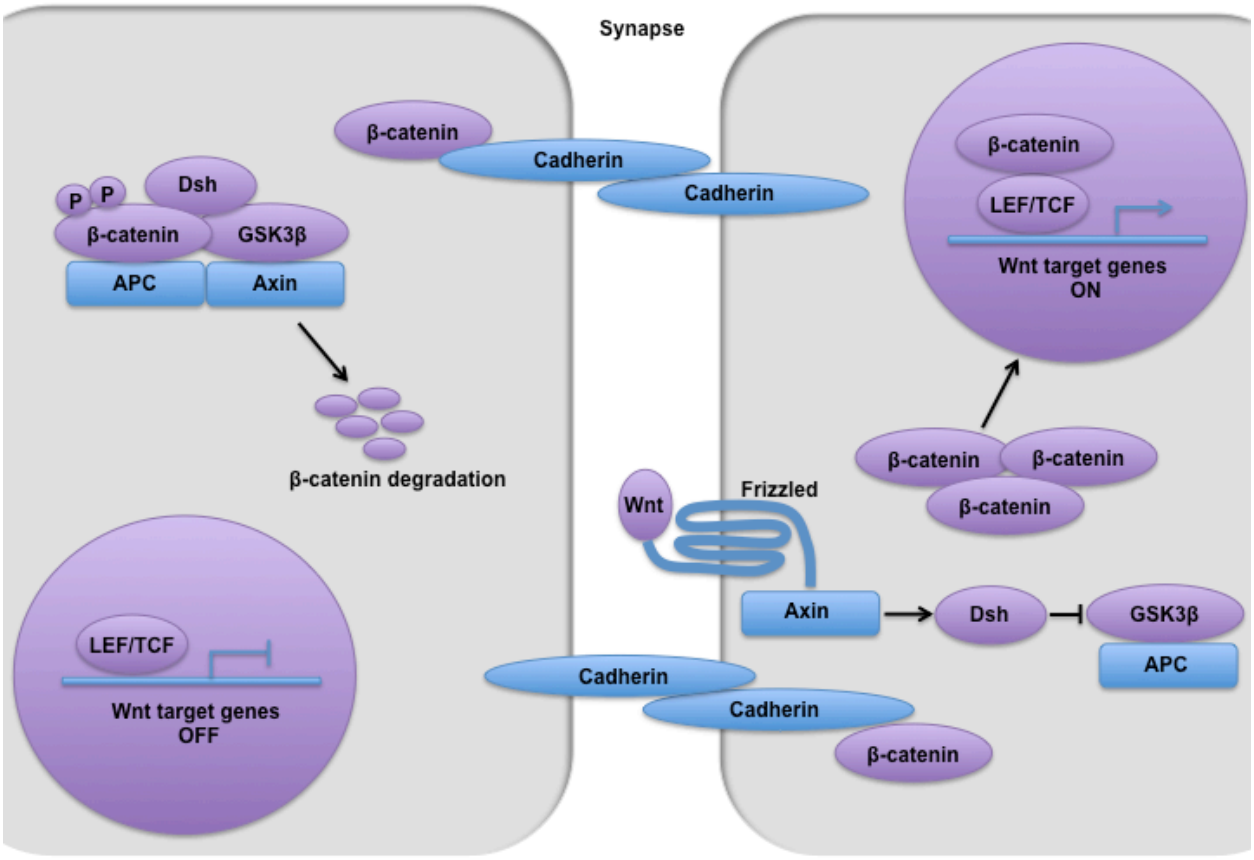

Figure 4-1. Wnt signalling at the synapse.

Initiation of Wnt signalling stabilizes β-catenin/Armadillo by preventing assembly of the destruction complex, resulting in the translocation of β-catenin/Armadillo into the nucleus and subsequent transcription of Wnt targeted genes.

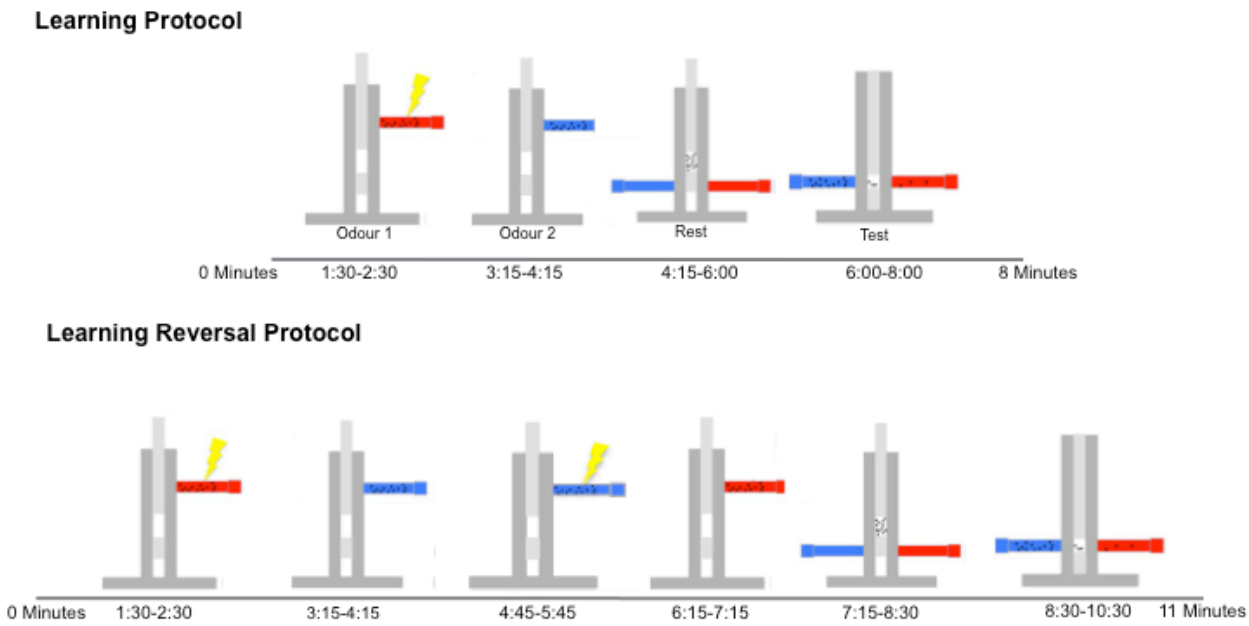

Figure 4-2. Learning and learning reversal protocol.
In the traditional learning protocol, flies are exposed to two odours sequentially one of which is paired with a foot-shock and immediately tested to evaluate if the flies were able to learn to avoid the odour paired with the foot-shock. The learning reversal protocol is similar to the learning protocol in that flies are presented with 2 odours, one of which is paired with a foot-shock but instead of testing the flies immediately, instead flies are then 're-trained' where the flies are presented with the same 2 odours again, but the second odour is now paired with a foot-shock. Following 're-training' the flies are then tested in order to determine if they were able to 're-learn'.

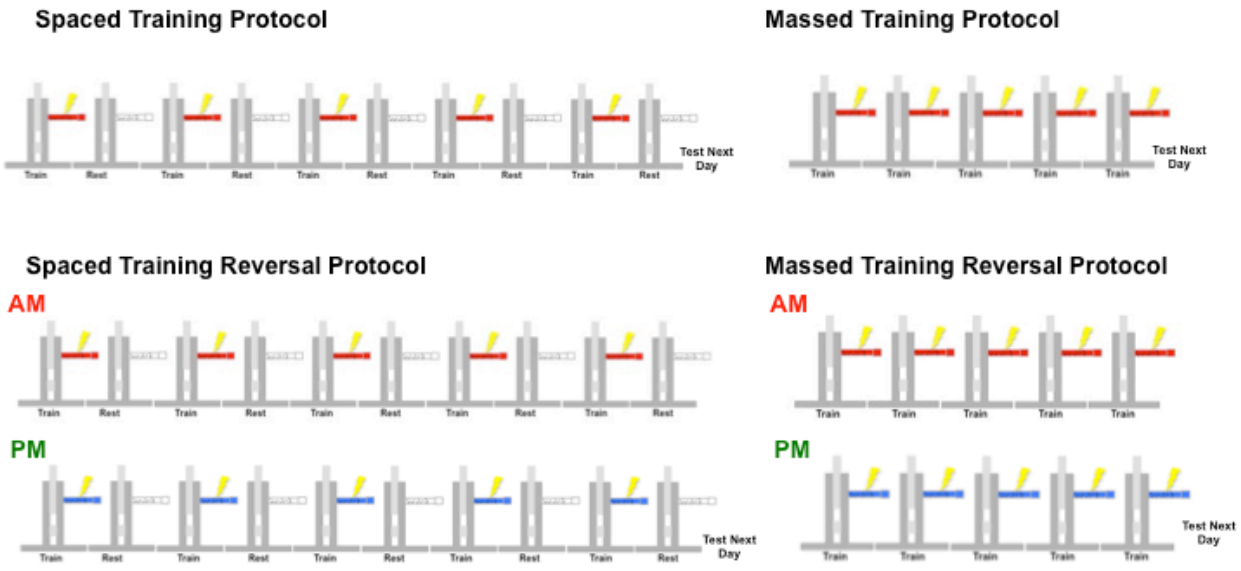

Figure 4-3. Long-term memory and long-term memory reversal protocol.
In the traditional spaced or massed training paradigm, flies undergo 10 training sessions, either with or without a 15 minutes rest period between training sessions, where they are exposed to two odours, one of which is paired with a foot-shock. Following the 10 training sessions flies are left at 18°C overnight and their 1-day memory is tested the following day. In the long-term memory reversal paradigm, flies under go 10 sessions of training where one odour is paired with a foot-shock, but following completion of training, flies are then ‘re-trained’ and undergo 10 sessions of training where the opposite odour is now paired with a foot-shock.

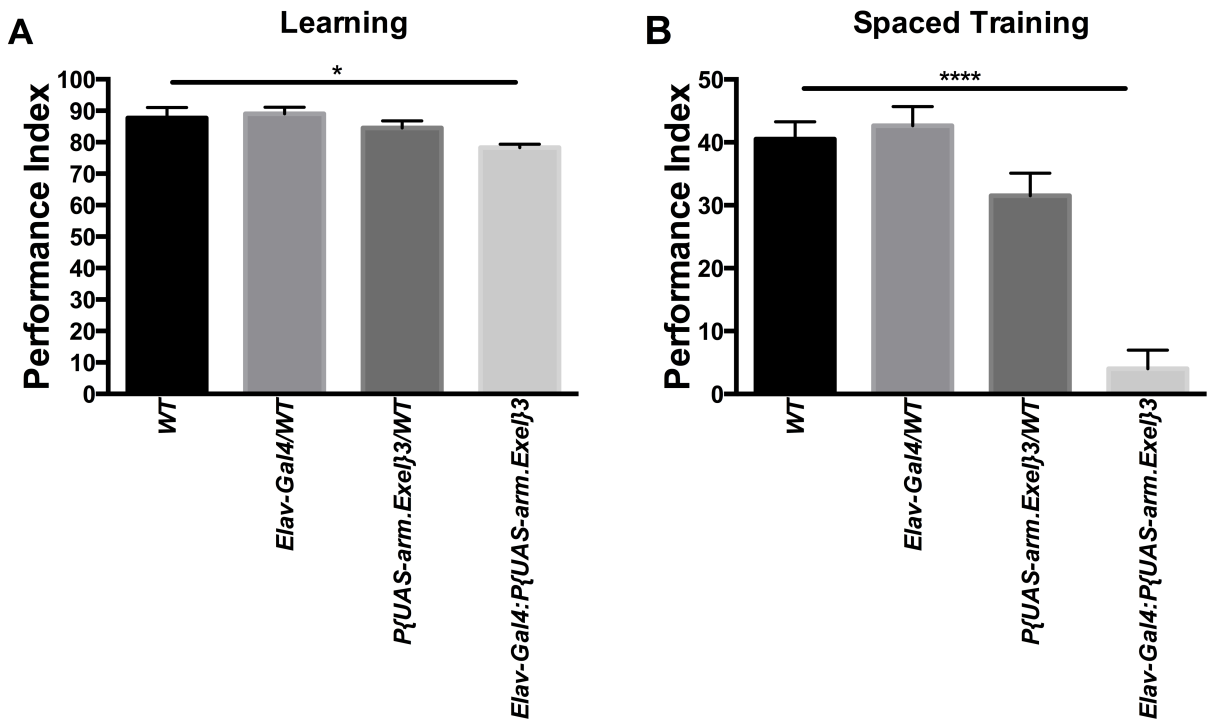

Figure 4-4. Pan-neuronal overexpression of armadillo/β-catenin results in learning and long-term memory defects.

(A) Learning was significantly lower in *Elav-Gal4:P(UAS-arm.Exel)3* flies compared to *WT* flies (ANOVA P= 0.0401;N=7).

(B) *Elav-Gal4:P(UAS-arm.Exel)3* flies exhibit significant defect in long-term memory compared to *WT* flies (ANOVA P<0.0001;N=8). All graphs depict mean ± S.E.M.

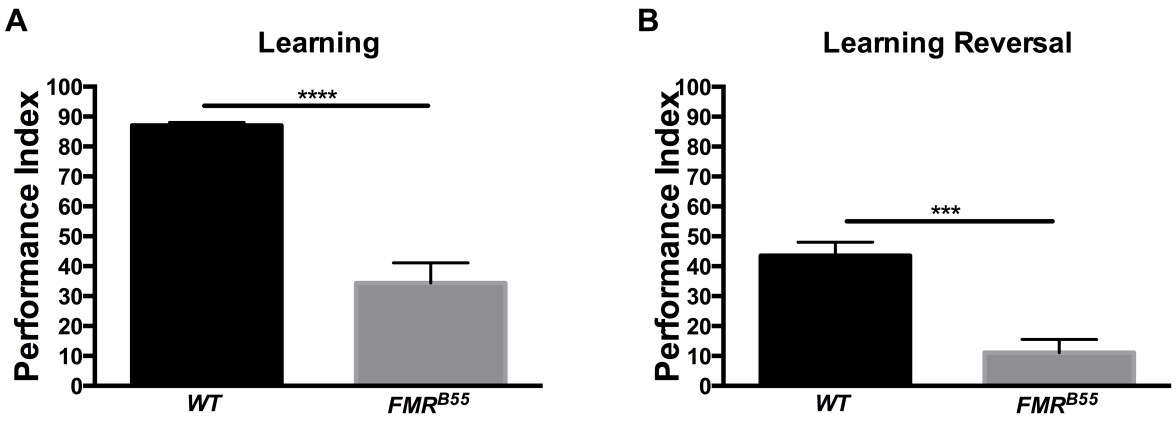

Figure 4-5. The Fragile X Syndrome mutant FMR^{B55} exhibits impaired learning reversal.
(A) *FMR^{B55}* flies exhibit significantly lower learning compared to *WT* flies (T-test P<0.0001;N=5).
(B) Learning reversal is significantly lower in *FMR^{B55}* flies compared to *WT* flies (T-test P=0.0002;N=8).
All graphs depict mean ± S.E.M.

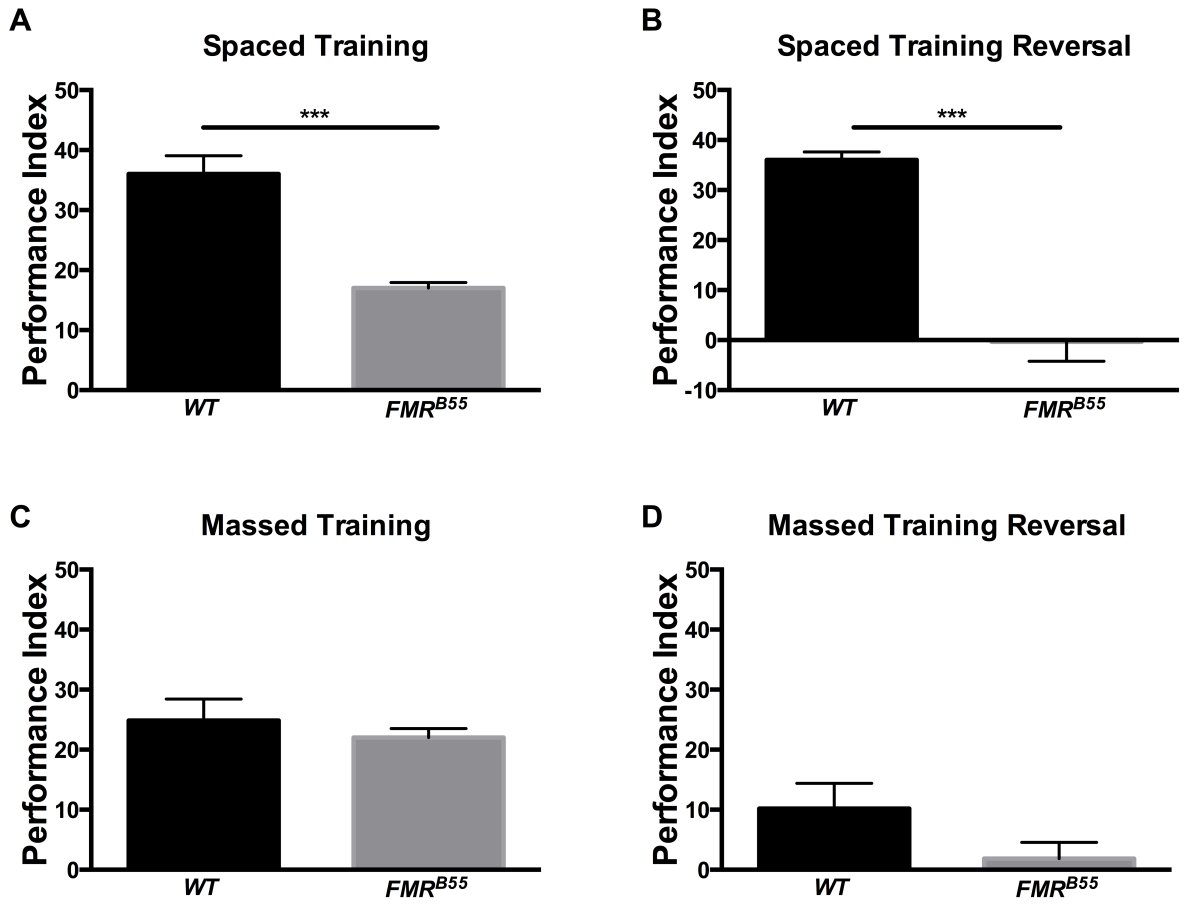

Figure 4-6. The Fragile X Syndrome mutant FMRB55 exhibits long-term memory reversal defects.
(A) *FMR*^{B55} flies exhibit a defect in one-day memory following spaced training (T-test P=0.0001;N=6).
(B) One-day memory following spaced training reversal is significantly lower in *FMR*^{B55} flies compared to *WT* flies (T-test P<0.0001;N=6).
(C) *FMR*^{B55} flies exhibit no significant defect in one-day memory following massed training compared to *WT* flies (T-test P=0.4863;N=6).
(D) One-day memory following massed training reversal was not significantly different in *FMR*^{B55} flies compared to *WT* flies (T-test P=0.1287;N=6). All graphs depict mean ± S.E.M.

CHAPTER 5- General Conclusions

Fragile X Syndrome is the most common form of inherited intellectual disability and the largest single genetic cause of autism. Fragile X Syndrome is caused by the loss of the Fragile X Mental Retardation Protein (FMRP) due to the expansion of CGG trinucleotide repeats in the 5'UTR of the *FMR1* gene resulting in methylation and silencing of the gene (*2*). FMRP is an RNA-binding protein that functions in the metabolism of neuronal mRNAs. FMRPs major function is to regulation the translation of target mRNAs, the absence of which results in a pathological increase in protein synthesis. FMRP functions in translation regulation by modulating mRNA stability, mRNA transport, and translation repression or activation of target mRNAs.

The development and modulation of neuronal networks requires the precise temporal and spatial regulation of gene expression. In absence of FMRP several physiological processes required for the establishment of neural circuits are disrupted including neurogenesis, axonal pathfinding, neuronal development, and synaptic plasticity. As a result dendritic spine dysmorphologies are a neuropathological hallmark of Fragile X Syndrome. Abnormalities in neuronal architecture result in perturbation of information processing, which relies on intracellular signalling pathways for signal transduction between processing centres. In absence of FMRP several signalling pathways are mis-regulated including mGluR, mTOR/PI3K/Akt, cAMP, and Wnt (*31, 33, 34, 327*).

The transmission and processing of sensory information occurs in genetically pre-determined neuronal networks established during development. Abnormal gene expression within complex gene networks results in abnormalities in information processing due to perturbations in neural connectivity and ultimately dysfunction within complex neuronal networks (*304*). Our results suggest that FMRP is required developmentally to regulate cAMP signalling in the establishment of neuronal networks that mediate dSO sensory processing and avoidance behaviour, specifically FMRP is required in the MB for higher-order processing, where loss of FMRP results in neuronal structural and functional defects (*205*). Furthermore we show that cyclic adenosine monophosphate (cAMP) is required for avoidance and identify the cAMP cascade as a key signalling pathway underlying avoidance behaviour dysfunction in FXS. Through

pharmacological intervention targeting the misregulated cAMP pathway we show that defects in dSO avoidance behaviour exhibited by loss of FMRP can be ameliorated thus demonstrating the ability to treat a developmental abnormality post-development.

Although we were able to identify a signalling pathway misregulated in absence of FMRP and implicate its dysfunction in the defective dSO avoidance behaviour exhibited by Fragile X Syndrome flies, it is likely that other signalling pathways also contribute to the suspected defect in the processing of sensory information exhibited. Intracellular signalling pathways do not exist as discrete cascades, rather they interact through sharing signalling intermediates and act in the regulation of one another. This provides a challenge in identifying potential therapeutic targets given the degree of interaction between the pathways. Although we targeted the cAMP pathway for pharmacological rescue and demonstrate that Fragile X Syndrome flies exhibit increased dSO avoidance behaviour following 5-day treatment with lithium likely due to increase in cAMP levels as prolonged lithium administration results in an increase in cAMP, these results also implicate Wnt signalling dysfunction in dSO avoidance behaviour as lithium is also a GSK3 inhibitor (*501*). Future research will be required to identify other signalling pathways that mediate dSO avoidance and how dysfunction within these pathways contribute to deficits in dSO avoidance behaviour in order to fully understand the mechanism underlying defects and the most effective targets for pharmacological intervention.

Neuronal activity is crucial in the formation and refinement of neural circuits by initiating changes in synaptic morphology and strength of synaptic connections. The Wnt signalling pathway mediates changes in synaptic connections in response to neuronal activity in mature neurons. Armadillo/β-catenin is key regulator of bidirectional changes in synaptic structure and strength and considered to be the “hub” of synaptic plasticity as it functions in activity-dependent synaptic remodeling and regulation of activity related Wnt targeted genes (*502*). Armadillo/β-catenin forms complexes with the cell adhesion molecule cadherin. Cadherin-armadillo/β-catenin complexes are regulated by neuronal activity and function in the organization of cytoskeleton components and connections between pre- and post-synaptic terminals (*446, 503*). Neuronal activity also increases FMRP expression, function, and its localization at synaptic sites, where FMRP negatively regulates Armadillo/β-catenin (*155, 188*). Armadillo/β-catenin regulates the

organization of cytoskeleton components and mediates connections between pre- and post-synaptic terminals in an FMRP-dependent manner.

Our results show that the pan-neuronal over-expression of Armadillo/β-catenin results in a significant defect in both learning and long-term memory. In absence of FMRP the mTOR/PI3K/Akt signalling pathway is misregulated resulting in an increase in Akt activity, which enhances the stabilization of Armadillo/β-catenin thereby blocking changes in synaptic plasticity suggesting that in the absence of FMRP, Armadillo/β-catenin contributes to pathophysiology of Fragile X Syndrome (*503*).

We also demonstrate that FMRP-deficient flies exhibit a significant deficit in learning reversal and long-term memory reversal. We anticipate that the over-expression of Armadillo/β-catenin may also be one of many mechanisms underlying the impairment in learning reversal and long-term memory reversal exhibited by Fragile X Syndrome. This hypothesis still requires additional experiments to verify, including examining how the over-expression of Armadillo/β-catenin independent of FMRP influences learning reversal and long-term memory reversal.

Although there is a significant amount of research verifying that armadillo/β-catenin functions in synaptic plasticity, Armadillo/β-catenin also interacts with multiple signalling proteins such as catenin, PI3K, and APC (*504*). Cadherins can recruit and bind to a second family of catenins, p120-catenins which can activate Rac activity (*505-507*). Rac also functions in mediating cytoskeleton structure in an FMRP-dependent manner and has been implicated in learning and memory reversal (*499, 500*).

The complex nature of signalling pathways that exhibit dysfunction in absence of FMRP provides a significant challenge in identifying effective therapeutic targets.

Further research will be required to determine how the simultaneous the loss of FMRP and over-expression of armadillo/β-catenin contributes to Fragile X Syndrome pathology, and whether targeting Armadillo/β-catenin dysfunction would re-establish synaptic plasticity in FMRP deficient synapses.

Fragile X Syndrome is a complex neurodevelopmental disorder characterized by misregulation of protein synthesis and dysfunction within multiple signalling pathways. The complexity and extent of interactions between dysfunctional signalling pathways in Fragile X Syndrome provides a challenge in identifying effective therapeutic targets. Current pharmaceuticals in preclinical or clinical trials target only one aspect

of misregulation in Fragile X Syndrome. Pharmaceuticals currently in clinical trial phases are aimed at reducing activity of signal transduction from mGluR receptors, including mGluR blockers, GABA agonists, AMPA activators, and GSK3 inhibitors (*508*). Only targeting one aspect of misregulation will likely not treat the multitude of dysfunction.

Here we highlight two signalling pathways underlying Fragile X Syndrome neuropathology, the cAMP signalling pathway and the Wnt signalling pathway. Although they are separate pathways, both have similar functions. The cAMP and Wnt signalling pathways both function in neurogenesis, neuronal development, and synaptic plasticity.

We identify cAMP as an effective therapeutic target for ameliorating sensory processing defects present in Fragile X Syndrome. Moreover we were able to ameliorate processing defects by targeting cAMP misregulation through multiple mechanisms; AC stimulation, PDE inhibition, and administration of a cAMP analog. Clinically this would provide flexibility in a course of treatment as there are multiple mechanisms to target cAMP dysregulation, providing a greater opportunity for clinical efficacy.

We also identify the overexpression of Armadillo/β-catenin as a possible mechanism contributing to Fragile X Syndrome pathology.

Collectively our results highlight that there are multiple pathways to dysfunction and many ways in which to treat it. It is likely that the most effective treatments will be targeted at treating multiple mechanisms of dysfunction underlying Fragile X Syndrome. Furthermore we also identified existing gaps in our understanding of the processing of olfactory information processing as well as the role of Armadillo/β-catenin in FXS.

Future research should examine role of FMRP in the antennal lobe, lateral horn, and olfactory receptor neurons in mediating dSO avoidance behaviour by using tissue specific drivers to knockdown and overexpress FMRP in each region both throughout development and acutely in adults. Further studies could also localize the requirement of cAMP in mediating dSO avoidance behaviour by using tissue specific drivers to knockdown and overexpress cAMP levels by manipulating AC and PDE activity in the antennal lobe, lateral horn, mushroom body, and olfactory receptor neurons. Moreover blocking neurotransmitter release using the temperature sensitive dominant-negative mutant of dynamin, *UAS-Shi^{ts}* in the antennal lobe, lateral horn, and mushroom body could be used to determine which structures

are essential for information processing involved in modulating avoidance behaviour. These results would provide additional insight into information processing required for dSO avoidance and possibly allow for the identification of new therapeutic targets.

Here we also show that the overexpression of Armadillo/β-catenin results in significant learning and long-term memory defects. This result is a stepping-stone in providing new insight into the pathology of FXS. Further research should utilize tissue specific drivers to determine the spatial and temporal requirements of Armadillo/β-catenin in learning and long-term memory, as well as in learning and long-term memory reversal. To further elucidate the role of Armadillo/β-catenin in FXS pathology, generating a cross between Armadillo/β-catenin mutants and FXS mutants would provide insight into genetic interactions between these two alleles.

Results from these experiments would provide novel insight into the pathology of FXS and aid in the development of new treatment strategies and therapeutic targets.

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