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A NEUROPROTECTIVE ROLE FOR MIR-1017,

A NON-CANONICAL MIRNA

by

Matthew Anthony de Cruz

A Thesis
Submitted to the Graduate School,
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and the Department of Biological Sciences
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in Partial Fulfillment of the Requirements
for the Degree of Master of Science

December 2017

A NEUROPROTECTIVE ROLE FOR MIR-1017,

A NON-CANONICAL MIRNA

by Matthew Anthony de Cruz

December 2017

Approved by:

____

Dr. Alex S Flynt, Committee Chair
Assistant Professor, Biological Sciences

____

Dr. Fengwei Bai, Committee Member
Associate Professor, Biological Sciences

____

Dr. Shahid Karim, Committee Member
Professor, Biological Sciences

____

Dr. Janet R Donaldson
Chair, Department of Biological Sciences

____

Dr. Karen S. Coats
Dean of the Graduate School

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ABSTRACT

A NEUROPROTECTIVE ROLE FOR MIR-1017,A NON-CANONICAL MIRNA

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December 2017

miRNAs are post-transcriptional regulators of gene expression, with numerous being involved in neurobiology. Within the human genome a quarter of the identified miRNA loci derive from a class of miRNAs termed tailed mirtrons. Despite the identification of this large population of miRNA, no functional studies have been conducted to identify their role. In this study we examined the highly expressed and deeply conserved Drosophila 3' tail mirtron, miR-1017, as a candidate to elucidate tailed mirtron functionality. We identified acetylcholine receptor transcripts, Dα5 and Dα2, as bona fide targets for miR-1017. Interestingly, Dα2 is also the host transcript for miR-1017. We utilized the GAL4/UAS system, to observe the miR-1017 expression pattern; furthermore we witnessed a disrupted feedback loop in the miR-1017 null background resulting in higher Dα2 transcriptional activation. This could be phenocopied with the acetylcholine receptor (AchR) activity antagonist, Donepezil, which likewise resulted in higher GFP expression. Together this suggests that Dα2 transcription is modulated by acetylcholine neurotransmission. Consistent with a role in dampening AchR activity miR-1017 mutant flies exhibit a neurodegeneration due to excitotoxicity. Ectopic expression of miR-1017 within an Alzhiemers disease fly model dampened the pathologenesis and improved neurological function and lifespan. Therefore revealing miR-1017, a 3' tailed mirtron, as a significant

neuroprotector within *Drosophila*. The coupled expression of Dα2 and miR-1017 works as a negative feedback loop that limits activity dependent transcription of Dα2.

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CHAPTER I – INTRODUCTION

1.1 *miRNAs as Gene Regulators*

Over the last two decades miRNAs have widely become recognized as common post-transcriptional regulators of gene expression[1]. miRNAs have been identified in Plants, Animals and Viruses and have numerous roles including physiological response and cell differentiation during development[2,3]. Furthermore when miRNAs are dysregulated it can cause serious health problems including cancer[4,5]. Animal miRNAs are 22 nucleotides (nt) in length and only require 6-8 nt of complementarity to a target mRNA transcript[6]. The seed region of the mature miRNA, nt 2-8, is responsible for complementation to the mRNA[7]. Once bound to Argonaute (Ago) proteins and their target transcripts, miRNAs perturb ribosomal translation and cause mRNA degradation[8].

In 1993 the first miRNA was identified and hypotheses ensued suggesting that small RNA molecules could cause posttranscriptional gene silencing. The seminal studies in *C. elegans* examined heterochronic genes in larval development, specifically looking at *lin-14*[9]*.* Through the use of Northern blotting they unexpectedly identified two small transcripts, *lin-4L* (61nt) and *lin-4S* (22nt), unbeknownst to them that this was the first identification of a pre-miRNA and mature miRNA respectively. After sequencing it was identified that the *lin-4L* transcript had a secondary structure based on complementary sequences within the transcript, this identified the first defining feature of miRNAs as having a hairpin structure. Furthermore they identified that the 61nt and 22nt transcript

had sequence complementarity to the 3' UTR of *lin-14* and recognized that *lin-4* could negatively regulate *lin-14* translation by RNA-RNA interactions[10].

Since then thousands of miRNAs have been identified in many organisms and studies have begun characterizing functional roles. Studies in *Drosophila* have identified important miRNAs like the Bantam miRNA, which stimulates cell proliferation and prevents apoptosis[11]. In recent years, miRNAs have been implicated as important regulators of homeostasis. Of which several studies link miRNAs that serve as neuroprotectors within aging brains[12]. miR-181 and miR-223 have been identified to regulate glutamate receptors, thereby directing the postsynaptic receptiveness to glutamate[13]. Furthermore, miR-485 has been identified to regulate presynaptically, by tuning expression levels of the synaptic vesicle protein SV2A, thus regulating the GluR2 receptor expression pattern[14]. In human studies, comparative analysis in neuroblastoma, cortical development and neuronal differentiation of embryonic stem cells, shows that miR-214 and miR-7 modulate neuronal differentiation and neurite growth[15]. Furthermore perturbed and misregulated miRNA expression patterns have been implied to aid neurodegenerative disorders[16,17].

1.2 *miRNA Biogenesis*

Encoded within the human genome are thousands of miRNAs, many of which are deeply conserved[18, 19]. A common feature among all miRNAs is that they are derived from hairpin structures[20]. However, there are numerous classes of miRNAs; canonical members require RNase III enzyme mediated excision and

processing, while non-canonical members transit through alternate biogenesis pathways[21].

Production of canonical miRNAs occurs after synthesis of primary miRNA (pri-miRNAs) transcripts by RNA polymerase II. Pri-miRNAs are identified by the microprocessor complex component Pasha, which leads to subsequent excision by Drosha, an RNase III domain-containing enzyme[22]. After excision the newly cleaved precursor-miRNA (pre-miRNA) are bound by Exportin-5 and transported to the cytoplasm in a Ran-GTP dependent manner[23]. Once within the cytoplasm another RNase III enzyme Dicer, processes the pre-miRNA to produce an RNA duplex[24]. The RNA duplex becomes incorporated into an Ago protein, one RNA strand, termed the “mature” miRNA, is preferentially loaded into an Ago complex and guides Ago to complementary mRNA targets [25]. The loaded Ago protein is ready to regulate a target transcript’s expression (Figure 1.1)[26].

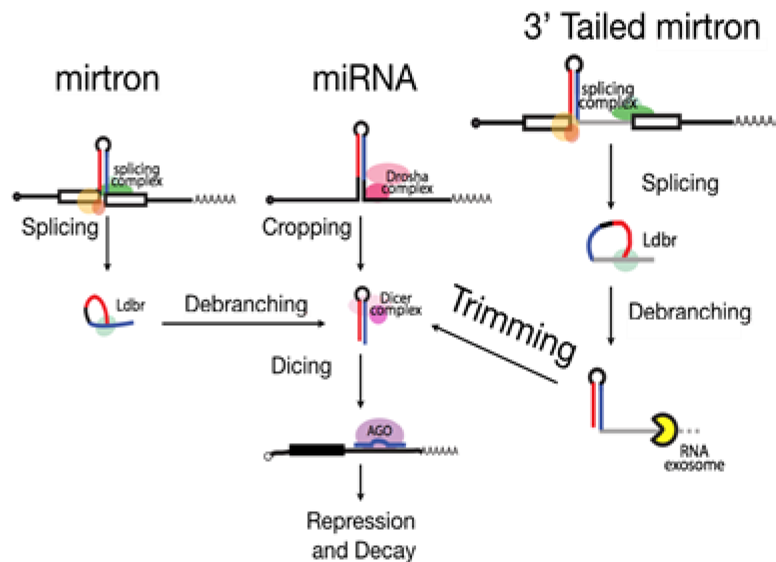

Figure 1.1 miRNA Biogenesis

Canonical and atypical biogenesis pathways of 3 classes of miRNA

One variety of non-canonical miRNA is mirtrons[27]. Mirtrons, which are found within introns, are independent of Drosha cleavage and are produced by the spliceosome[28]. Mirtrons can be further categorized into conventional mirtrons and tailed mirtrons. Conventional mirtrons are short introns between exons, in which the splice sites directly meet the base of both hairpin arms[29]. After splicing and lariat debranching, the hairpin can continue onto Dicer processing and Ago loading (Figure 1.1). Tailed mirtrons possess an extended nucleotide tail attached to one of the hairpin arms, adjacent to either the 5' or 3' splice site.

For tailed mirtron synthesis, a precursor-mRNA (pre-mRNA) must be transcribed by RNA polymerase II[30]. After transcription of the pre-mRNA, the spliceosome is recruited to cleave out introns, at the 5' GU and 3' AG sites, to produce a mature exonic mRNA[31]. Before a hairpin containing intron can continue through the biogenesis pathway they need debranching by the lariat debranching enzyme[32]. Once debranched, mirtrons fold into a hairpin structure and are ready for tail removal. For 5' tailed mirtrons, it is not known whether the tails are cleaved by endonucleases or trimmed by exonucleases. However studies in *Drosophila* showed that 3' tails can be trimmed by an exonuclease, the RNA exosome[33]. After tail removal, the hairpin is dicer-processible and the tailed mirtron continues through the canonical pathway (Figure 1.1).

1.3 *Tailed mirtrons: an unexplored area*

Over the past two decades, identification of miRNAs has become increasingly easier with the use of RNA sequencing tools to collect small RNA reads. Within the human genome, reports have identified 478 mirtron loci, of

which 410 belong to the 5' tailed mirtron class[34]. This non-canonical population of miRNA encompasses a quarter of the miRNA loci in humans. miRNAs are being identified in the thousands for some model organisms and functional roles are being characterized for abundant canonical miRNA species. However despite the large population of tailed mirtrons in human, there has been limited to no research conducted to explore the functional roles of tailed mirtrons.

miR-1017 is a 3' tailed mirtron which resides within an intron of the nicotinic acetylcholine receptor alpha-2 subunit, commonly known as nAchRα2 or Dα2 subunit. Interestingly, the TargetScan algorithm, a miRNA-mRNA targeting database, predicts miR-1017 can regulate its own host gene, Dα2. Amongst all the annotated tailed mirtrons, miR-1017 is by far the most highly expressed and highly conserved, being present in all 12 of the sequenced *Drosophilids*[35]. Due to the high expression level and deep species conservation of miR-1017, we hypothesize that miR-1017 has a significant role in *Drosophila* biology. Therefore miR-1017 is a prime candidate to explore whether tailed mirtrons possess a functional role.

CHAPTER II – Methods

2.1 ***Fly strains***

w^{1118}, Dα2-GAL4 (48952), UAS-mCD8::GFP (32184), mir-1017^{KO}/TM3 (58889), Elav-GeneswitchGAL4 (43642), UAS-ArcAβ42 (33773), UAS-LUC-mir-1017/TM3 (41208) were obtained from the Bloomington stock center.

2.2 ***Construction of luciferase reporters***

microRNA binding sites were PCR-amplified, gel purified and ligated into Psicheck 2 vector (Promega) digested with *Not*I and *Xho*I. The Dα2 ORF fragment and Dα5 UTR fragment were ligated downstream of the *Renilla reniformis* reporter. Dα2-F 5' CTAGGCGATCGCTCGACGAGCTCGCGGCCGCC TCAAAGATCAAATTCTAACCACCAACG 3' and Dα2-R 5' TTATTGCGGCAGC GGCCGACTAGTCTCGAGTTGTTGTAGAGCACGATGTCGG 3'. Dα5-F 5' CTAGGCGATCGCTCGACGAGCTCGCGGCCGCGGATAAGCGAGGCGTTTT CATACAAA and Dα5-R 5' TTATTGCGGCAGCGGCCGACTAGTCTCGAGGGG GAATGTGGCGTAATCACTTAG 3'.

Mutant microRNA binding sites were generated by site-directed mutagenesis using splicing by overlap extension (SOEing) PCR. Two sets of primers were designed to amplify DNA from regions upstream and downstream of the mutated segment. These two PCR products were used in a SOEing PCR with the outside primers to create a product containing the mutated microRNA binding sites. Dα2-Mutant-F 5' GTCACATCTAGGGCTGTGCCATCCGAGCACA TCTG 3' and Dα2-Mutant-R 5' GCACAGCCCTAGATGTGACGCCGCCATACT

CC. Dα5-Mutant-F 5' CAATTTGTTCTAGGGCTACTCGTAGTAGGAAACGTAG GTG 3' and Dα5-Mutant-R 5' GAGTAGCCCTAGAACAAATTGAAGTTTTCCTAA AAGGTTTGG 3'.

2.3 *Luciferase assays*

S2 *Drosophila* cells were plated 1 hour before transfection and cotransfected in quadruplicate with 50μl Grace's insect medium (Gibco), 1μl TransIT-Insect reagent (Mirus), supplemented with 100ng of *Ub-GAL4*, 200ng of *UAS-DsRed-miR-1017*, and 200ng of Psicheck vector. We performed all assays 72 h after transfection with the dual luciferase assay (Promega) on a Synergy H1 microplate reader (BioTek).

2.4 *RT-qPCR*

Total RNA was extracted from ~30 fly heads, per condition, with TRI reagent (MRC). Total RNA was isolated by chloroform phase separation and DNAse treated. cDNA was synthesized by random primers and Revert AID RT (Thermo scientific). RT-qPCR was performed using SYBR green (Thermo scientific). Primers used to assay Dα2 mRNA levels were F 5' AGTACGTGG TCACCACCATGAC 3' and R 5' TCTCACAGCTGGACTTGAAGATGG 3'. Measurements were normalized to ribosomal 18s RNA F 5' CAAAAAGTTGTGG ACGAGGCCAAC-3' and R 5' GTCCGATCACCGAAATTAAGCAGC 3'.

2.5 *Drug feeding*

Donepezil (ACROS organics) was dissolved in water and added at a final concentration of 10μM per gram of standard fly food. Mifepristone (ACROS organics) was dissolved in 100% ethanol and added at a final concentration of

100μM per gram of standard fly food. Food was stored overnight to ensure even distribution of the drug before use.

2.6 *Lifespan assay*

Approximately 100 flies of each genotype were reared at 26°C, under a 12h: 12h light: dark cycle. Groups of approximately 20 flies were collected at eclosion and aged separately. Flies were transferred to fresh food vials every 4 days, and the numbers of dead flies were counted daily.

2.7 *Climbing assay*

Approximately 40 flies of each genotype were placed in an empty plastic vial. Flies were tapped to the bottom and the number of flies within the top, middle, or bottom of the vial were scored after 15 seconds. Experiments were repeated five times, and a representative result shown.

2.8 *Immunocytochemistry*

Adult brains of each genotype were dissected in PBS and fixed in 4% paraformaldehyde for 20 min. Brains were washed five times with PBS supplemented with 0.1% Triton-X100 (PBT). Brains were blocked with PBT and 5% Goat serum for 1 h at 4°C. Brains were incubated in primary antibody, rabbit anti-Caspase 3 (1:100, Cell Signalling, #9603S), overnight at 4°C. Brains were washed three times with PBT. Brains were mounted with DAPI and imaged by confocal microscopy.

CHAPTER III - miR-1017 transcriptional regulation of AchRs

3.1 ***miR-1017 targets Ach receptor subunits***

miR-1017 resides within the 4th intron of Dα2, an acetylcholine (Ach) receptor subunit gene. Predicted targets of interest generated by TargetScan were identified as the Dα2 host transcript and a second nicotinic acetylcholine receptor alpha-5 subunit, known as Dα5 or nAchRα5. The *Dα2* opening reading frame contains a miR-1017 8mer site across 8 of the 12 sequenced Drosophilids, whilst the *Dα5* 3' UTR contains a 8mer site in 9 of the sequenced Drosophilids. To examine whether miR-1017 had the ability to regulate its own host transcript, as well as the Dα5 transcript, we created constructs harbouring either wildtype or mutated sequences for the miR-1017 binding sites and fused each construct to the *Renilla* luciferase coding sequence. The mutant constructs were generated by SOEing PCR to introduce transversional mutations, of purine to pyrimidine and vice versa, to the predicted binding sites (Figure 3.1A). A previously reported *yan* wildtype and double mutant luciferase construct were used as known controls[33]. We cotransfected S2(R+) cells with either wildtype or mutant *Renilla* constructs, and ectopically expressed miR-1017 as it is not endogenously expressed in the S2 culture, using *ub-Gal4 and UAS-DsRed-mir-1017*. We confirmed *miR-1017* could inhibit the *Renilla luciferase* sensors, whilst the mutant constructs exhibit a clear derepression of the *Renilla luciferase* sensors (Figure 3.1B). Revealing miR-1017 can repress the *Dα2* ORF 5 fold (Student's T-test; P < 0.001) and the *Dα5* 3'UTR 2 fold (Student's T-test; P < 0.01).

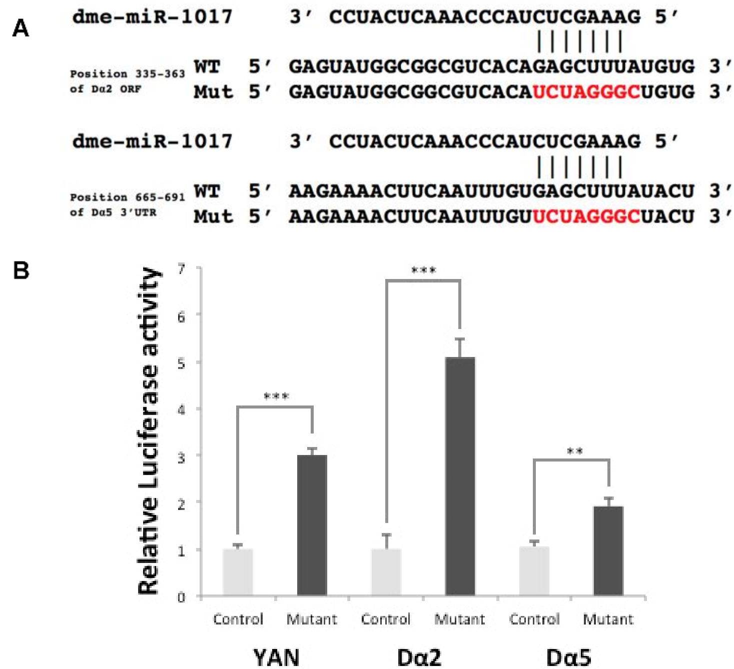

Figure 3.1 Validation of miR-1017 binding sites

miR-1017 acts by regulating acetylcholine receptor transcripts. (A) Predicted target sites for miR-1017 in the Dα2 opening reading frame and the Dα□ 3'-UTR. Red, mutations introduced for mutant *Renilla* constructs. (B) Luciferase reporter assay. Control indicates wild type binding sites for miR-1017. Student's T-test: * and ** indicates <0.001 and <0.01 respectively.**

To examine if miR-1017 regulates similarly *in vivo*, we acquired *mir-1017Δ* flies, which were generated by the Cohen group during their efforts to delete miRNAs from the fly genome[36]. As previously stated, miR-1017 has been identified via northern blotting in the male and female heads[33]. Therefore RNA was extracted from Wild type and *mir-1017Δ* heads, from Day 2 and Day 25 male and female heads. We performed RT-qPCR analysis on Dα2, the host transcript, to see if miR-1017 exerts similar ability *in vivo*. Indeed, the level of the Dα2 mRNA was increased ~5 fold and ~2 fold in RNA isolated from day 2 and day 25 *miR-1017* mutant males respectively, compared to wild type males (Figure 3.2,

Student's T-test; $P < 0.001$, < 0.05). Day 2 *miR-1017* mutant females exhibit a ~1.7 fold increase compared to wildtype (Student's T-test: $P < 0.05$) whilst day 25 *miR-1017* mutant female show no significant difference Figure 3.2).

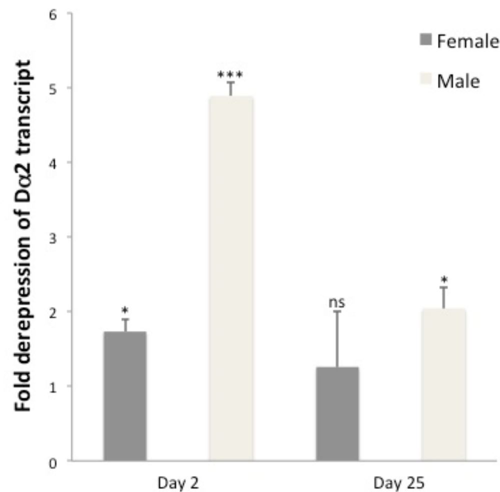

Figure 3.2 RT-qPCR reveals *in vivo* regulation of Dα2 transcript

RT-qPCR revealing *in vivo* derepression of Dα2 transcript levels from *miR-1017* mutant males and females, aged day 2 or 25. Values were normalized against wild type flies. Student's T-test: * and * indicates <0.001 and <0.05 respectively.**

3.2 *Identifying the expression pattern of miR-1017*

To elucidate the expression pattern of miR-1017 we utilized the GAL4/UAS system. The GAL4/UAS system has become an important tool to study gene expression profiles in *Drosophila,* Zebrafish, cell culture and many other organisms[37, 38]. The GAL4/UAS system is widely used in *Drosophila,* with thousands of fly stocks. The system involves two factors: the GAL4 gene, which encodes the yeast transcriptional activator protein GAL4, and an Upstream Activation Sequence (UAS), an enhancer sequence that GAL4 specifically binds to activate transcription of a gene of interest or a reporter gene[39]. In fly lines the

GAL4 gene is frequently fused to tissue specific endogenous gene promoters, which allows for transcription of the GAL4 protein within those specified cells.

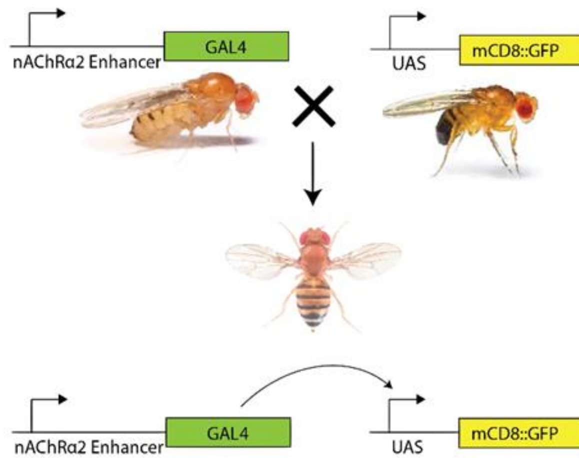

Figure 3.3 Genetic tools to visualize the Dα2 expression pattern

Utilization of the GAL4/UAS system used to visualize the miR-1017 host transcript

Within our system we utilized two fly lines, Dα2-GAL4 and UAS-mCD8::GFP, to visualize specific tissues within the *Drosophila* brain which express Dα2 and therefore miR-1017. The Dα2-GAL4 construct drives GAL4 expression under the control of Dα2 transcriptional enhancer sequences. When activated by GAL4, the UAS-mCD8::GFP transgene transcribes a membrane tethered GFP fusion protein, leading to an easily identifiable Dα2 positive cell. As both Dα2-GAL4 and UAS-mCD8::GFP stocks are homozygous, female Dα2-GAL4 and male UAS-mCD8::GFP flies were collected and crossed together (Figure 3.3). The progeny produced were dissected to inspect GFP expression in the *Drosophila* brain.

Using confocal microscopy we observed GFP positive cells in the suboesophageal (SOG) region, which included both cells within the region and associated surrounding neuropils (Figure 3.4A&B). The SOG region has a role in

processing gustatory signals, in which the region is entangled with sensory neurons, extended from the proboscis and maxillary palp[40]. After identifying GFP labelling within neuropils, we examined whether the expression was isolated to central nervous system (CNS) neurons, or whether expression included projections for the peripheral sensory neurons by examining the proboscis and maxillary palp in 3-day-old pupa. GFP positive cells were identified in both the proboscis and maxillary palp; therefore suggesting miR-1017 may have a role within both the central and peripheral nervous system (Figure 3.4C). Lastly GFP expression was observed within the larval CNS (Figure 3.4D). GFP expression observed within the CNS, which may implicate that miR-1017 could regulate its predicted targets during development of the *Drosophila* head.

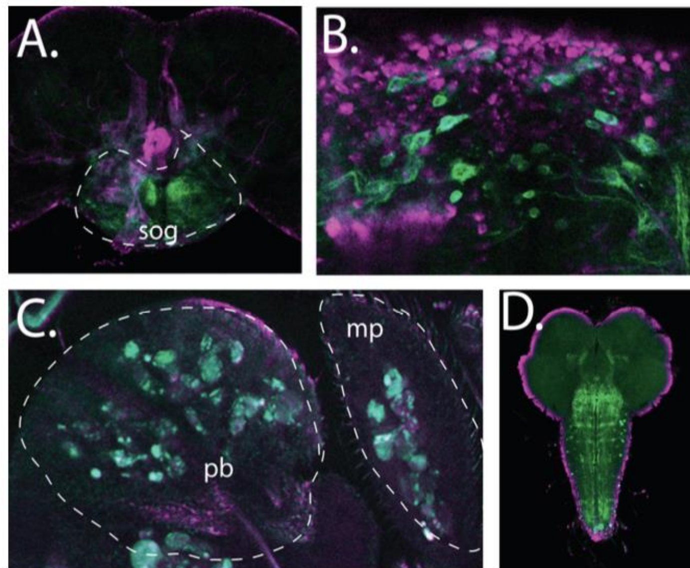

Figure 3.4 Visualization of the Dα2 expression pattern in the CNS

Confocal images showing expression of GFP under Dα2-GAL4 control. A) Expression in the adult CNS in the SOG. B) Magnification of SOG C) Expression in proboscis and maxillary palp in 3 day pupa. D) Expression in larval CNS. DAPI in violet

After examining the Dα2 GFP expression, we speculated whether there might be a phenotypic manifestation in those cells in a miR-1017 null background. To assess whether there is a phenotypic difference we incorporated the GAL4/UAS system within the mutant background (Figure 3.5). To achieve a homozygous system, fly stocks containing balancer chromosomes were used. Balancer chromosomes cannot participate in crossing over events during meiosis, therefore once our desired transgenes have recombined onto the same chromosome they were stabilized within the stock. The balancer chromosomes used in our experiment was MKRS/TM6B, both balancers have dominant marker phenotypes allowing for easy identification throughout the genetic cross.

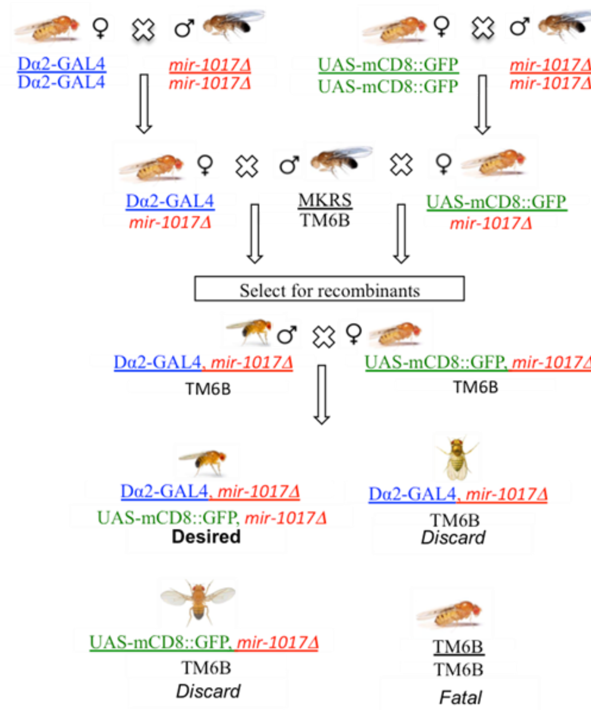

Figure 3.5 Incorporating the GAL4/UAS system into *mir-1017Δ* background

The genetic cross used to establish the GAL4/UAS system within the *mir-1017* null background.

All desired transgenes are located on the 3rd chromosome. Crossing over during meiosis only occurs within female flies; therefore female progeny from the first cross were brought forth to the next cross with the balancer chromosome flies[41]. The *mir-1017Δ* mutant line does not present a phenotypic selective marker; therefore to insure a successful recombinant we performed PCRs taken from DNA extracted from the fly legs. The flies which have the recombinant chromosomes, Dα2-GAL4,*mir-1017Δ* and UAS-mCD8::GFP*,mir-1017Δ,* were crossed together to create our transgenic GAL4/UAS system within the *mir-1017Δ* background.

The wild type females exhibit a low expression of Dα2 GFP, whilst mutant female flies show an increased GFP expression (Figure 3.6). The mutant females had increased GFP suggesting a disruption of a feedback loop, resulting in higher activation of the Dα2 enhancer sequences. As GFP expression is a direct readout of Dα2 transcriptional we sought to determine if the increased GFP patterning was attributed to an increase in Ach receptor activity. To test this we treated wild type females with donepezil, an inhibitor of acetylcholinesterase (AChE). Inhibition of AChE reduces the degradation of Ach and therefore will induce an increased Ach receptor activity. Our findings show the donepezil treated female exerts a high Dα2 GFP expression pattern (Figure 3.6). These findings indicate that Dα2 transcriptional activation may occur by an activity dependent manner, caused by heightened Ach receptor activity.

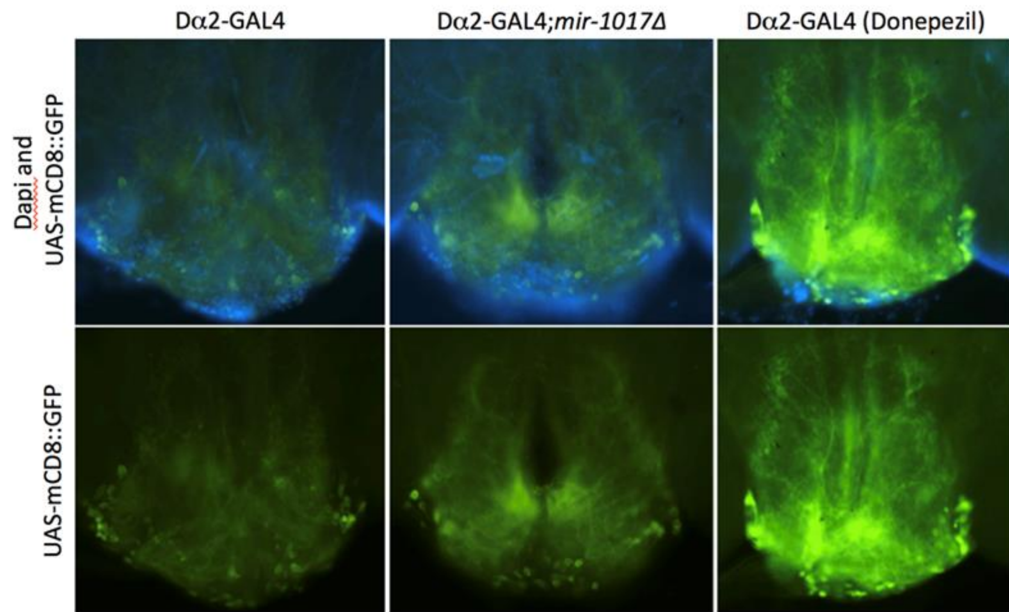

Figure 3.6 Dα2 transcription is regulated by AchR activity

Dα2 GFP expression pattern reveals Dα2 transcriptional activation is dependent on acetylcholine receptor activity.

CHAPTER IV – The neuroprotective role of miR-1017

4.1 ***miR-1017 neuroprotective role within Drosophila***

Previous studies of Alzheimer's disease (AD) fly models have shown elevated levels of miR-1017[42]. One of the defining features of AD is increased firing of background action potentials[43]. Furthermore increased receptor activity can cause reactive oxygen species to develop, which subsequently leads to neuronal cell death[44]. These Ach receptors have become a focal point for pharmaceutical drug therapies, including Donepezil an AChE inhibitor, in an attempt to prevent or dampen AD symptoms[45]. With our knowledge that miR-1017 regulates Ach receptor activity, we began to hypothesize if the elevated miR-1017 could be a cellular attempt to repress the receptor activity. Therefore to test whether miR-1017 has a role in preventing a neurotoxic state we performed neurological assays with *mir-1017Δ* and wild type flies.

To assess whether male and female *miR-1017* mutant flies undergo neurodegeneration, we performed a lifespan analysis of male (n=151) and female (n=150) mutant flies, comparing them to wild type flies (n=135), and monitored the rate of death daily. *mir-1017Δ* males showed a sharply reduced adult lifespan, with survival declining rapidly between days 20-30. *mir-1017Δ* females exhibit a reduced adult lifespan, but not as severe as the male mutants (Figure 4.1, Cox proportional hazard ratio test; $P < 0.001$ comparing wild type, *miR-1017Δ* male and female).

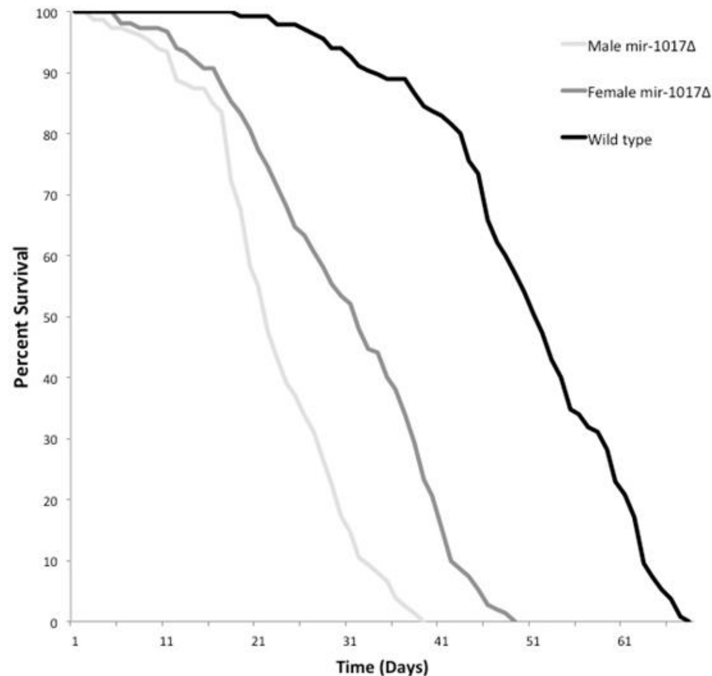

Figure 4.1 *miR-1017* mutant reduced lifespan

Male and female *miR-1017* mutants demonstrate a severely reduced lifespan compared to wild type. Cox proportional hazard ratio test; *P* <0.001 comparing wild type, *miR-1017Δ* male and female.

Furthermore *miR-1017Δ* mutants present an early-onset neurological deficit when compared to wild type. By day 20, 50-65% of male mutants (n=47) were impaired in their performance in a climbing assay (Figure 4.2, ANOVA: *P* <0.001), and 40-50% female mutants (n=48) were impaired compared to wild type (n=58) (Figure 4.2, ANOVA; *P* < 0.05). Male mutants show a greater deficit compared against female mutants (Figure 4.2, ANOVA; *P* <0.01). On day 30 both mutants exhibit severe neurological deficits with males (100%) and females (87.5%) impaired climbing ability compared to wild type (Figure 4.2, ANOVA, *P* < 0.001). Furthermore male mutants climbing ability is poorer than the female mutants (ANOVA; *P* <0.01). Reduced adult lifespan and neurological deficits are characteristics of *Drosophila* models of neurodegeneration[46].

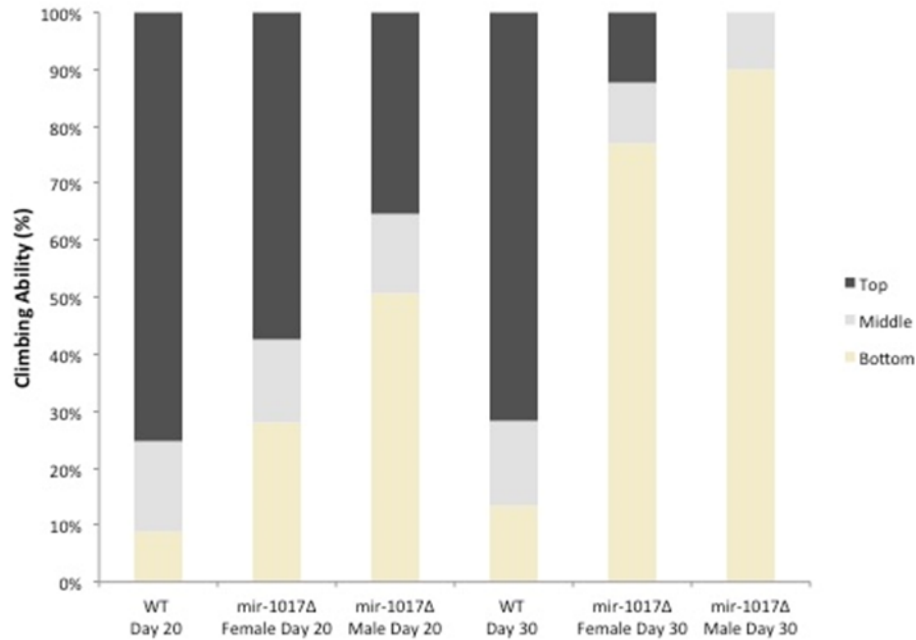

Figure 4.2 *miR-1017* mutants are neurologically impaired

Climbing assay analysis reveals that neurological deficits arise early in *miR-1017* mutant males and females, suggesting an early-onset neurodegeneration.

Moreover we used an antibody against the active form of Caspase 3, to identify whether *mir-1017* mutants were undergoing apoptotic cell death. Both male and female *mir-1017* mutants have a greater population of apoptotic cells by day 30 compared to the wild type (Figure 4.3). These findings suggest that *miR-1017* mutants are presenting an excitotoxic neurodegeneration.

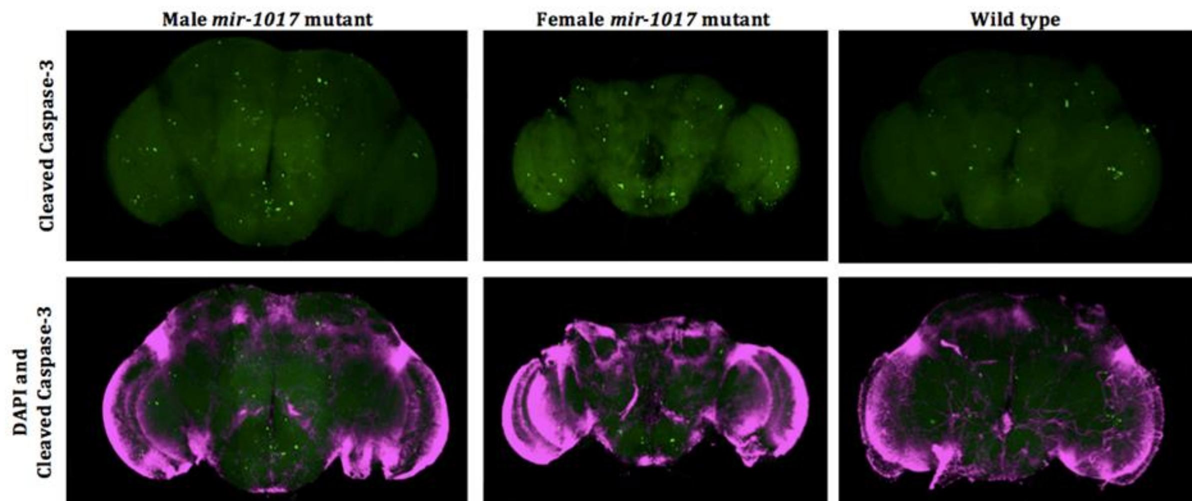

Figure 4.3 *miR-1017* mutants exhibit an increased amount of apoptosis

Caspase-3 antibody stain on day 30 presents a higher population of apoptotic cells in *miR-1017* mutants compared to wild type.

4.2 *Neuroprotection by miR-1017 within a neurodegeneration model*

To test whether miR-1017 has a neuroprotective role against an excitotoxic state we ectopically drove miR-1017 in an AD fly model and compared it to the control group, to do this we utilized the GAL4/UAS system. To establish a standardized life expectancy for the AD fly model, two fly lines were utilized, Elav-Geneswitch:GAL4 and UAS-ArcAβ42. Elav is a protein exclusively expressed within neurons, making GAL4 expression localized only to neuronal cells. The Geneswitch system utilizes the fusion of the ligand-binding domain of the human progesterone receptor with the transcriptional activation domains of GAL4[47]. The Geneswitch system was chosen to allow for inducible expression of the AD transgene after eclosion. Therefore activation of GAL4 required induction by the steroid mifepristone, which was easily introduced to the adult flies' diet[48].

To ectopically drive miR-1017 in AD fly model a homozygous AD transgenic stock had to be created. To achieve this we used the SpHid;LgHid/Sm;TM6B balancer chromosome stock (Figure 4,4). SpHid;LgHid/Sm;TM6B stock is second (Sm) and third chromosomal (TM6B) balancers, which contains a copy of the heat shock activated Hid genes, which is a pro-apoptotic protein[49]. UAS-ArcAβ42 (2nd chromosomal) and Elav-Geneswitch:GAL4 (3rd chromosomal) were crossed, the desired transgenes were on separate chromosomes therefore gender of the selected progeny was unimportant. Progeny was crossed with SpHid;LgHid/Sm;TM6B, the resulting progeny were heated shocked to select for flies in possession of Sm;TM6B. Progeny produced that were carriers of both transgenes have an increased depth of phenotypic red eye therefore those flies were selected for crossing to UAS-miR-1017.

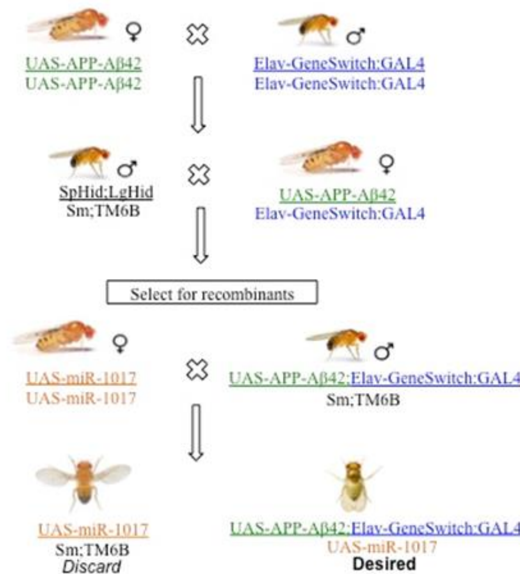

Figure 4.4 Introducing ectopically driven miR-1017 into an AD fly model

The genetic cross used to implement ectopically driven miR-1017 in an AD fly model

To understand whether ectopically driven miR-1017 could dampen or rescue the AD phenotype, we performed a lifespan analysis. Indeed miR-1017 improved survival of the AD fly model (Figure 4.5, Cox proportional hazard ratio test; $P < 0.001$). The AD fly model (n=129) severely declined between days 45-55, whilst the miR-1017-AD fly model (n=163) exhibited a declining lifespan between days 50-60. However 25% of miR-1017-AD flies surpassed the lifespan of the AD fly model. Both induced AD lines were significantly different to their uninduced control counterparts (Figure 4.5, Cox proportional hazard ratio test; P <0.001).

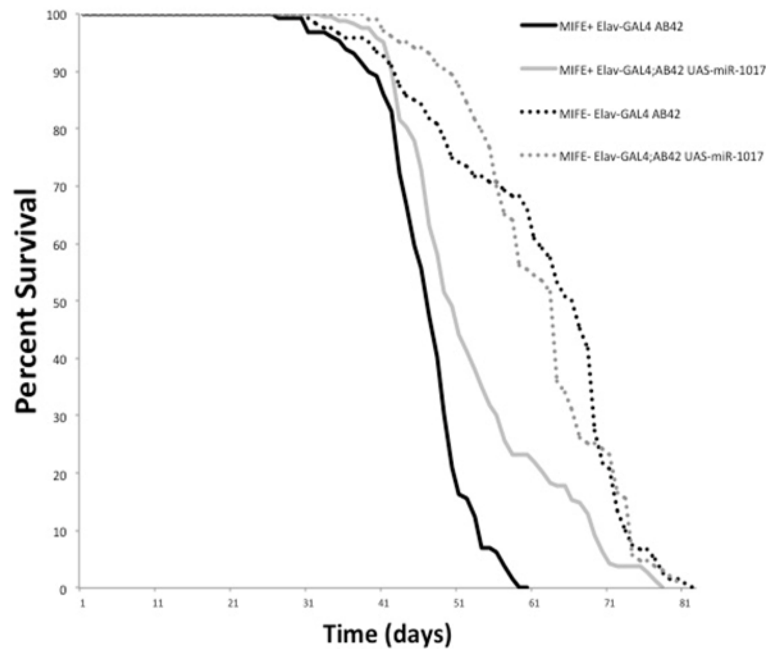

Figure 4.5 Ectopic miR-1017 extends lifespan of AD fly model

Lifespan analysis presents an extension of lifespan of AD flies when miR-1017 is ectopically driven. Mife+/- indicates mifepristone induction/uninduced. Cox proportional hazard ratio test; *P* <0.001 comparing AD against miR-1017 AD fly model's.

Some studies have shown that transgenes can alter lifespan, however our uninduced lines show no reduced lifespan, of which their comparable lifespan is not significantly different (Figure 4.5, Cox proportional hazard ratio test; $P = 0.71$)

Furthermore ectopically driven miR-1017 can rescue climbing ability of the AD fly model. Day 25 genotypes performed well in the climbing assay. However the AD fly model (n=43) exhibited a slight reduction in climbing ability with approximately 20% of flies showing neurological deficits compared to the miR-1017-AD fly model (Figure 4.6, ANOVA; $P < 0.01$). By day 45, the AD fly model presented severe neurological deficits, with 94% of flies performing poorly in the climbing assay (Figure 4.6, ANOVA; $P < 0.001$). In comparison 55% of the miR-1017-AD fly model performed poorly in the climbing assay (Figure 4.6, ANOVA; $P < 0.001$ compared to the AD fly model). Neither uninduced genotype performed poorly, and were not significant different (Figure 4.6, ANOVA; $P = 0.27$). These findings indicate that miR-1017 can dampen the symptoms exhibited from an excitotoxic neurodegenerative state. Further supporting the role of miR-1017 as a cellular response to regulate neurological receptor activity.

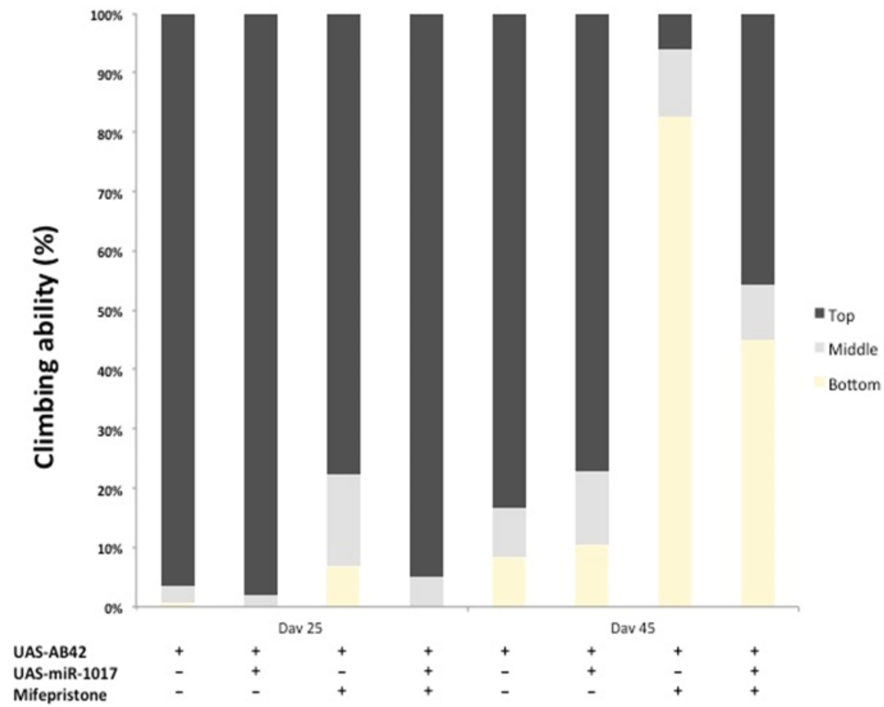

Figure 4.6 Ectopic miR-1017 improves neurological function

Climbing assay analyses demonstrate a deterioration of the AD fly model climbing ability, which ectopic miR-1017 improves the climbing ability of the AD fly model.

CHAPTER V – Discussion

Despite the high population of tailed mirtron loci within the human genome, no research has been reported that examines the roles of these miRNAs. The reason for the lack of research conducted may be attributed to the fact that mirtrons are not highly expressed or well conserved. However this miRNA species may in fact give rise to species-specific evolution. There are numerous abundant miRNAs that are broadly expressed across animals because they are essential for development, however mirtrons seem to be better conserved amongst close relatives[50]. When we examine *Drosophila* miR-1017, we can clearly see that the 3' hairpin arm, is deeply conserved. When considering how miR-1017 became a functionality important miRNA within the *Drosophila* species, it's worth noting that we have identified it regulates AchR activity. Ach is the neurotransmitter in the CNS for insects, whilst in animal's glutamate is the primary CNS neurotransmitter[51]. Therefore when diverging from other insects, *Drosophila* may have incurred a mutation which gave rise to the birth of miR-1017. Whilst we know that *miR-1017* mutants are viable and produce offspring, they exhibit a reduced lifespan and neurological deficits. Therefore the birth of miR-1017 may have given rise to a well-maintained neurotransmission and extended lifespan, which would improve fitness and benefit the evolution of the *Drosophila* species. Using *Drosophila's* miR-1017 as a candidate, we have for the first time identified the function and phenotype of one of these miRNA.

Of the 241 predicted binding sites of miR-1017, 86.7% of the predicted targets are expressed within *Drosophila* neurons (GSE1060). Of the 209

predicted target genes, 18 (8.6%) of those are known to be involved in neurotransmission. Our findings suggest that miR-1017 acts to regulate Ach receptor transcripts and thereby modulating Ach synaptic transmission. Luciferase assays and RT-qPCR data revealed derepressed transcript levels in the *miR-1017* mutant constructs and flies, therefore identifying Dα5 and Dα2 as bona fide targets of miR-1017 (Figure 3.1&2). The GFP expression pattern of Dα2 transcription indicates males have highly active transcription of the Dα2 transcript (Data not shown). When considered with the qPCR data which revealed a near 5 fold depression of the Dα2 transcript in the male mutants, we can hypothesize that transcription in males is much higher, and that Dα2 is being regulated post-transcriptionally by miR-1017. In comparison, the females exhibited low GFP patterns, suggesting a much lower activation for Dα2 transcription. The mutant GFP phenotype presented a slightly higher Dα2 GFP expression pattern, consistent with the qPCR data, which may suggest disruption of a feedback loop. Our hypothesized mechanism is that increased Dα2 transcript levels and therefore Ach receptors, results in an increase in Ach neurotransmission, which may in turn promotes transcription of the Dα2 transcript. This was supported by our donepezil treatment, which phenotypically showed that increased Ach receptor activity induced by donepezil increased the Dα2 GFP pattern, presenting a higher Dα2 transcriptional activation (Figure 3.6).

This could be further supported by the phenotypic observations made in the *miR-1017* mutants, which exhibit a neurodegeneration. Both male and female

miR-1017 mutants had a reduced lifespan, performed poorly in the climbing assays and presented a higher number of apoptotic cells compared to wild type controls (Figure 4.1&2). Furthermore the male *miR-1017* mutants display a greater neurological deficit than females, potentially due to differentiate transcriptional expression of Dα2. Our phenotypic studies support our hypothesis that miR-1017 serves as a neuroprotective miRNA.

In *Drosophila,* another neuroprotective miRNA has been identified as miR-1000. miR-1000 modulates VGlut mRNA levels, in turn regulating glutamate synaptic activity. Interestingly they hypothesized that miR-1000 expression was an activity-dependent mechanism, in which photoreceptor activity regulated miR-1000 expression[52]. With this in mind, we could hypothesize that miR-1017 expression is controlled by an activity dependent mechanism. Our *miR-1017* mutant study has revealed an increase in Ach receptor transcripts, which appears to be caused by increased Ach receptor activity, leads to an excitotoxic state. However if we consider that a destabilization or an increase in Ach neurotransmission in a wild type model would lead to transcription of Dα2 and thereby miR-1017. The expression of miR-1017 can then act post transcriptionally to down regulate Ach transcripts, and therefore balance Ach receptor activity, thereby suggesting an activity dependent mechanism (Figure 5.1). When we treated the GAL4/UAS wild type females with donepezil, an inhibitor of acetylcholinesterase, we observed a higher GFP expression, which indicates that Dα2 transcription is induced by the increased AchR activity caused by the donepezil treatment.

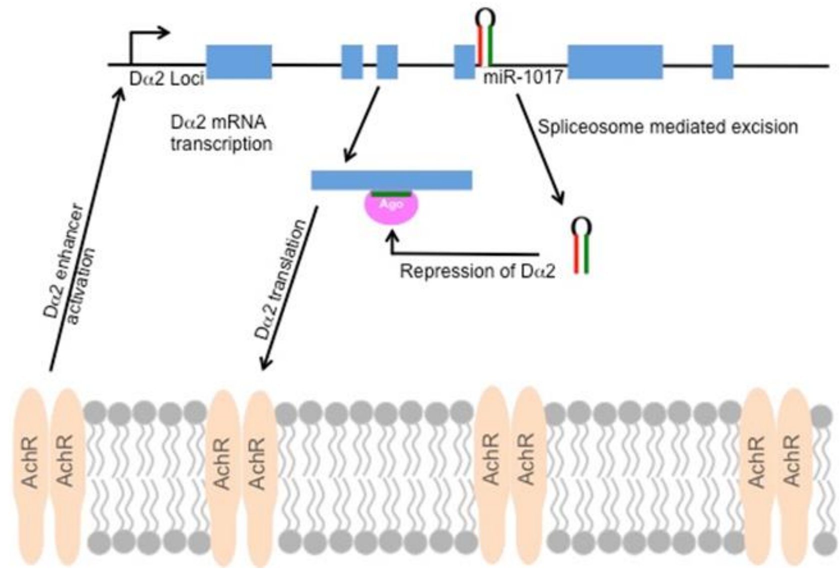

Figure 5.1 Proposed mechanism for AchR transcription

Proposed mechanism for AchR transcription (Positive feedback loop) and miR-1017 post-transcriptional regulation of AchR transcripts (Negative feedback loop).

One of the most common neurodegenerative diseases is Alzheimer's disease. The commonly associated pathology is the aggregation of amyloid-β plaques in the synaptic cleft[53]. Amyloid-β plaques cause a neurotransmitter dysregulation, which induces an increased firing of background action potentials[54]. The increased ion flux associated with spurious channel activity results in reactive oxygen species, which in turn induces neuronal apoptosis[55]. Pharmaceutical options to treat AD include Donepezil (AChE inhibitor) and Memantine (glutamate receptor inhibitor), these drugs attempt to improve the neurological integrity of AD patients, however no pharmaceutical drug has been proved to be curative. To be understand AD, creation of an AD fly model has allowed for an *in vivo* examination of protein-protein interactions and a better understanding of unknown AD mechanisms[56]. Interestingly, when we ectopically

expressed miR-1017 in an AD fly model, we were able to alleviate the pathogenesis, therefore supporting the repressible activity of miR-1017 in an excitotoxic state (Figure 4.5&6). Despite presenting a reduced lifespan and poor climbing ability, the AD fly model did not present a Caspase-3 phenotype. This may suggest that the ectopically driven Aβ42 plaques may induce a caspase independent apoptosis[57].

To conclude, the importance of this study has elucidated a class of miRNA that has yet to be studied. Tailed mirtrons, which encompass a quarter of the miRNA loci in human, have previously been overlooked. Here we demonstrate a phenotype and role of a 3’ tailed mirtron within *Drosophila,* therefore identifying their significance as a miRNA.

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