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Regulation of Synaptogenesis by the miRNA Pathway and FMR/P Bodies

Jacqueline Rochelle Furlong

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Regulation of Synaptogenesis by the miRNA Pathway and FMR/P Bodies

A Thesis

Presented to

the Faculty of Natural Sciences and Mathematics

University of Denver

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Jacqueline R. Furlong

August 2015

Advisor: Dr. Scott A. Barbee, Ph.D.
ABSTRACT

Post-transcriptional regulation of mRNA is facilitated by different mechanisms, such as microRNA (miRNA) induced gene silencing or fragile X mental retardation protein (FMRP) mediated repression either independent of or acting through cytoplasmic RNA Processing bodies (P bodies). DPTP99A, Lar, and Wg have known functions during synaptogenesis and may be targets of miR-8. Here, we provide evidence that miR-8 regulates \textit{DPTP99A in vitro}. Non-endogenous miR-8 expressed using an UAS driver regulates Lar. Endogenous miR-8 may regulate DPTP99A \textit{in vivo}. Here we show that FMRP is capable of colocalizing with the P body components: DCP1, HPat, and Me31B, but not CCR4. We also show that RNAi against HPat and Me31B but not CCR4 and DCP1 are required for FMRP’s repression of a translational reporter \textit{in vivo}. This functional analysis provides additional insight into another aspect of FMRP’s and P bodies’ ability to cooperatively control repression of mRNA targets.
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CHAPTER ONE: FORWARD

Neurons are polarized cells containing a nucleus within the cell body with dendritic and axonal outgrowths. In humans, axonal outgrowth can span up to a meter. Neurons communicate to other cells via chemical synapses that form between axon terminals and dendritic spines. Once formed, the synapse also undergoes remodeling via anterograde and retrograde signals that remodel and fine-tune the synapse via local translation events. Thus, axon terminals are a focus of protein translation during development, synaptic formation, synaptic fine-tuning, and signaling (McNeill and Van Vactor 2012). Neurons are specialized cells with a polarized morphology that is extremely evident in axons creating synapse with muscular targets. Neurons also shuttle messenger RNA (mRNA), protein-coding RNA sequences transcribed from DNA, down axons for local translation at the terminal. The molecular process by which mRNAs are transported vast distances along the axon to the synapse is of interest because misregulation at this point can cause numerous disruptions in the functional ability of neurons. Mechanisms regulating transcriptional control have been well defined, but post-transcriptional regulation has only recently become of interest.

microRNAs (miRNAs) are short-noncoding RNAs that are predominantly 21-23 nucleotides in length which have the ability to bind to various targets via mismatch base pairing and directly target mRNAs for translational repression or degradation by
associating with Ago1 (*Drosophila*) in the RNA Induced Silencing Complex (RISC), a multi-protein complex protein complex involved in posttranscriptional gene silencing. miRNAs have been shown to associate with RNA Processing bodies (P bodies), which also function to coordinate the translational regulation of genes. The ability of a single miRNA to bind to multiple mRNA targets throughout growth is key during the rapid pace of development and makes them excellent candidates for coordinating gene expression.

P bodies are key regulators of axon terminal growth in *Drosophila*. For one, they are believed to transport and store RNA at the synapse. To prevent overgrowth, pools of mRNAs required during development and activity dependent growth must be switched from being translationally active into an inactive state by storage within P bodies. Furthermore, P bodies house machinery involved in translational repression and degradation and also have a role in microRNA-mediated repression. Translational repressors, which have the ability to bind to multiple targets, help coordinate the expression of gene transcripts during the rapid pace of development at neuronal synapses.

Fragile X mental retardation protein (FMRP) is found throughout the body and is especially abundant in the brain. FMRP is a conserved RNA binding protein that negatively regulates translation and interacts with the miRISC complex and P bodies. Furthermore, P bodies, miRNAs, and FMRP are believed to control the translation of mRNAs encoding for products that are required to grow, remodel, and maintain synapses. Pathways controlling synaptic plasticity are implicated in numerous pathologies underlying human neurological disorders, such as age-related memory loss, mental retardation, autism, and addiction.
My thesis is broken into two portions. The first project involves validating DPTP99A, Lar, and Wingless (Wg) as targets of miR-8 in vitro and in vivo. These mRNAs have previously been identified as targets of miR-8 using bioinformatics approaches (Nesler et al. 2013). miR-8 is of interest because it has previously been shown to be regulated by acute synaptic depolarization activity, and is in controlled by activity dependent growth (Nesler et al. 2013). The second project involves looking into how FMRP functionally interacts with protein components of P bodies, specifically CCR4, DCP1, HPat, and Me31B, to control translational repression. These proteins work in the degradation and/or translational repression pathway. Present in all cells, CCR4 is the catalytic component of the 3'-to-5' deadenylase complex and current evidence suggests that it has a unique postsynaptic function (J. Chen, Chiang, and Denis 2002; Cougot, Babajko, and Séraphin 2004; Cougot et al. 2008). DCP1 is part of a complex which functions by removing the 5’ cap from mRNA (Cougot, Babajko, and Séraphin 2004; Parker and Sheth 2007). HPat and Me31B, a DEAD-box helicase, present in postsynaptic granules, both function as activators of decapping (by increasing the efficiency of decapping activity) and are involved in translational repression pathways (Barbee et al. 2006; Cougot et al. 2008; Parker and Sheth 2007). These four components of P bodies are of interest because they associate with FMRP, and are also components of neuronal granules in flies and mammals (Barbee et al. 2006; Cougot et al. 2008). Therefore, FMRP, miRNA, and P bodies work in concert to regulate the translational pathways involved during development and maintenance of neural synapses (Cheever and Ceman...
2009). The mechanisms underpinning the exact interactions between FMRP, miRNA, and P bodies are yet to be determined.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Fly husbandry, strains, handling, and establishing genetic crosses

2.1.1 Fly food and storage

Unless otherwise specified, flies were grown on standard Bloomington stock food media. Fly stocks for experiments were grown at 25 °C. Both male and female larvae were used (in roughly an equal ratio) in all experiments described below.

2.1.2 Fly Strains

An isogenized w1118 line (referred to here as Iso31) was used as a wild-type control for most experiments. Fly stocks used for over-expression (OE) assays were DPTP99A OE (y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}Ptp99A[EY07423] (BDSC)) and Lar OE (UAS: Lar w[*]; P{w[+mC]=UAS-Lar.K}P4B (I and III) (BDSC)). These were crossed with ptc-Gal4 to verify antibody specificity during a gain of function studies. Fly stocks expressing Lar RNAi (y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00822}attP2 (I and III) (BDSC)), DPTP99A RNAi (y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}Ptp99A[EY08245] (BDSC)), and Wg RNAi (y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]= TRiP. HMS00794}attP2 (BDSC)) were crossed with ptc-Gal4 to verify the antibodies specificity with a loss of function. UAS: mCherry-miR-8 / CyO Line 2 and UAS: mCherry-miR-8 / Tm3 (Sb) Line 3 over-expressing mCherry-
miR-8 (a gift from D. Van Vactor) were crossed to ptc-gal4 to determine if non-endogenous miR-8 can regulate DPTP99A and Lar. w-; FRT42D/CYO was crossed to w-; miR8 Δ1 (*,mW+) (a gift from S. Cohen) was used to create a recombinant fly with the genotype: w; miR8 Δ1 (*, mW+), FRT42D/CyO. To analyze the genetic mosaic, w-; FRT42D, miR-8 Δ1 /CyOGFP; hs-FLP/Tm6TBSB was crossed with w-; FRT42D. armi: LacZ/42D armi/LacZ; +/+.

RT-qPCR analysis was conducted with Iso31 and a cross between miR8 Δ1 and miR8 Δ2 (a gift from S. Cohen).

2.2 Genetic mosaics using neomycin-based selection for FRT42D and the FLP recombinase system with lacZ (β-galactosidase)

2.2.1 Neomycin-based selection for an FRT42D miR-8 Δ1 recombinant

In order to create the w; miR-8Δ1 (*, mW+), FRT42D/CyO fly, virgin heterozygous flies were selected and crossed with FRT42D/CyO males on selective neomycin antibiotic media. A negative control was setup to ensure the neomycin selection killed non-resistant flies. The neomycin liquid stock solution (25 mg/ml) was mixed with Drosophila media (100 µl of stock solution per 10 ml of food and vortexed for uniform consistency. To determine the volume of food, the height of the food in the vial was measured using the conversion factor of 17 mm = 10 ml, which was then used to calculate the appropriate amount of neomycin to add to the media. After 2-3 days of egg laying at 25°C, the flies were transferred to a new vial, and the old vial was heat shocked at 37°C for 60 minutes every 12 hours until pupae adhered to the sides of the vial. Heat shocking can be performed with or without the adults from the initial cross present. The mini-white gene was used to select for flies with red eyes, and a single pair mating
established stock lines. The stock flies were PCR tested to ensure that the FRT42D was present.

2.2.2 Fly strains used to make a genetic mosaic

In order to create a genetic mosaic, three stable fly strains were generated from pre-existing stocks. The first fly line: w-; FRT42D, miR-8 Δ1 [mw]/CyO crossed to w-; Kr/CyOGFP ; D/Tm6TbSb, the F2 displaying ;FRT42D, miR-8 Δ1 /CyOGFP; D/Tm6TbSb were selected for and a single pair mating was performed to ensure a stable line. The second fly: w-; Kr /CyOGFP; D/Tm6TbSb was crossed with w-;; hs-FLP/hs-FLP, the F2 displaying w-; Kr/CyOGFP; hs-FLP/Tm6TbSb were selected for and a single pair mating was performed to ensure a stable line. The third fly: w-; FRT42D, miR-8 Δ1 /CyOGFP; D/Tm6TbSb was crossed with w-;Kr/CyOGFP; hs-FLP/Tm6TbSb, the F2 displaying w-; FRT42D , miR-8 Δ1 /CyOGFP; hs-FLP/Tm6TbSb were selected for and a single pair mating was performed to ensure a stable line. The final fly cross to analyze the genetic mosaic was: w-; FRT42D, miR-8 Δ1 /CyOGFP; hs-FLP/Tm6TBSB was crossed with w-; FRT42D. armi: LacZ/FRT42D armi/LacZ; +/+

2.2.3 FLP recombinase system using lacZ (β-galactosidase)

A genetic mosaic was made using the FRT/FLP-recombinase system. Males from w-; FRT42D, miR-8 Δ1 /CyOGFP; hs-FLP/Tm6TbSb was crossed with virgin females from w-; FRT42D. armi: LacZ/FRT42D armi/LacZ; +/+. Fly crosses were established and allowed to lay for 48 hours. They then were transferred every 12 hours into a new vial in order to synchronize the egg laying with the growth of larvae. The first vial with the initial cross was discarded because the eggs had been laid over a 48-hour period. The
vials, were heat shocked for 60 minutes in a 38 °C water bath 24 hours after transferring flies from the vial. Once the larva developed to the wandering 3rd instar stage, they were dissected and their wing discs were collected for immunohistochemistry (IHC).

2.3 Collecting wing imaginal discs

With the aid of a dissecting microscope, wing discs were collected from mature, third instar larvae crawling on the sides of the food vial. Forceps were used to grab the larvae at the mid-section. With another set of forceps, the larvae were grabbed underneath the first forceps to tear away the posterior half of the larvae to discard. Forceps were used to grab the mouth hooks and push down on the head, inverting the larvae and exposing the innards. Extra tissues, such as fat and brain, were removed, exposing the wing discs to the media used for staining. The discs were then mounted using VECTASHIELD Mounting Media with DAPI (Vector Labs, stored at -4° C) or DAPI Fluoromount-G® (SouthernBiotech, stored at -20° C) and imaged within a week of storage.

2.4 DNA and RNA extraction for from Drosophila

Genomic DNA was extracted from the Iso31 control line. Roughly 5-10 adults were selected of both sexes and placed in 1X PBS. An electric tissue homogenizer (Qiagen TissueRuptor) was used to collect genomic DNA for PCR amplification of the Lar gene. About 5-10 3rd instar larvae were used for RNA extraction from Iso31 and miR-8 Δ1/Δ2 knockout was also used to verify the presence of miR-8 in the wing imaginal disc during this specific stage of development.
2.5 Immunohistoochemistry methods

2.5.1 Immunohistochemistry in Drosophila larvae

Larva were microdissected in a 12-well plate in 1X PBS using Number 5 forceps and fixed in 3.5 or 4% paraformaldehyde for 10 minutes at room temperature. Cuticles with attached wing discs were then washed 3 times in 1X PBS for 10 minutes on a shaker, followed by 3 washes in 1X PBS + T (0.01% Triton X-100) for 10 minutes. Specimens were blocked in 1X PBS with 2% BSA and 5% normal goat serum (NGS) for 30 minutes. About 200 microliters of primary monoclonal or polyclonal antibody diluted in block solution was used to stain the wing imaginal wing discs. The specific antibodies and concentrations were: DPTP99A (1:10, Developmental Studies Hybridoma Bank (DSHB), 4C71B7-s), Lar (1:5, DSHB, 9D82B3-s), Wingless (Wg, 1:10, DSHB, 4D4-s), mCherry (1:100, ABCAM, ab167453), lacZ (β-galactosidase) (1:300, Life Technologies, A11123). Primary antibodies were left on the specimen overnight at 4°C (alternatively, 2 hours at RT) on a shaker. After incubation, the specimens were washed six times with PBS+T for 10 minutes on a shaker. Specimens were incubated in secondary antibodies (Alexa Fluor® 488, ABCAM, and Alexa Fluor® 568, Jackson Labs, 1:500) at room temperature for 1 hour. The specimen was washed twice in PBS+T for 10 minutes, followed by one 10-minute wash in PBS. The wing discs were located adjacent to the trachea and were careful removed from the cuticle and placed on the slide. Roughly 10-15 discs were mounted on a single slide with a drop of VECTASHIELD or 15 microliters of DAPI Fluoromount-G®. Cover slides were affixed on the VECTASHIELD slides with clear nail polish. For DAPI Fluoromount-G®, no nail polish was needed, as the
glass hardened the gel which kept the coverslip in place. Once the preps were mounted, they were stored at -20° C prior to imaging.

2.5.2 Immunohistoochemistry in S2-DRSC cells

100 µl of S2-DRSC cells transfected with GFP-FMRP fusions were plated on a poly-L-lysine treated cover slip (MatTek). The cells were allowed to settle and adhere to the cover slip for at least 1 hour at room temperature in a humid chamber. Cells were fixed with paraformaldehyde (2%) by flooding the dish with 1 ml of fixative, with care to avoid pipetting directly on the cells. The paraformaldehyde was gently poured off and another 1 ml of fixative was added to the dish and allowed to incubate in at room temperature in a humid chamber for 10 minutes. The fixative was removed and quickly washed twice with 2 ml of 1X PBS and then immediately poured off. The cells were then washed three times with 2 ml of 1X PBS for five minutes in a humid chamber. Cells were permeabilized with 1 ml of 1X PBS+ 0.1% Triton X-100 for 15 minutes. The dish was flooded with 1 ml of block (2% BSA, 5% NGS in PBS) and incubated for 30 minutes at room temperature in a humid chamber. Rat anti-Hpat antibody was used as a primary antibody and diluted in block (A gift from Akira Nakamura, 1:2000) and incubated overnight at 4°C (alternatively 1 hour at room temperature) in a humid chamber. The solution was poured off and quickly washed twice with 2 ml of 1X PBS and then immediately poured off. The cells were then washed three times with 2 ml of 1X PBS for five minutes in a humid chamber. Anti-rat fluorescent secondary antibody was diluted in block and added to the dish and incubated for 1 hour at room temperature in a humid chamber (Alexa Fluor® 568, Jackson Labs, 1:500). The solution was poured off and
quickly washed twice with 2 ml of 1X PBS and then immediately poured off. The cells were then washed three times with 2 ml of 1X PBS for five minutes in a humid chamber. All washes and incubations at room temperature were slowly agitated on a shaker. 100-200 µl of mounting media was added to cover the cells. Cells were covered by carefully placing a cover slip with forceps. Dishes were immediately imaged or stored overnight at -20°C prior to imaging.

2.6 miRNA target predictions

miR-8 target predictions were performed by TargetScan Fly Release 6.2 (http://www.targetscan.org/fly). The TargetScanS algorithm searches the 3'UTR of mRNA's for conserved motifs present in at least 12 species of Drosophila that match the seed region of 148 mature Drosophila melanogaster miRNAs to predict regulatory targets. The 3'UTRs of DPTP99A, Lar, and Wg each have one exact match for the miR-8 (position +2-8:AAUACUG, residues 2–8) seed sequence.

2.7 Cell culture, transfection methods, and the dual-luciferase reporter assay system

2.7.1 Cell culture

Cells were grown in Schneider’s or M3+BYPE complete media (5% heat inactivated FBS, 1:100 Pen/Strep and 1:100 Fungizone (Invitrogen)). When cells reached a confluency of 85-100%, they were passaged at a ratio of 1:4 to 1:5, roughly every four days. Cells were split using Schneider’s or M3+BYPE minus Fungizone (1:100 Pen/Strep, 5% FBS) at 1:2 in a total volume of 15 mL in suspension flasks.
2.7.2 Transfections

S2-DRSC-DRSC cells were transfected with FugeneHD (Promega) or Effectene (Qiagen) using three biological replicates in 6-well plates (CELLTREAT). FugeneHD transfections were conducted with a 3:1 (DNA to FugeneHD) ratio. 300 ng DNA was mixed in 100 microliters of FugeneHD and incubated for 10-30 minutes. For Effectene transfections, the buffer from the kit was used to bring up the final volume to 300 microliters. The ratios used were 1:8, DNA to enhancer, and 1:10, DNA to Effectene. Incubation times for Effectene were 3 minutes after the enhancer was added, followed by 7 minutes after the Effectene was added. The total volume for each well was brought up to 600 microliters and mixed thoroughly by pipetting up and down, then 550 microliters of Schneider’s incomplete media plus DNA and Effectene/Fugene was added drop-wise to each well. The transfected cells were incubated at room temperature for 72 hours and luminescence was measured by using a dual-luciferase reporter assay system. For transfections involving FMRP and RNAi, the concentrations of DNA or RNA in each well were: 50 ng of FLuc, 200ng of RLuc, 250 ng of λN or the λN-FMRP, and 2000 ng of RNAi (to non-control wells). For microRNA transfections, 50 ng of either a miRNA expression vector or empty vector control, 50 ng of FLuc, and 200ng of RLuc were added to each well. For colocalization experiments, 250 ng of mCherry and GFP fusions were added to each well. Note that transfections efficiency using FugeneHD was generally much lower then Effectene.
2.7.3 Dual-luciferase reporter assay system

After 72 hours of incubation, adherent cells were scraped from the bottom of the 6-well plate (Cell Treat) and 75 µl from each biological replicate were transferred in sets of three technical replicates into a 96-well white opaque plate (Costar). A dual-luciferase reporter assay system (Promega) was performed following the Promega protocol. 75 µl of Dual-Glo® Luciferase Reagent was added into each well and allowed to incubate for 10 minutes prior to taking the luminescence reading for FLuc. Dual-Glo® Stop & Glo® substrate was added to the Dual-Glo® Stop & Glo® Buffer (1:100) to create the Dual-Glo® Stop & Glo® Reagent. 75 µl of Dual-Glo® Stop & Glo® Reagent was added into each well and allowed to incubate for 10 minutes prior to taking the luminescence reading for RLuc. Luminescence was measured using a Synergy HT microplate reader (Biotech) to measure luciferase activity for each replicate.

2.8 Confocal microscopy

All confocal microscopy was done using a laser scanning Olympus FluoView FV1000. For wing imaginal discs, the 20x oil immersion objective (N.A. 0.85) was used and z-stack was created with imaging distance set at 0.8 microns. For imaging cells in culture, semi-adherent cells transfected with GFP fusions or co-transfected with GFP and mCherry fusions were imaged using the 100x objective (N.A. 1.4) with the 2x-4x digital zoom. Fluorescence Recovery After Photo-bleaching (FRAP) was done on select puncta in GFP-FMRP transfected cells using the Fluoview SIM scanner. The cells were photo-bleached at 100% laser transmission for 1.5 seconds and then images were acquired every 3.9 seconds for approximately 5 minutes (78 frames).
2.9 Bacterial Methods

Bacterial cells were either made in the lab for plasmid transformations, NEB DH5-alpha chemically competent cells for low efficiency cloning, or One Shot® TOP 10 chemically competent cells for high efficiency cloning. Chemically competent cells were taken from the -80 and placed on ice to thaw. Once thawed, 1 micro liter of the plasmid was inserted into the microcentrifuge tube containing 50 microliters of dH5Alpha cells and incubated for 30 minutes on ice. The bacterial cells with plasmid DNA were heat shocked for 30 seconds at 42 °C then returned to ice for 2-3 minutes. After incubation in ice, 250 microliters of S.O.C. media for NEB cells or 250 microliters of LB broth were placed in the microcentrifuge tube. The bacteria with plasmid were shaken horizontally at 37° C. An agar plate with a selective antibiotic was then used to plate out roughly 100 microliters of bacteria. The plate was then placed in a 37 °C incubator overnight. In the morning, the plate was checked for isolated colonies and placed in the 4 °C cooler.

Isolated colonies from the plate were chosen and placed in LB broth with selective antibiotic and shaken horizontally at 37 C to mini/midi/maxi preps according to the Omega Biotek/Promega protocol. Glycerol stocks were made at the same time by taking 500 microliters of the media from the bacterial culture grown overnight from an isolated bacteria by combining it with 500 microliters of glycerol (50%) and vortexing for 3 seconds then placing tube in the -80°C freezer. Once made, glycerol stocks were used to skip the transformation step by scraping of the ice with a flamed inoculating loop and placing into media with a selective antibiotic.
2.10 RT-PCR

RNA was isolated from wing imaginal discs in Iso31 and a miR-8 Δ1/Δ2 mutant. Reverse transcriptase was added to create cDNA (Qiagen) Real Time Polymerase Chain Reaction (RT-PR) was conducted using bantam and miR-8 primers. The experiment was done in replicates of three and samples were averaged. SYBR green, which binds to the minor groove of double stranded DNA, was used for a DNA binding dye, and fluorescence was measured after each cycle (Qiagen). Melting point analysis was done to confirm primer specificity. Primers for U1 were also used but the data was discarded because U1 did not pass the quality control test, displaying multiple peaks during the melting point analysis. The experiment was done in replicates of three and samples were averaged to calculate fold change. The average of miR-8 expressed in the mutant was subtracted from that expressed in Iso31, the same was done for bantam. The fold change was determined using the following equation: \(2^{(C_{T1} - T_{T2})}\), with \(C_{T1}\) = bantam and \(C_{T2}\) = miR-8 (Fraga Meulia & Fenster, 2008).

2.11 Statistical analysis

Statistical analysis was conducted using Prism v6.0 (GraphPad software). Statistical significance was considered at \(p<0.05\). Each specific test is indicated in the figure legend. A Student’s t-test was used for samples compared to only one other sample. For multiple comparisons a one-way Anova with a Tukey’s post-hoc was used. Where indicated, data have been normalized to controls and shown as mean ±SEM.
2.12 Fluorescence Recovery After Photo-bleaching and analysis

Bright, distinct puncta were selected in S2-DRSC cells for FRAP using a circular region of interest (ROI) and targeted with 100% laser transmission for 1.5 seconds and then images were acquired every 3.9 seconds for approximately 5 minutes (78 frames). To avoid photobleaching, the laser transmission was set at 2.5% or lower throughout imaging, accept during FRAP. ImageJ version 1.48 was used to measure the relative fluorescence intensity in ROIs of puncta targeted by FRAP (Fp), control puncta, and a background region (Fb) to account for background fluorescence. ROIs in the time lapse images were selected using the 'Intensity v Time Monitor' tool of ImageJ ('Plugins' → 'Time_Series_Analyzer' → 'Time Series Analyzer V3'). The control ROI was a distinct puncta away from the ROI targeted for FRAP. The background ROI was selected in an area with no puncta. Fluorescence intensity was normalized in the Fp as follows: Fp – Fb. Recovery of the Fp at each time lapse point was calculated by: Fp /Fp (initial). Prism v6.0 (GraphPad software) was used to curve fit the fluorescence intensity of the Fp with a one-phase exponential (Zheng et al. 2011).
CHAPTER THREE: MIR-8’s REGULATION OF SYNAPTOGENESIS

3.1 Introduction

3.1.1 Biogenesis of microRNAs and their interaction with the RISC complex

microRNAs (miRNAs) are a class of conserved, endogenously expressed, short noncoding RNA molecules that are 21-23 base pairs in length (Aravin et al. 2003; Bartel 2009; R. C. Lee 1993; Lewis et al. 2003). Mature miRNAs function post-transcriptionally to target the 3'UTR of target mRNA by perfectly matching to a 5’ seed sequence of 6-8 nucleotides. They bind with imperfect complementation over the remainder of the miRNA, and target the mRNA for degradation or translational repression (Bagga et al. 2005; Bartel 2009; R. C. Lee 1993). In some instances, miRNAs can also bind to the 5’ UTR or coding region of mRNA (Bushati and Cohen 2007). miRNA biogenesis begins in the nucleus of the cell when RNA polymerase II transcribes a primary (pri)-miRNA from DNA (Y. Lee et al. 2002; Li, Lin, and Jin 2008, Figure 1). The pri-miRNA has one or multiple hairpin structures that form one or more loops, each consisting of a stem and terminal loop. During normal RNA polymerase II co-transcriptional processing, pri-miRNAs undergo 5' cap insertion, splicing, and polyadenylation, producing one or more mature miRNA (Carthew and Sontheimer 2009; Kim, Bellini, and Ceman 2009, Figure 1). Before leaving the nucleus, the pri-miRNA is cut into a ~70 nucleotide pre-miRNA by
the Microprocessor complex—comprised by Drosha, and DGCR8—then transported into the cytoplasm by Exportin 5 (Y. Lee et al. 2003; H. Siomi and Siomi 2010; Yi et al. 2003; Zeng and Cullen 2004, Figure 1). This type of pre-miRNA processing follows the canonical pathway, but there are some instances where Drosha processing is bypassed and miRNA processing undergoes noncanonical pathways (Westholm and Lai 2011).

Mitrons are an example of noncanonical miRNA biogenesis, where splicing at the intron-exon boundary is used to circumvent processing by Drosha (Ruby, Jan, and Bartel 2007; Westholm and Lai 2011). Once the pre-miRNA has entered the cytoplasm, Dicer cuts off the stem loop, forming a 21-23 nucleotide long double stranded miRNA duplex with a 5’ and a 3’ miRNA (H. Siomi and Siomi 2010, Figure 1). The duplex unwinds, and one microRNA is preferentially loaded into the RNA Induced Silencing Complex (RISC) complex through its association with Argonaute 1 (AGO1 in flies) forming the miRISC complex (H. Siomi and Siomi 2010, Figure 1). The miRNA functions in the miRISC complex as a guide by seed matching to its target mRNA (Bartel 2009; H. Siomi and Siomi 2010). In order for mRNA degradation or gene silencing to occur, GW182, a component in P bodies, functions in the miRISC pathway through its binding with Ago1 and recruiting CCR4 and DCP1, P body components which function in 3’-to-5’ decay via deadenylase, and as an enhancer of 5’ decapping, respectively (Behm-Ansmant et al. 2006; Fabian et al. 2011).
Figure 1: The canonical pathway of miRNA biogenesis from transcription to translational repression or degradation. The miRNA gene is transcribed into a pri-miRNA. The Drosha complex cleaves off the stem loop, creating a pre-miRNA duplex. Exportin 5 transports the pre-miRNA from the nucleus into the cytoplasm. Dicer cuts off the stem loop, forming a miRNA duplex. One strand from the duplex is preferentially loaded into the RISC complex to form miRISC for translational repression or degradation of mRNA.
3.1.2 The abundance of microRNAs and their mRNA targets

microRNAs are abundant molecules, and have numerous predicted targets based on sequence analysis (Consortium et al. 2010; Enright et al. 2003; Lewis et al. 2003; Stark et al. 2003). Many miRNAs exhibit a stage, organ, or tissue-specific expression during development, allowing them to coordinate various development processes (Bartel 2004; Sempere et al. 2004; Zeng and Cullen 2003). miRNA misexpression studies show that mutant flies display pronounced phenotypes, presumably in response to the misregulation of a large number of transcripts (Bejarano et al. 2012). miRNAs are shown to be abundant in the nervous system of flies and mammals (Enright et al. 2003; Miska et al. 2004; Sempere et al. 2003, 2004). Neurons are polarized cells that carry out local translation at their synapses by drawing upon the pool of mRNAs that reside in their dendrites, axons, and growth cones (Y. Li, Lin, and Jin 2008; McNeill and Van Vactor 2012). Research indicates that there is a significant link between miRNA function and learning in both flies and mammals (Y. Li, Lin, and Jin 2008). As many as 20-40% of miRNAs in the brain are developmentally regulated in mammals (Sempere et al. 2004).

More is known about transcriptional control of miRNAs in vertebrates than in Drosophila, but many miRNAs are conserved between mammals and Drosophila, so it follows that the transcriptional regulation may also be conserved. Moreover, in flies, many miRNAs are predicted, and in some instances confirmed, to be involved in regulating processes such as the asymmetric cell division of neuroblasts, transcription factors involved in neuronal differentiation, axon growth and guidance, target turning, and synapse formation (Presutti et al. 2006). Many targets are conserved in vertebrates.
with comparable functions (Enright et al. 2003; Karres et al. 2007; Olsson-Carter and Slack 2010). It is estimated that >60% of human protein-coding genes are conserved targets of miRNA regulation (Friedman et al. 2009). Multiple miRNAs are implicated in the regulation of synaptic plasticity, which indicates that miRNAs function in neuronal circuit formation, refinement, and function (McNeill and Van Vactor 2012).

3.1.3 Activity-dependent miRNA regulation of genes controlling pre- and post-synaptic morphology by fine-tuning protein expression in vertebrates

Current research indicates that miRNAs are important regulators of spatial and temporal gene expression, which induces long lasting change in synaptic structure and physiologic function caused by neural stimulation (McNeill and Van Vactor 2012). miRNAs play an important role in long-term synaptic plasticity, however, only a few confirmed activity-regulated miRNAs have validated mRNA targets. A few examples from different species exist in the literature. In mice, a genome-wide screen showed that the miR-29a/b cluster was significantly up-regulated in response to neurostimulants, causing its target, Arpc3 which is a component of the ARP2/3 actin nucleation complex, to be down-regulated (Lippi et al. 2011). Therefore, miR-29a/b fine tunes structural plasticity and controls dendritic spine morphology by regulating actin network branching (Lippi et al. 2011). In rats, miR-132 was identified in a genome-wide screen as a predicted target of the transcription factor cAMP- response element binding protein (CREB) (Vo et al. 2005). miR-132 is up-regulated in response to by brain derived neurotrophic factor (BDNF) and synaptic activity (Vo et al. 2005). Expression miR-123’s target, GTPase-activating protein p250GAP, involved in regulating the Rac1/PAK1
pathway, is expressed contrariwise to miR132 levels and enhances spine formation (Impey et al. 2010; Wayman et al. 2008). CREB has a widely accepted function in regulation of axon outgrowth and dendritic development, current data suggests that CREB controlled miRNAs also have a role in regulating neurogenesis (Impey et al. 2010; Vo et al. 2005; Wayman et al. 2008). In addition, the rat CNS-specific miR-379–410 cluster, is downstream from the transcription factor myocyte enhancing factor 2 (Mef2), which is necessary and sufficient for activity-dependent transcription of this miRNA cluster (Fiore et al. 2009). One miRNA in this cluster, miR-134, is dendritically localized and has the ability to regulate spine morphogenesis by regulating its target, Pumilio2, which is a conserved translational repressor (Fiore et al. 2009). Another study in rat hippocampal neurons using a microarray-based screen, showed long-term potentiation (LTP) was stimulated by up-regulation of miR-188 (K. Lee et al. 2012). miRNA-188-dependent post-transcriptional control regulates dendritic spine development and synapse structure by negative regulation of semaphorin-3F receptor, neuropilin-2 (Nrp-2) (K. Lee et al. 2012). These interactions all indicate that there is a gene regulatory network involved during neuronal stimulation that positively or negatively regulates the transcription of genes encoding for miRNAs, whose expression negatively correlates to their target mRNAs expression, resulting in long-term changes in synaptic plasticity.

3.1.4 The neuromuscular junction of Drosophila as a model for activity-dependent growth

Synaptic plasticity is a requirement for learning and memory and involves refinement of synapses. Long-term changes may be induced by rapid activity dependent
gene transcription, which induces protein synthesis. The *Drosophila melanogaster* neuromuscular junction (NMJ) has been utilized as a model system to study activity-dependent synaptic growth *in vivo* (Ataman et al. 2006). *Drosophila* have glutamatergic synapses at the NMJ similar to the glutamatergic synapses found in the vertebrate CNS, making it a powerful model system (Johansen et al. 1989; Menon et al. 2013). Inducing activity in the larval NMJ with high K+ or Channelrhodopsin-2 (ChR2) (light-induced spaced depolarization) results in rapid activity-dependent growth which results in change in both synapse structure and function (Ataman et al. 2006, 2008). Activity at the NMJ induces ghost bouton formation, which are presynaptic axon terminal extensions to postsynaptic target muscles that are initially devoid of active zones and post-synaptic specializations (Ataman et al. 2006, 2008). Ghost boutons are not the result of mature boutons retracting, but are indicative of rapid *de novo* formation of immature undifferentiated boutons (Ataman et al. 2008). Therefore, glutamatergic synapses at the larval NMJ show rapid modifications in presynaptic structure and physiology in response to activity (Ataman et al. 2008). Ghost boutons are believed to be analogous to those detected in the dendritic spines of cultured hippocampal neurons (Ataman et al. 2008; J. Yao, Qi, and Chen 2006). Using this model system to study activity-dependent changes has already elucidated novel mechanisms underlying structural plasticity at the NMJ (Freeman et al. 2011; Nesler et al. 2013; Pradhan et al. 2012).
3.1.5 Activity-dependent regulation miRNAs and their target mRNA predictions in
*Drosophila*

Subsequent work on *Drosophila* in our lab using a sequential miRNA microarray and real-time quantitative PCR (RT-qPCR)-based screen found five mature miRNAs (miRs-1, -8, -289, -314, and -958) from the larval CNS to be significantly down-regulated after acute spaced high K+ depolarization paradigm (Nesler et al. 2013). Three of these miRNAs (miRs-8, -289, and -958) were misexpressed in an overexpression assay (OE), by cloning a primary miRNA (pri-miRNA) construct for each activity-regulated miRNA in larval motor neurons, which resulted in negative regulation of activity-dependent growth. No significant increase in ghost bouton formation was found at the NMJ (Nesler et al. 2013). Reduction of function (ROF) experiments using transgenic “sponges” with 10 repetitive sequences of the miR-8 seed sequence, demonstrated an increase in growth, when compared to controls, due to miR-8’s expression being reduced by the transgenic sponge (Nesler et al. 2013). Not only was growth up-regulated in response to miR-8’s ROF, the synapse also increased responsivity to stimulation, and exhibited significant growth after three treatments of spaced stimulation, as opposed to the standard five treatments in controls (Nesler et al. 2013). Next, in order to predict the targets of activity-regulated miRNAs, *in silico* target analysis was performed. Thirty-three mRNAs are predicted to be co-regulated by miRs -8, -289, -958 (Nesler et al. 2013). Functional annotation cluster analysis was conducted on the 33 mRNAs, but none were found to be significantly neuronally enriched (Nesler et al. 2013). Comparing miRs -8 and -289 revealed 32 statistically significant mRNA targets that mapped to the neuron-
related clusters, with 10 (31%) having an annotated function in controlling axon development, guidance, and/or growth (Nesler et al. 2013). Based on this preliminary data, miR-8 was chosen as a candidate for this project, because it is: (1) a highly abundant miRNA in the larval CNS (our unpublished observation); and (2) an activity regulated miRNA that induces a phenotype in the NMJ during ROF or OE assay.

3.1.6 miR-8’s role in developmental regulation and NMJ morphology

miR-8 is the sole homologue in Drosophila of the miR-200 family in bilaterian animals, with nearly identical sequences to vertebrates, given research findings a potentially broader impact (Hyun et al. 2009; Kennell et al. 2008). This abundant miRNA has characterized regulatory functions in Drosophila larvae during development. miR-8 expression is reduced via cues from ecdysone, an insect maturation hormone that regulates body size, and its target, u-shaped (USH), is reciprocally upregulated. USH is a conserved microRNA/ target axis that regulates insulin signaling and expression levels (H. Jin, Narry Kim, and Hyun 2012). Research also shows that miR-8 activates phosphoinositide-3 kinase (PI3K), a protein involved promoting metabolism and cellular growth, and represses USH (Hyun et al. 2009). miR-8 mutants have a reduced body size because loss of function fails to activate PI3K or negatively regulate USH, which functions as PI3K inhibitor to suppress cell growth (Hyun et al. 2009). Another target of miR-8 is atrophin, a transcriptional corepressor, associated with histone deacetylase activity and expressed in nervous tissue; miR-8 mutants display elevated atrophin levels with behavioral defects and elevated apoptosis in the brain indicative of neurodegenerative disorders (Karres et al. 2007). During the embryonic stage, RP3 motor
axons require miR-8 for the earliest stages of muscle target recognition (Lu et al. 2014). Localization of multiple synaptic cell adhesion molecules (CAMs), such as Fasciclin III (FasIII) and Neuroglian (Nrg), are reliant on miR-8 expression, indicating it controls the initial states of synaptic site assembly, and is a key regulator of cell adhesion proteins pre- and post-synaptically (Lu et al. 2014). As described earlier, miR-8 functions during the larval stage of development in the NMJ, where miR-8 functions pre- and post-synaptically to modulate neuromuscular junction formation and in response to activity. (Loya et al. 2009; Nesler et al. 2013). Another direct target of miR-8 is Enabled (Ena), a member of the highly conserved Ena/VASP (Vasodilator-Stimulated Phosphoprotein) protein family, which functions as an actin-assembly cellular mechanism responsible for coordinating synaptic morphology (Loya et al. 2014). Based on the literature, miR-8 has a significant role throughout development, especially synaptically.

3.1.7 Predicted targets of miR-8: DPTP99A, Lar, and Wg

Our proposed model is that an upstream transcription factor is negatively or positively regulated in response to activity at the NMJ, which in turn down-regulates miR-8’s expression. This coordinates the up-regulation in the expression of miR-8’s target mRNAs, encoding for proteins involved during synaptic plasticity, culminating in activity-dependent growth at the NMJ. The mechanisms underpinning our working model are largely unknown and we are now working to validate predicted targets of miR-8 as bona fide targets in vivo (Figure 2). Based on bioinformatic and functional gene ontology (GO) cluster analysis of target mRNAs, we elected to focus on DPTP99A, Lar, and Wg for this project, due to their significant neuronal enrichment and as predicted targets of
miR-8 (Nesler et al. 2013). As proof of concept, an *in vitro* luciferase reporter assay was performed that confirmed two putative targets, Lar and Wg, were capable of repression by miR-8 (Nesler et al. 2013). Moreover, disruption of Lar and Wg function prevents activity-dependent ghost bouton formation (Ataman et al. 2008; Nesler et al. 2013).
Figure 2: A working model for miRNA-mediated control of activity-dependent synaptic growth at the larval NMJ. In an unstimulated motor neuron, activity-regulated miR-8 negatively regulates translation of its target mRNAs (*DPTP99A*, *Lar*, and *Wg*) that transcribe for proteins involved in the control of activity-dependent synaptic growth. In contrast, our working model proposes that acute spaced stimulation results in the rapid down-regulation of mature miR-8 levels, resulting in up-regulation in translation of *DPTP99A*, *Lar*, and *Wg* mRNAs, resulting in rapid activity-dependent synaptic growth.
3.1.8 DPTP99A, Lar, and Wg function during axon pathfinding and synaptogenesis

Neurons arise from undifferentiated cells, undergoing morphologically stages of outgrowth, guidance and synapse formation to compose an integrated, structural network which forms the nervous system (Johnson and Van Vactor 2003). Axon outgrowth occurs during development, when an axon growth cone responds to specific guidance cues to connect to its target muscle (Van Vactor et al. 1993). Some growth cones exhibit multiple arborizations over various substrates until target recognition has been achieved, upon which they specialize into presynaptic structures that then undergo pruning to form a mature synapse (Van Vactor et al. 1993).

DPTP99A and Lar are in the family of conserved receptor protein tyrosine phosphatases (RTPT) whose expression is constrained to axons in the larval central nervous system (CNS) during development (Mohebiany et al. 2013; Tian, Tsoulfas, and Zinn 1991). There is evidence that cross-talk between DPTP99A and Lar may occur as these two proteins send cooperative and competitive axon guidance signals to motor axon growth cones seeking target recognition (C J Desai et al. 1997). Historically, RTPTs have been referred to as orphan receptors because they are known to interact with numerous proteins in vitro to coordinate axon guidance and synaptogenesis, but their ligands have remained, in large, a mystery. Years after their discovery, only now are RTPTs functions and their ligands becoming elucidated, (Fox and Zinn 2005; Mohebiany et al. 2013).

Located in the type III subfamily of RTPTs, DPTP99A is required for axon guidance and pathfinding but does not display a loss of function phenotype in mutants (Johnson and Van Vactor 2003). Double mutants for DPTP99A and type Ila class
RTPTs, Lar or DPTP69D, display a highly synergistic penetrant phenotype (C J Desai et al. 1997; Chand J. Desai et al. 1996; Johnson and Van Vactor 2003). There is much that remains unclear about DPTP99A’s regulation throughout development and ligands. Found in the conserved type IIa subfamily of RPTPs, Lar has distinct roles in synaptic formation and regulation of the size and shape of the active zone. Lar mutants have a partially penetrant motor axon guidance defect, causing the motor axon to stop short or grow past its target (Johnson and Van Vactor 2003; Johnson et al. 2006; Kaufmann et al. 2002; Mohebiany et al. 2013). In addition, Lar also functions to regulate the morphology of the synapse; Lar mutants have a reduced synapse size, along with reduced terminal branch complexity (Kaufmann et al. 2002). Lar is a better-characterized RPTP, having known ligands. Two identified ligands are in the heparan sulfate proteoglycan (HSPGs) family: Syndecan (Sdc) activates Lar to control axon pathfinding and pre-synaptic growth, and Dally-like (Dlp), which inhibits Lar, controlling active zone morphology (Fox and Zinn 2005; Johnson et al. 2006; Kaufmann et al. 2002). Furthermore, recent work in our lab has shown that miR-8 is capable of regulating Lar in vitro (Nesler et al. 2013). Still, much is unknown about RPTPs in regards to pathways underlying translational control of synaptogenesis, which are important to understanding neuronal physiology.

Wingless (Wg) is a well characterized protein from the conserved Wnt signaling pathway involved in morphogenesis and pattern formation (Packard et al. 2002). Briefly, the absence of the Wg ligand from its receptor Frizzled (Fz) causes the β-catenin homolog Armadillo (Arm) to be phosphorylated and targeted for ubiquitination and
subsequent degradation by a multi-protein 'destruction complex' (Bejsovec 2013). In contrast, the presence of the Wg ligand, Dsh binds to Fz, disrupting the destruction complex; this prevents Arm from degradation, causing accumulated Arm to translocate into the nucleus. Once in the nucleus, Arm catalyzes gene expression by binding to the transcription factor Pangolin (Pan) (Bejsovec 2013). However, less is known regarding Wg’s regulatory function at the larval synapse during growth. Wg is expressed in glutamatergic synapses and is secreted by synaptic boutons, which catalyze retrograde signals involved in proper active zone formation and nascent post-synaptic boutons (Packard et al. 2002). Wg loss of function severely impedes synapse formation and creates defective postsynaptic specializations (Packard et al. 2002). Wg is secreted in response activity-dependent regulation, causing a postsynaptic increase in Wg protein levels (Ataman et al. 2008). This postsynaptic increase does not decrease presynaptic Wg levels, indicating that Wg signaling is a crucial effector regarding activity-dependent synaptic plasticity (Ataman et al. 2008). In addition, we have also shown that miR-8 is capable of regulating Wg in vitro (Nesler et al. 2013).

3.2 Results

3.2.1 miR-8’s repression of DPTP99A and Lar in vitro

In order to experimentally verify that DPTP99A is a target of miR-8 in vitro, the 3’UTR of DPTP99A was cloned into the firefly luciferase (FLuc) reporter vector to perform a luciferase-based reporter assay (Figure 3A). The 3’UTR of Lar was previously cloned into the Fluc reporter vector, and we confirmed using an in silico-based approach (Nesler et al. 2013). The 3’UTR of DPTP99A has one binding site for miR-8 and Lar has
one predicted miR-8 binding site (Figure 1A). We co-transfected S2-DRSC cells in three biological replicates with an empty vector or a miR-8 overexpression vector. A dual luciferase assay 72 hours after transfection confirmed both DPTP99A and Lar expression levels were significantly repressed relative to the control, 49% and 39% respectively (p < 0.0001), by miR-8 binding (Figure 2B). This data experimentally confirmed our bioinformatic analysis, suggesting miR-8 may target DPTP99 (and Lar) in vitro to induce translation repression.
Figure 3: miR-8 is capable of repressing DPTP99A reporter expression in vitro. (A) Schematic representation of reporter constructs with firefly luciferase (FLuc) followed by miR-8 (residues 2–8) binding sites (red boxes) within the entire 3’ UTR of DPTP99A and Lar. TargetScanFly 6.2 was used to predict the number of binding sites in the 3’UTR of each gene. (B) FLuc DPTP99A and Lar plasmids were co-transfected into S2-DRSC cells with an empty vector (nonsense RNA) or miR-8. Renella luciferase (RLuc) was used as a transfection control because it contained no predicted miR-8 binding sites. Error bars indicate the mean ± SEM. STATISTICS: Student’s t-test. **** p<0.0001.
3.2.2 Validating the specificity of the DPTP99A, Lar, and Wg antibodies

Since miR-8 is capable of repressing the expression of the DPTP99A, Lar, and Wg reporters in vitro (Nesler et al., 2013, Figure 2B), our next step was to confirm that miR-8 works in vivo to regulate DPTP99A, Lar, and Wg expression. We performed immunohistochemistry (IHC) on microdissected wing imaginal discs from control (Iso31) 3rd instar larvae to stain with monoclonal antibodies from Developmental Studies Hybridoma Bank (DSHB). First, we determined the optimal antibody dilution for fluorescence while imaging (Figure 5A-A’, Figure 6A-A’, and Figure 7A-A’). We then verified that the antibody was targeting the correct proteins in order to rule out non-specific binding. Next, we tested for antibody’s specificity. We hypothesized that for DPTP99A, Lar, and Wg staining would be upregulated in an overexpression construct. Ptc-Gal4 was used to drive overexpression of DPTP99A and Lar along the anterior-posterior boundary of the wing disc, axis (Figure 4). A Wg overexpression vector was not immediately available for this experiment. In each instance, DPTP99A and Lar overexpression increased the intensity of the staining along this axis (Figure 5B-B’ and Figure 6B-B’). We also hypothesized that staining would show downregulation of DPTP99, Lar, and Wg when mRNA transcripts were targeted by a short-hairpin RNAi construct. A reduction of function experiment was conducted by crossing ptc-Gal4 to short hairpin RNAi lines against DPTP99A, Lar, and Wg along the same compartment (Figure 4). The reduction of staining was very robust for DPTP99A (Figure 5C-C’). Lar’s staining was also reduced with RNAi, but not as strongly as DPTP99A’s (Figure 6 C-C’). The Wg protein is expressed as a stripe along the wing hinge, in the dorsal-ventral
compartment of the disc (Michael 2001, Figure 4) and RNAi against Wg showed robust reduction in the patch crossing the hinge (Figure 7C-C’). Based on this data, the antibodies were deemed specific, and experiments were continued to verify that these proteins are \textit{bona fide} targets of miR-8 \textit{in vivo}. 
Figure 4: Compartment boundaries in the wing imaginal disc. Wing imaginal disc from wandering 3^{rd} instar larva with anterior, posterior, dorsal, and ventral compartments labeled. A= anterior compartment. P= posterior compartment. D= dorsal compartment. V= ventral compartment. Ptc-Gal 4 is expressed along the anterior-posterior compartment (Purple arrows, yellow line). Wingless (Wg) is expressed along the dorsal-ventral compartment (blue line between green arrows).
Figure 5: Anti-DPTP99A specifically targets DPTP99A in the wing imaginal disc. (A and A’) Wing imaginal disc from Iso31 stained with an antibody against DTP99A in order to determine the optimal dilution ratio. This ratio was determined to be 1:10. (B and B’) Ptc-Gal4 is used to drive overexpression of DPTP99A between the anterior-posterior compartments. (C and C’) Ptc-Gal4 is used to drive expression of the transgenic RNAi construct against DPTP99A. Scale bar=100 µm (top panels) and 50 µm (inset).
Figure 6: Anti-Lar specifically targets Lar in the wing imaginal disc. (A and A’) Wing imaginal disc from Iso31 stained with an antibody against Lar to determine optimal antibody dilution (determined to be 1:10). (B and B’) Ptc-Gal4 is used to drive overexpression of Lar between the anterior-posterior compartments. (C and C’) Ptc-Gal4 is used to drive expression of the transgenic RNAi construct against Lar. Scale bar=100 µm (top panels) and 50 µm (inset).
Figure 7: Anti-Wg specifically targets Wg in the wing imaginal disc. Wing imaginal disc from *Iso31* stained with an antibody against Wg (optimal concentration is 1:10). Ptc-Gal4 is used to drive expression of the transgenic RNAi construct against Wg. Scale bars =100 µm (top panel) and 50 µm (inset).
3.2.3 Exogenous miR-8’s regulation DPTP99A, Lar, and Wg in vivo

Once the specificity of the antibody was confirmed, we next wanted to determine if exogenous miR-8 would down-regulate DPTP99A, Lar, and Wg in the wing imaginal disc. In order to test this experimentally, we crossed ptc-Gal4 with a fly line that coexpressed mCherry and miR-8. This construct contains the pri-miR-8 sequence cloned in the 3’ UTR of mCherry and is expressed along the anterior-posterior compartment of the wing imaginal disc. DPTP99A did not exhibit a reduction in this expression domain (Figure 8). In contrast, Lar expression was reduced in this region where miR-8 was overexpressed (Figure 9). Due to the Wg antibody requiring a significant amount of optimization in order to verify its specificity, miR-8 repression experiments have not yet been completed due to time constraints. Since miR-8 is highly expressed during development (Lagos-Quintana et al. 2001) there is a possibility that overexpression of miR-8 in the wing imaginal disc would be insufficient to cause downregulation in translation of the DPTP99A transcript (Figure 8). Thus, the next step was to test if endogenous miR-8 loss-of-function can regulate Lar, and DPTP99A.
Figure 8: Exogenous miR-8 is not capable of repressing DPTP99A in vivo. Wing imaginal disc using ptc-Gal4 as an overexpression driver for coexpression of mCherry-pri-miR-8 stained with anti-DPTP99A. The mCherry-pri-miR-8 transcript is processed, which produces mature miR-8 via the canonical pathway. (A and A’) Panel and inset show DPTP99A staining throughout the wing disc. (B and B’) Overexpression of miR-8 in the anterior-posterior compartment, mCherry fluorescence has not been enhanced with antibodies. (C and C’) Merged image of the red and green channel. Scale bars=100 µm (upper panels) and 50 µm (insets).
Figure 9: Exogenous miR-8 is capable of repressing Lar in vivo. Wing imaginal disc using ptc-Gal4 as an overexpression driver for coexpression of mCherry-pri-miR-8 stained with anti-Lar. The mCherry-pri-miR-8 transcript is processed, which produces mature miR-8 via the canonical pathway. (A and A’) Panel and inset show Lar staining throughout the wing disc. (B and B’) Overexpression of miR-8 in the anterior-posterior compartment, mCherry fluorescence has not been enhanced with antibodies. (C and C’) Merged image of the red and green channel. Merged image of the red and green channel. Scale bars=100 µm (upper panels) and 50 µm (insets).
3.2.4 RT-PCR to verify endogenous presence of miR-8 in the wing imaginal disc

In order to do experiments with endogenous miR-8 in the wing imaginal disc, we first needed to determine that miR-8 was present in the wing disc at sufficient levels to induce regulation, and that the loss-of-function actually reduced miR-8 levels in mutants. We used primers for bantam and miR-8 for quantitative real time PCR (RT-qPCR). Bantam is a ubiquitous miRNA that is present in the wing imaginal disc (Brennecke et al. 2003). Using RT-qPCR, we compared the levels miR-8 RNA present in Iso31 controls to miR-8 present in a miR-8 mutant (both were normalized to levels of bantam). RT-qPCR confirmed relative expression in mutants was downregulated by two fold when compared the control (Figure 10 and Table 1). The miR-8 mutant sample was a mixture of heterozygotes and homozygotes larva, which explains why miR-8 expression was not completely absent in the mutant data (Figure 10). This result confirmed the presence of miR-8 in the wing imaginal disc.
Table 1: RT-qPCR for bantam and miR-8

<table>
<thead>
<tr>
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<th>Bantam (Ct values)</th>
<th>miR-8 (Ct values)</th>
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<tbody>
<tr>
<td>Iso31 (control)</td>
<td>30.08</td>
<td>27.78</td>
</tr>
<tr>
<td>miR-8Δ1/miR-8Δ2(mutant)</td>
<td>30.59</td>
<td>26.37</td>
</tr>
<tr>
<td>Fold change</td>
<td>0</td>
<td>1.92</td>
</tr>
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</table>

Table 1: miR-8 is expressed endogenously in the wing imaginal disc and is downregulated in miR-8 mutants. RT-qPCR data from wing imaginal discs in Iso31 and miR-8 Δ1/miR-8Δ2 mutants (a mixture of homozygous and heterozygous). Primers for bantam and miR-8 were used to test the relative expression of each in the wing imaginal disc tissue. Melting point analysis was done which identified single peaks for each primer, indicating there is specific primer binding occurring. Bantam, shown to be highly enriched in wing imaginal discs (Brennecke et al. 2003), was used as a normalizing control and the reason for a 0 fold change in bantam levels. miR-8 levels were normalized to bantam levels in each sample. Levels in miR-8 mutants were normalized to Iso31 controls. SYBR green was used for a DNA binding dye. Fold change was determined by ddCT.

Figure 10: miR-8 is expressed endogenously in the wing imaginal disc and is downregulated in 8Δ1/miR-8Δ2 mutants. RT-qPCR data from the wing imaginal disc in Iso31 and 8Δ1/miR-8Δ2 mutant showing relative change in comparison to controls (expression is 26% of controls).
3.2.5 Endogenous miR-8’s regulation DPTP99A, Lar, and Wg in vivo

Based on our finding that miR-8 is present in the wing imaginal disc (Table 1), we constructed a genetic mosaic to determine whether endogenous miR-8 is robust enough to replicate the regulation of Lar with miR-8 overexpression (Figure 9). We used the FRT/FLP system to induce mitotic clones after heat-shock (Xu & Rubin, 1993). Using anti-β-galactosidase (LacZ) (Life Technologies), anti-Lar (DSHB), and anti-DPTP99A antibodies (DHSB), a scanning confocal microscope was used to image the wing imaginal discs. LacZ somatic twin-spot clones were not present in the wing imaginal discs of DPTP99A and Lar (Figure 11 And Figure 12). However, the specificity of the LacZ antibody was not determined and could potentially be non-specific. Due to this, also we looked for twin-spots in DPTP99A’s and Lar’s staining in the wing imaginal disc tissue. Lar exhibited uniform staining across the wing imaginal disc (Figure 12). In contrast, DPTP99A appeared to have a homozygous region where the anti-DPTP99A’s staining was downregulated in one area and upregulated in the other, which we believe could be twin-spot clones (Figure 11). This staining pattern was never observed in any other wing disc experiment. Although this data is promising, further optimization needs to be done in order to verify that the twin-spots observed with DPTP99A are actually from a mosaic animal and not abnormalities from the experiment.
Figure 11: Analysis of DPTP99A expression levels in a potential genetic mosaic with miR-8 null clone spots. (A-A’) Anti-DPTP99A staining. Anti-DPTP99A exhibits non-uniform staining in regions where DPTP99A is potentially upregulated (inset). (B-B’) Anti-beta Galactosidase (LacZ). Anti-LacZ exhibits uniform staining with no indication of recombination by the LacZ marker. (C-C’) Merged image of the red and green channel. Scale bars=50 µm (upper panels) and 25 µm (inset).
Figure 12: Analysis of Lar expression levels in a potential genetic mosaic with miR-8 null clone spots. (A-A’) Anti-Lar staining. Anti-Lar exhibits uniform staining with no indication of Lar being up- or downregulated. (B-B’) Anti-beta Galactosidase (LacZ). Anti-LacZ exhibits uniform staining with no indication of recombination by the LacZ marker. (C-C’) Merged image of the red and greed channel. Scale bars=50 µm (upper panels) and 25 µm (inset).
3.3 Conclusions

The data presented here supports the hypothesis that miR-8 is involved in the regulation of DPTP99A and Lar. My initial research aim was to determine if DTP99A was a target of miR-8 \textit{in vitro} and to collect preliminary data \textit{in vivo} regarding the role of miR-8 in regulating DPTP99A, Lar, and Wg. These aims have been partially accomplished, and more detailed conclusions are discussed according to each specific aim.

3.3.1 miR-8’s regulation of DPTP99A

Our bioinformatic data predicted DPTP99A as a target of miR-8, and this prediction was validated \textit{in vitro}. Transfected S2-DRSC cells confirm DPTP99A is a \textit{bona fide} target of miR-8, which is a novel finding and support the bioinformatic target predictions (Nesler et al. 2013, Figure 3). Exogenous miR-8 does not display a phenotype with DPTP99A in the wing imaginal disc when over-expressed using Ptch-Gal4 (Figure 8). Since miR-8 is known to be endogenously abundant (Table 1), adding experimentally overexpressed transcripts may not induce a phenotype. Analyzing the genetic mosaic indicated that there were twin-spot clones in the wing imaginal disc, despite the LacZ antibody not showing twin-spot clones. Numerous wing discs from all other experiments were stained with DPTP99A, but none of these discs showed upregulation in DPTP99A’s staining remotely similar to what we observed in the genetic mosaic (Figure 11). This indicates that we are not seeing non-specific binding in the twin spot studies and instead indicates our hypothesis is correct.
Another potential reason the overexpression of miR-8 did not cause an observable phenotype is that an intermediary protein may be involved in the regulation of DPTP99A in the wing imaginal disc. However, bioinformatic data indicates that there is one direct binding site for DPTP99A (Figure 3A), so this scenario is unlikely. In addition, positive data from DPTP99A in cell culture (Figure 3B), and from the genetic mosaic (Figure 11), support the idea that miR-8 directly regulates DPTP99A. This fits into our working model that miR-8 is able to repress this protein in vivo (Figure 11). DPTP99A has a well established function in axon pathfinding, but its role in regulating activity-dependent growth is unclear (Sun et al. 2000). More work needs to be done in order to provide supporting evidence that activity-dependent regulation of miR-8 results in inverse expression of DPTP99A and growth at the NMJ.

3.3.2 miR-8’s regulation of Lar

*In vitro* and *in vivo* data confirm Lar as a *bona fide* target of miR-8. Replication of the *in vitro* assay showed that miR-8 represses Lar (Figure 3). Furthermore, exogenous miR-8 represses Lar *in vivo* (Figure 6). Data collected from genetic mosaics does not show any recombination, as indicated by the presence of twin-spot clones. This could be due to a negative result indicating that miR-8 does not regulate Lar *in vivo*. However, as mentioned previously, the LacZ positive control was unable to generate homozygous clones in the tissue, which makes validating this as a true negative result extremely difficult. Further optimization is required, however evidence collected thus far indicate that miR-8 may be involved in Lar’s regulation *in vivo*. It is well known that Lar has a function in axon pathfinding, but its role in regulating activity-dependent growth is
relatively unknown (Krueger et al. 1996). Knockdown studies using RNAi exhibit Lar has a function during activity dependent growth because reduction of Lar disrupts activity dependent growth at the NMJ (Nesler et al. 2013). This data supports our working model (Figure 2). However, Lar has yet to be confirmed as a downstream target of miR-8 in response to activity. Further research is required to show that activity down-regulates miR-8 at the NMJ in conjunction with synaptic growth at the NMJ due to Lar’s upregulation.

3.3.3 miR-8’s regulation of Wg

Unfortunately, the data confirming the anti-Wg antibody specificity is recent, and we were unable to establish crosses for twin-spot studies due to time constraints. Additional complications here could arise due to the limited expression domain of Wg (Figure 7). Previous research established Wg is an activity-regulated protein at the NMJ (Ataman et al. 2008). It has also been established that miR-8 is down-regulated in response to activity and that Wg is required to control activity-dependent axon terminal growth (Ataman et al. 2008; Nesler et al. 2013). Connecting these two pathways would be of interest because it would help validate our working model that hypothesizes activity causes miR-8 to be downregulated and inversely correlates to an increased expression of Wg at the NMJ.

3.3.4 Determining the functional relationship between miR-8 and DPTP99A, Lar, and Wg

These three proteins remain of interest as downstream targets of neuronal miR-8. Further research needs to be done to determine the mechanistic interaction in vivo on the activity dependent growth, and miR-8’s regulation during neurogenesis. Collecting this
data will provide further evidence for an activity dependent gene regulatory network in
*Drosophila* that is conserved in mammalian systems.

### 3.3.4 Research implications

Memory formation is typically measured by nascent synaptic growth, post-development. Understanding how synapses are grown and maintained is important to understand learning and memory processes. As the human lifespan increases, due to advances in technology, healthspan is also a pivotal component to the aging process. Neurological diseases are devastating to individuals and their families, further knowledge of the mechanisms underpinning synapse formation and regulation may provide novel solutions to increase quality of life in the elderly via prevention and therapeutic strategies for neurodegenerative disorders. Finally, understanding how activity dependent growth works at the molecular and cellular level may lead to discovering therapeutic approaches involved in axon regeneration and repair. Although axon regeneration is much broader than the research presented here, understanding basic responses to activity that cause growth in *Drosophila* may lead to greater understanding of the mechanisms regarding growth post-development.

### 3.4 Future directions

Further work should be done to conclude experiments in the wing imaginal disc. A cross with ptc-Gal4 to mCherry-miR-8 overexpression constructs should be completed to test if miR-8 overexpression has an effect on Wg in the wing imaginal disc. Next, crossing ptc-Gal4 to miR-8 sponge constructs could be done on all three candidate proteins to determine if loss of function displays a phenotype in the wing imaginal disc.
Optimization of the FRT/FLP protocol should be done in order to confirm that recombination is actually occurring, since anti-LacZ does not display twin-spot clones. Trying different antibodies for LacZ is one possible solution in addition to acquiring a fly strain to use as a positive control with LacZ overexpression and a negative control with RNAi against LacZ.

A preliminary test for DPTP99A at the NMJ should use RNAi against DPTP99A to ascertain if there is an activity-dependent phenotype similar to what was previously preformed experimentally for Lar and Wg (Nesler et al., 2013). Since the antibodies against DPTP99, Lar, and Wg are very weak, with dilutions of 1:5-1:10, alternative approaches to studying these proteins must be used. A Tubulin84-GFP construct has been made as a vector to insert the 3’UTRs for DPTP99A, Lar, and Wg 3. This will allow us to better visualize their interaction with miR-8 in the wing imaginal disc. Lar has already been cloned from cDNA and is ready to be inserted into the Tubulin84-GFP vector. *DPTP99A* and *Wg* require ordering new primers for cloning into the pAc5.1 vector from cDNA. Once this is done, flies could be made with a tubulin promoter that will place GFP fused mRNAs (DPTP99A, Lar, and Wg) under translational control of elements in the 3’UTR. This method should be used to study the expression of DPTP99A, Lar, and Wg in response to miR-8’s regulation in the wing imaginal disc instead of relying on antibody staining. Finally, translation of these targets could be examined at the NMJ using transgenic reporters. A UAS-destabilized EGFP has been made that will signal targets undergoing translation to the membrane. We could use this fusion to test if activity causes an observable phenotype at the NMJ by using high potassium or ChR2
spaced simulation with larva. This protocol has been previously used in our lab to detect if miR-8 is regulated by activity in the NMJ (Nesler et al., 2013). This construct will allow us to observe whether local translation of these proteins is occurring at the NMJ. If local translation is occurring in synapses, genetic approaches should be used to tie in miR-8’s activity-dependent regulation of axon terminal growth by testing if 1) these proteins are downstream of miR-8 and 2) whether miR-8 is responsible for regulating growth at the NMJ resulting from DPTP99A, Lar, and Wg protein expression.
CHAPTER FOUR: FMRP’S INTERACTION WITH THE P BODY
COMPONENTS CCR4, DPC1, HPAT, AND ME31B

4.1 Introduction

4.1.1 Fragile X syndrome and RNP related pathologies

Fragile X syndrome (FXS) is the number one cause of inherited mental retardation in humans, effecting 1 in 5,000 males and roughly half as many women, as it is an X-linked dominant trait (Coffee et al. 2009). The predominant feature in individuals affected by FXS is an abnormally low IQ between 20-70, with 15-50% of individuals showing characteristically autistic mannerisms, such as poor visual contact and repetitive behaviors (Fisch, Simensen, and Schroer 2002; Penagarikano, Mulle, and Warren 2007). FXS is predominantly caused by a trinucleotide expansion in the CGG sequence within the 5’-UTR of the fragile X mental retardation protein (FMRP), resulting in a loss of FMRP expression (Santoro, Bray, and Warren 2012; Verkerk et al. 1993). However, in some instances, FXS is cause by mutations or deletions within \textit{FMR1} (P. Jin, Alisch, and Warren 2004). Neurons require local regulation of mRNA translation to control a variety of processes, ranging from axon guidance, synaptogenesis, and synaptic plasticity (Jung, Yoon, and Holt 2012). Since FXS also affects cognition and short-term memory, the structure of the nervous system is of interest (Penagarikano, Mulle, and Warren 2007). The neuroanatomy of FXS is typified by long, thin dendritic spines along with an
increased spine density, indicative of impaired spine maturation and synaptic pruning (Irwin, Galvez, and Greenough 2000). FMR1 is an mRNA binding protein that is highly expressed in neurons (Bhakar, Dölen, and Bear 2012). In mammals, FMRP has two paralogs, FXR1 and FXR2 (M. C. Siomi et al. 1995; Zhang et al. 1995). In contrast, Drosophila has one FMRP gene making it an excellent genetic model organism to study FXS. In flies, null mutants exhibit abnormal neuronal architecture and synaptic physiology, impairment of long-term memory, and model of behavioral abnormalities (Santoro, Bray, and Warren 2012). Since the structural framework of FXS, evident in aberrant dendritic morphology, indicates that there is a disruption in local regulation of translation, understanding the mechanistic machinery underlying local regulation of mRNA translation which controls a variety of processes ranging from axon guidance, synaptogenesis, and synaptic plasticity in neurons is pivotal to elucidating potential therapeutic strategies for FXS (Jung, Yoon, and Holt 2012).

4.1.2 Normal, permutation, and mutations leading to expression or loss of FMRP

As described earlier, FMRP is transcribed from the FMR1 gene. At the molecular level, FXS is caused by a DNA expansion in the 5’UTR of this gene that results in loss of FMRP expression. In a healthy population, the CGG repeat is polymorphic and ranges from 6-54 repeats (Santoro, Bray, and Warren 2012, Figure 13). In the premutation the number of CGG repeats is between 55 and 200, with an increased number of the FMR1 gene present with a subsequent reduction in FMRP (Santoro, Bray, and Warren 2012, Figure 13). Although premutation alleles do not cause FXS, they may result in adult onset disorders, such as fragile X–related primary ovarian in- sufficiency (FXPOI) and fragile
X–associated tremor/ataxia syndrome (FXTAS) (Santoro, Bray, and Warren 2012, Figure 13). Roughly 20% of premutation carriers are affected by FXPOI; with some women developing the ovarian insufficiency as teenagers (Sherman 2000). FXTAS is an adult-onset (50-70 years at age of onset) neurodegenerative disease; individuals with this disease exhibit as progressive intention tremor, ataxia, and, in some instances, increased cognitive decline (Jacquemont et al. 2004). Finally, the full mutation affects individuals with CGG repeats of more than 200 by methylation of the CGG repeats and the FMR1 promoter, hypoacetylation of histone, and chromatin condensation resulting in transcriptional gene slicing that is induced by epigenetic changes and a complete loss in FMRP (Santoro, Bray, and Warren 2012, Figure 13).
Figure: 13: DNA expansions in the CGG trinucleotides repeat results in normal, premutation, or full mutation allelic function of the *FMR1*. (A) Normal alleles (<55 repeats) for *FMR1* results in proper transcription of mRNA and translation of FMRP. (B) Premutation alleles (55-200 repeats) significantly increase the transcription of *FMR1* and reduced FMRP expression. RNA toxicity caused by elevated levels of FMR1 messenger RNA (mRNA) causes fragile X–associated tremor/ataxia syndrome (FXTAS) and fragile X–related primary ovarian insufficiency (FXPOI). (C) Full mutations (>200 repeats) induce epigenetic changes in the CGG repeats and the *FMR1* promoter, in addition to transcriptional gene silencing of FMRP. Fragile X syndrome (FXS) symptoms are caused by the absence of FMRP. Image from Santoro, Bray, and Warren 2012.
4.1.3 FMRP’s function and role in mediating repression

FMRP is highly conserved mRNA-binding protein and can act as regulator of protein synthesis. At the cellular level, FMRP loss of function causes an absence in protein synthesis-dependent synaptic plasticity and synaptic physiology. FMRP is expressed in numerous tissues and is especially abundant in the brain where it regulates a substantial mRNA population, binding ~4% of all mRNAs in the mammalian brain (Ashley et al. 1993). FMRP has conserved functional RNA binding motifs: two hnRNP-K homology (KH) and an arginine-glycine-glycine (RGG) box (Ashley et al. 1993). The KH2 domain of FMRP binds tightly to RNA complex termed a loop–loop pseudoknot, or “kissing complex” (Darnell et al. 2005). Recently, the KH2 domain of human FMRP has also been shown to bind to and regulate translation through distinct RNA recognition elements (RRE) in target mRNA (Ascano et al. 2012). The RGG box functions in G-quadruplex loop recognition in vitro and in vivo (Bassell and Warren 2008). Determinants within the RGG domain drive association of FMRP with target mRNA by forming cytoplasmic granules, messenger ribonucleoprotein (mRNP), along with several other RNA-binding proteins to negatively affect post-transcriptional translation (Laggerbauer et al. 2001; Z. Li et al. 2001; Mazroui et al. 2002). mRNPs containing FMRP are active and oscillate between polyribosomes and cytoplasmic granules (Mazroui et al. 2002). The majority of cytoplasmic FMRP is associated with elongating polyribosomes via messenger mRNP where it is believed to repress general translation from occurring through its binding to the ribosome and precluding transfer RNA (tRNA)
and translational elongation factors from binding to the ribosome, which induces stalling (E. Chen et al. 2014; Corbin et al. 1997; Wang, Bray, and Warren 2012).

4.1.4 FMRP associates with RNP components

FMRP can undergo subcellular localization in response to a nuclear localization signal (NLS) on FMRP that signals for importation into the nucleus where it can bind directly to mRNA (Eberhart et al. 1996; Kim, Bellini, and Ceman 2009). FMRP also contains a nuclear export signal (NES) which recruits motor proteins, kinesin-1 and cytoplasmic dynein, in order to undergo nucleocytoplasmic shuttling from the nucleus into the cytoplasm (Eberhart et al. 1996; Feng et al. 1997; Fridell et al. 1996; Ling et al. 2004). In neurons, FMRP containing RNP granules travel along dendrites or axons via the neurospecific kinesin KIF3C, a member of the anterograde motors kinesin II family, to translocate mRNAs from the nucleus to specific regions of spines or growth cones, where local protein synthesis occurs (Davidovic et al. 2007). In this instance, FMRP works as an adapter to shuttle the FMRP-RNP granules to dendrites or axons and is an excellent marker for RNP granule. Both fly and mammalian neurons exhibit a functional interaction in a significant percentage of FMRP-containing RNPs which transit in axons and dendrites that colocalize with conserved P bodies (Barbee et al. 2006; Cougot et al. 2008; Pradhan et al. 2012). In Drosophila, the P body component Me31B and dFMR1 coimmunoprecipitate from head extract, hence validating a physical interaction between P bodies and FMRP (Barbee et al. 2006). There is also a genetic interaction between Me31b and dFMR1 in vivo, loss–of-function alleles for Me31B rescues the rough eye phenotype observed when dFMR1 is over-expressed in photoreceptors (Barbee et al. 2006).
In sensory neurons, overexpression of Me31B leads to aberrant branching of terminal dendrites, a shared phenotype with dFMR1 (Barbee et al. 2006). Together, this data suggests specific populations of neuronal P bodies may have a unique function in controlling translation and neuroplasticity.

4.1.5 FMRP’s synaptic regulation of protein synthesis post stimulation

Once synapses are established, localized translation endows local sites with independent protein expression decisions and distinct forms of activity-dependent synaptic plasticity, along with dendritic spine morphology (Kindler et al. 2005). As already described, mRNAs undergo nucleocytoplasmic shuttling to dendritic translation sites in mRNP complexes where it is crucial that they translationally dormant en route and at their final destination until depolarization events release the mRNA from RNP granules into translational active pools, inducing de novo protein synthesis (Kindler et al. 2005; Krichevsky and Kosik 2001). FMRP functions during rapid, activity-regulated transport of mRNAs required during synaptogenesis. Based on the current evidence for activity-regulated FMRP, the “metabotropic glutamate receptor (mGluR) theory” is one of the most prevalent models which proposes how FXS causes behavioral and synaptic defects in neurons as a direct result of abnormal mGluR-dependent protein synthesis (P. Jin, Alisch, and Warren 2004, Figure 14). Briefly, cytoplasmic FMRP dimerizes and is transport into the nucleus via its nuclear localization signal (P. Jin, Alisch, and Warren 2004, Figure 14). FMRP binds to mRNA and associates with proteins to form a FMRP-mRNP complex (Figure 14). The nucleus exportation signal on FMRP signals for transport of the FMRP-mRNP complex from the nucleus into the cytoplasm (Figure 14).
Once in the cytoplasm, the FMRP-mRNP complex can either directly bind to ribosomes in the cell body or associate with proteins in the RNA-induced silencing (RISC) complex and then associate with ribosomes (Figure 14). Both RISC dependent and independent complexes work to mediate regulation of de novo protein synthesis in the cell body of the neuron. Alternatively, in response to synaptic stimulation, mGluR activation may cause either complex to undergo nucleocytoplasmic shuttling via protein motors to dendrites to regulate local protein synthesis of distinctRNAs. The absence of FMRP, whose function is to normally repress synapse-localized mRNAs following mGluR activation are inappropriately translated in response to synaptic activity, increasing protein synthesis (Bhakar, Dölen, and Bear 2012). In mice, mGlur stimulation in FMR1 mutants resulted in impaired dendritic mRNA localization and transport, causing an altered developmental morphologic plasticity similar to FXS (Dictenberg et al. 2008). Another study done in FMR1 knockout mice showed that neurotransmitter-induced stimulation of activity neither induced rapid formation of polysomes nor local synaptic protein synthesis unlike their wild-type controls, resulting in immature dendritic spine formation akin to that observed in FXS (Greenough et al. 2001). All of this suggests that FMRP has a pivotal role in regulating mechanisms that promote synapse maturation and pruning.
Figure 14: Model of fragile X mental retardation proteins function in a neuron. Cytoplasmic FMRP (green hexagon) dimerizes and is transported into the nucleus via its nuclear localization signal. FMRP binds to mRNA to form a ribonucleoprotein (mRNP) complex by association with RNA transcripts (hairpin structure) and proteins to form and FMRP-mRNP complex. The nucleus exportation signal on FMRP signals for transport of the FMRP-mRNP complex from the nucleus into the cytoplasm. Once in the cytoplasm, the FMRP-mRNP complex can either directly bind to ribosomes (purple ovals) in the cell body or associate with proteins in the RNA-induced silencing complex (RISC, red star) and then associate with ribosomes. Both RISC dependent and independent complexes work to mediate regulation of de novo protein synthesis (string of blue circles) in the cell body of the neuron. Alternatively, in response to synaptic stimulation, metabotropic glutamate receptor (mGluR, orange oval) activation may cause either complex to undergo nucleocytoplasmic shuttling to dendrites to regulate local protein synthesis of distinct RNAs. Image from: P. Jin, Alisch, and Warren 2004.
4.1.6 FMRP associates with the miRNA pathway and P body components

Another possible function for FMRP is through the miRNA-guided RNA silencing pathway that suggest FMRP can regulate specific mRNAs using the miRNA pathway (P. Jin, Alisch, and Warren 2004). Research indicates, that RISC machinery and FMRP associate. It is well established that a component of RNA Processing bodies (P bodies), GW182, is responsible for binding to Argonaute proteins and recruiting the CCR4/Not complex as a docking site for deadenylase activity on the poly(A) tail (Braun et al. 2011; Chekulaeva et al. 2011). Other observations indicate that GW182 plays a protective role for Argonaute bound miRNA (B. Yao et al. 2012). In Drosophila, AGO1 is required for dFmr1-mediated regulation of synaptic plasticity (P. Jin, Alisch, and Warren 2004). In mammalian cells, FMRP, via its KH2 domain, has also been shown to act in miRNA-mediated repression and possibly in an AGO-independent manner by performing as an miRNA acceptor protein for the ribonuclease Dicer (Caudy et al. 2002; Ishizuka et al. 2002; Plante et al. 2006). In this instance P bodies components are believed to aide in this interaction because GW182 recruits the deadenylase complex, CCR4/Not, for degradation of target mRNA (Fabian et al. 2011). In addition, phosphorylated FMRP, typically associated with stalled ribosomes, also interacts with precursor miRNAs (pre-miRNAs) but not Dicer (Cheever and Ceman 2009). Dissimilarly, unphosphorylated FMRP associates with Dicer but not pre-miRNAs (Cheever and Ceman 2009). In addition to other observations, these findings suggest FMRP somehow plays a modulatory role for converting specific pre-miRNAs into mature miRNAs (X.-L. Xu et al. 2011; Xia-Lian Xu et al. 2008). Another piece of
evidence that supports FMRP’s interaction with miRNA-mediated repression of mRNA is its association with the RNA helicase MOV10 by allowing bidirectional modulation of repression based on proximity of FMRP to miRNA binding elements on the mRNA, allowing helicase activity by preventing AGO association (Kenny et al. 2014). Our lab has also shown a genetic interaction between HPat and the catalytic subunit of the deaneylase complex, CCR4, and the miRNA pathway, via AGO1 (Pradhan et al. 2012). Thus, multiple lines of evidence suggest that there is an interaction between FMRP, P bodies, and the miRNA pathway that involve negative regulation of their mutual mRNA targets.

4.1.7 RNP components and their function

As previously described, FMRP is known to interact with RNP components. Pools of translationally inactive mRNAs are incorporated into RNP, which are comprised of two subsets: P bodies and stress granules (Ramaswami, Taylor, and Parker 2013). Gene expression is controlled via a dynamic cycling of cytoplasmic mRNA between polysomes, P bodies, and stress granules in a dynamic exchange that influences translation and decay pathways (Ramaswami, Taylor, and Parker 2013). Stress granules accumulate in cells undergoing limited translational initiation or stress and contain mRNA associated with mRNA binding proteins and translation initiation factors (Buchan and Parker 2009; Ramaswami, Taylor, and Parker 2013). P bodies contain aggregates of mRNA and function in the degradation or translational repression pathway (Parker and Sheth 2007; Sheth and Parker 2003; Sossin and Desgroseillers 2006). The first process can occur via deadenylation followed by decapping of mRNA; the second process
induces repression by storing mRNA within the P body. Decapping is initiated by the DCP1/DCP2 decapping complex when the 5' cap is removed, exposing the transcript to 5'-to-3' degradation by the endonuclease Xrn1 (Teixeira et al. 2005). Alternatively, 3'-to-5' degradation via deadenylation, which is catalyzed by CCR4/Not, a conserved complex in 3'-to-5' exonuclease activity (Teixeira et al. 2005). Ribosomes are not found in P bodies and are thought to compete with P bodies for positive and negative regulation of translation (Teixeira et al. 2005). Moreover, P bodies are also found in neuronal synapses which may be due to their role in mRNA turnover in order to regulate local translation events (Sossin and Desgroseillers 2006). Proteins functioning in decapping or deadenylation machinery are of interest because of their role in post-transcriptional repression. P bodies have core elements that are conserved across species and can be broken into three groups: 1) mRNA decapping machinery, the DCP1/DCP2 complex, 2) activators of decapping, specifically HPat and Me31B, 3) 5'-to-3' exonuclease, Xrn1p (Cougot, Babajko, and Séraphin 2004; Parker and Sheth 2007). Because our primary focus is on proteins involved during decapping and deadenylation, CCR4 is another protein of interest in P bodies, due its role in 3'-to-5' deadenylation and due to Xrn1's absence in dendritic P bodies (Cougot, Babajko, and Séraphin 2004; Cougot et al. 2008). Specifically the function of the proteins of interest are as follows: DCP1 is of interest because it is an enhancer of decapping and is a unit within the decapping complex; CCR4 is a deadenylase, which allows the decapping machinery to function or can continue with 3'-to-5' decay on its own; HPat for its role in tying deadenylation and decapping together
and as an enhancer of decapping; and Me31B, as a positive control in some experiments because of its known function in FMRP association and is an enhancer of decapping.

4.1.8 Neuronal P body components: CCR4, DCP1, HPat, and Me31B

Numerous lines of evidence indicate that FMRP is abundant in the brain and is a RNA binding protein that aids in translational repression. FMRP also interacts with RNPs, specifically P bodies which are localized to synapses. Both P bodies and FMRP may coordinate their functions to induce negative translational repression. As previously described, Me31B causes aberrant dendritic branching during loss-of-function experiments, similar to those observed in FXS phenotypes, indicating these two pathways are somehow interconnected and that Me31B has a functional role in regulating dendritic growth via translational repression (Barbee et al. 2006). Previous research in our lab has shown that HPat and CCR4 mutants display a strong synaptic hyperplasia at the NMJ (Pradhan et al. 2012). These synaptic defects are associated with rearrangement of the axonal microtubule cytoskeleton suggesting that HPat negatively modulates pre-synaptic microtubule-based growth during NMJ development. In addition, both pre- and postsynaptic overexpression of HPat inhibits rapid ghost bouton formation induced by spaced depolarization (Pradhan et al. 2012). Furthermore, HPat genetically associates with CCR4 and the miRNA pathway (AGO1) to regulate bouton formation (Pradhan et al. 2012). Thus, it is speculated that HPat is necessary to target mRNAs regulating microtubule architecture and synaptic terminal growth to induce repression, assumedly in P bodies, through both general and miRNA-mediated regulatory mechanisms.
4.2 Results

4.2.1 FRAP on FMRP granules to test their dynamics

In order to study FMRP in cell culture, we developed EGFP- and mCherry tagged FMRP fusion proteins. Previous work showed that FMRP is a dynamic granule (Gareau et al. 2013). We wanted to first demonstrate the constructs were also dynamic, and not just static protein aggregates of FMRP. Drosophila Schneider 2 (S2-DRSC) cells were transfected with an EGFP version of wild-type FMRP, and Fluorescence Recovery After Photobleaching (FRAP) was used to measure the recovery of EGFP: FMRP in S2-DRSC cells five minutes post-FRAP. Initially, EGFP: FMRP puncta recovered fairly quickly (half time), then transitioned into a slower phase of recovery (Figure 15). This displays an initial rapid recovery of EGFP: FMRP recycled from the cytoplasm during the first 80 seconds (Figure 15). The fluorescence recovered began leveling out and approached a plateau after recovering two-thirds of its original fluorescence (Figure 15). Based on these results, FMRP has a robust mobile phase, confirming that the FRMP granules we observed were indeed dynamic granules capable of interacting with the surrounding environment and were similar to findings in published literature.
Figure 15: Fluorescence Recovery After Photobleaching (FRAP) of S2-DRSC cells transfected with EGFP:FMRP. Cells were imaged using a scanning laser confocal microscope. (A) FMRP granules were selected (arrow) and photobleached with the 488 laser set to 100% transmission for 1.5 seconds. The cells were then imaged every 3.9 seconds for 78 frames. (B) Statistical analysis was performed using a non-linear regression one phase exponential model in Prism with SEM. Plateau=69.39 seconds, half-life=81.22, R squared=0.7161.
4.2.2 FMRP’s colocalization with FMRP, CCR4, DCP1, HPat, and Me31B in vivo

Since FRAP confirmed that the FMRP are granules and not static protein aggregates, in vitro experiments were conducted to observe FMRP's colocalization with CCR4, DCP1, HPat, and Me31B. Plasmids were constructed with EGFP and mCherry fusions to determine colocalization of the puncta. S2-DRSC cells were co-transfected with mCherry:FMRP and EGFP:CCR4 (Figure 16 A-C), mCherry:FMRP and EGFP:DCP1 (Figure 16 D-F), mCherry:FMRP and EGFP:HPat (Figure G-I), and mCherry:FMRP and EGFP:Me31B (Figure 16 J-L). CCR4 displayed diffuse staining with no discrete puncta, but FMRP formed discrete granules like those observed during the FRAP experiments (Figure 15 and Figure 16 A-C). DCP1 and FMRP exhibited distinct puncta, with the majority of the DCP1 granules colocalizing strongly to FMRP (Figure 16 D-F). HPat and FMRP colocalized in few of the puncta with the majority of granules forming discrete puncta that did not overlap (Figure 16 G-I). Me31B and FMRP displayed extensive colocalization with few singular puncta that did not colocalize (Figure 16 J-L). Some puncta of FMRP appear to be immediately adjacent to P bodies as if they might be "docked" (Figure 16). These results indicate that FMRP interacts with DCP1, HPat, and Me31B in vitro.
Figure 16: FMRP’s colocalization with CCR4, DCP1, HPat, and Me31B \textit{in vitro}. Scanning confocal fluorescent images of S2-DRSC cells cotransfected with mCherry:FMRP and EGFP:CCR4 (A-C), mCherry:FMRP and EGFP:DCP1 (D-F), mCherry:FMRP and EGFP:HPat (J-K), and mCherry:FMRP and EGFP:Me31B. Scale bar = 5 \(\mu m\).
4.2.3 Repression of a translational reporter by tethered FMRP following CCR4, DCP1, HPat, and Me31B knockdown by RNAi.

Colocalization experiments indicate that these proteins interact in vitro. We hypothesize that knocking out genes for CCR4, DCP1, HPat, or Me31B might also disrupt FMRP function. This hypothesis is based on published and unpublished data from our lab (Pradhan et al. 2012, data not shown). Therefore, to experimentally test this we used RNAi against CCR4, DCP1, HPat, or Me31B by using a reporter system to measure the luminescence in response to different treatments. We fused FMRP, from Drosophila, to the λN protein which exhibits high binding affinity to five BoxB stem loop sites (5xBoxB) cloned into the 3’UTR of firefly luciferase (FLuc) reporter mRNA (Figure 17A). We transiently transfected S2-DRSC cells with the FLuc-5xBoxB reporter construct, Renilla luciferase (Rluc), a plasmid expressing λN peptide control or the λN-FMRP fusion; this set of transfections served as our positive control to show that the FMRP repression worked (Figure 17A). As we have previously observed, tethered FMRP causes an approximately 3-fold decrease in expression of the FLuc-5xBoxB reporter (Figure 17B; far left). In parallel, we used dsRNA against CCR4, DCP1, HPat, and Me31B, the FLuc-5xBoxB reporter construct, Rluc, and a plasmid expressing λN peptide control or the λN-FMRP fusion. RNAi against HPat or Me31B had a modest but significant de-repression from the λN-tagged FMRP morphology observed (Figure 17B). However, RNAi against CCR4 or DCP1 did not induce significant de-repression from the λN-tagged FMRP construct (Figure 17B). This could be a result of RNAi incompletely knocking down the function of each gene (we have not yet confirmed knockdown), which
would allow the remnant endogenous mRNA to transcribe enough protein for the FMRP:CCR4 or the FMRP:DCP1 to retain some or all of their functional interaction.
Figure 17: HPat and Me31B are required for FMRP’s repression in cell culture. (A) is a schematic showing the tethering assay where the λN peptide or λN fused FMRP binds to the 5xBoxB sequence. (B) A dual luciferase assay measuring FMRP’s repression and RNAi against CCR4, DCP1, HPat, and Me31B determine if derepression of FMRP’s repression occurs. FLuc values were normalized to RLuc. Error bars indicate the mean ± SEM. Statistics: One-way ANOVA. * p<0.05 ** p<0.01. n = 3 in each column.
4.3 Conclusions

4.3.1 Colocalization of FMRP with CCR4, DCP1, HPat, and Me31B

Based on our data FMRP appears to interact with DCP1, HPat, and Me31B in some capacity. Using FRAP, we have confirmed that granules observed here were similar to those previously published (Gareau et al. 2013). The FMRP granules were able to quickly recover a significant portion (67%) of their original fluorescence, exhibiting a robust mobile fraction (MF) (Figure 15). If the puncta observed were protein aggregates, it is expected that diffusion of cytosolic FMRP would have little to no effect on fluorescence recovery since aggregates are static structures and would be unable to uptake diffuse FMRP from the cytoplasm to recover fluorescence (Hubstenberger et al. 2013). The FMRP granules tested here exhibit a single recovery phase, indicating that it only has one bound and one diffuse state (Sprague and McNally 2005). This is unlike similar RNP granules found in C. elegans which display a biphasic recovery, suggesting that the there are two distinct phases of diffuse granules and bound granules (Hubstenberger et al. 2013). Once this was determined, an in vitro assay showed whether FMRP and P body granules were able to colocalize with each other. DCP1, HPat, and Me31B formed distinct punctate structures that all showed varying degrees of colocalization with FMRP (Figure 16). CCR4 did not show any colocalization and was very diffuse, not forming any distinct puncta (Figure 16). Potentially, in this system, CCR4 does not form visible granules and does not colocalize with FMRP in vitro. Expanding the number of cells imaged and finding percent colocalization in an increased population of cells would be useful. Looking at specific proteins to determine if any serve
as docking platforms is also of interest. We have also made constructs where the known functional domains of FMRP: RGG, PRiD, KH1, and KH2 have been deleted. Therefore, we can ask if deletions of these binding domains interferes with functional interactions observed during colocalization experiments.

4.3.2 Functional assay of FMRP with CCR4, DCP1, HPat, and Me31B

The in vitro data suggests FMRP only requires enhancers of decapping, HPat and Me31B, to induce translational repression in S2-DRSC cells (figure 17). This is an unexpected result because DCP1, which is able to colocalize with FMRP (Figure 16), was insignificant. This may indicate that DCP1 does interact with FMRP, but is not a major requirement for translational repression to occur. It could also mean that decapping/deadenylation is not involved in this system. It is possible that RNAi was inefficient and endogenous DCP1 and CCR4 was still robust enough to have an effect to allow FMRP to induce translational repression. Biochemical approaches would clarify how these complexes are interacting by confirming that the RNAi significantly reduced protein expression in this assay. More needs to be done in order to discover the biochemical interaction between these proteins and how they function in concert to induce translational repression. Finally, looking at local translation events in the cell is of interest because understanding the broader mechanisms underlying FMRPs interaction with P bodies has potential therapeutic implications.

4.4 Future directions

Further work on the project requires collecting additional data to confirm that the granules seen in the colocalization experiments (Figure 16) are indeed functional
granules and not protein aggregates. This could be experimentally validated by fixing S2-DRSC cells transfected with a GFP-FMRP fusion tag, and then staining with antibodies against CCR4, DCP1, HPat, and Me31B to confirm that the colocalization observed with GFP:FMRP fusions mCherry:CCR4, mCherry:DCP1, mCherry:HPat, and mCherry:ME31B can be validated by using another method. Preliminary data from fixed S2-DRSC cells confirm that anti-HPat and GFP:FMRP colocalize and validate the GFP and mCherry fusion colocalization data (Figure 18). The reciprocal of this experiment would use anti-FMRP antibodies with GFP-tag fusions with CCR4, DCP1, HPat, and Me31B. Alternatively, we could examine colocalization of endogenous protein using available antibodies (although this would be technically challenging). A future aim for this project is to add a biochemical component. First, we will confirm, using western blots on cell lysates acquired from the RNAi experiments, that RNAi against CCR4, DCP1, HPat, and Me31B was efficient, and that the observations are not a result of incomplete gene knockdown. Transfections require replication to show that the results observed can be repeated with fidelity. Immunoprecipitations using FLAG-tagged versions of FMRP to pull down the four P body components and using EGFP-tagged CCR4, DCP1, HPat, or Me31B will confirm that these proteins colocalize and validate intact complexes. The converse of this experiment can also be completed. Since all of these proteins are expressed endogenously in S2-DRSC cells, this is a more physiological experiment and may be used instead of overexpression constructs required for GFP-tagged experiments. Our aim is to verify these results using antibodies, but there is a caveat because data from antibodies used during this experiment might be unclear, due to
non-specific binding. In order to resolve this, the GFP.TRAP system may be used to target an endogenously expressed protein: 1) make a GFP fusion of endogenous protein using CRISPR; 2) make a stably expressing line of S2-DRSC cells, in which the endogenous gene is tagged with GFP, circumventing overexpression. In either of these systems pull-down assays using immuniprecipitations can be performed. This same system may be used in vivo with brain lysates. Finally, we have also made constructs where the functional domains of FMRP: RGG, PRiD, KH1, and KH2 have been deleted. Therefore, we can ask if deletions of these binding domains interfere with the functional observations during colocalization and transfection experiments. The same question can be asked for the biochemical aspect of this project, if deletions of RGG, PRiD, KH1, and KH2 binding domains interfere with FMRP’s function during immunoprecipitations with CCR4. DCP1, HPat, and Me31B.
Figure 18: Anti-HPat colocalizes with mCherry:FMRP fusions. Fixed S2-DRSC cells with anti-HPat and mCherry:FMRP to observe the colocalization of the puncta \textit{in vitro}. An HPat antibody along with tagged mCherry:FMRP was used to dye the granules in vitro. Images are shown in gray-scale, except the merged image. Colocalization of the puncta can be observed in yellow. Scale bar = 2 µm.


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