A microRNA cluster negatively regulates synaptic function at *Drosophila* larval neuromuscular junction

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Abstract

Proper synaptic growth and plasticity are critical for higher brain functions including memory and learning. We are interested in identifying and characterizing mechanisms that underlie the regulation of morphological and functional synaptic plasticity. To that end, we conducted a forward genetic gain-of-function screen aimed at identifying novel regulators of synaptic growth, using *Drosophila* larval neuromuscular junction (NMJ) as a model synapse. From this screen, we identified a well-conserved microRNA cluster, miR-310-313, whose expression affects synaptic plasticity at *Drosophila* NMJ. We generated a deletion of this miRNA cluster by imprecise excision of a P-element. Our results show that lack of miR-310-313 leads to a significant increase in evoked post-synaptic current and in quantal content, a measure of the efficiency of presynaptic neurotransmitter release. This phenotype can be rescued by overexpressing miR-310-313 during larval stage in nervous system, suggesting the temporal and tissue-specific requirement of this miR-310-313 cluster.

miRNAs repress expression of their targets via base-pairing with the 3’UTR of their mRNAs. Computational algorithms predicted Khc-73, a member of Kinesin Superfamily proteins (KIFs), as one of the highest-scoring targets for miR-310-313. We hypothesize that miR-310-313 cluster regulates synaptic function by downregulating Khc-73. In support of our hypothesis, overexpressing Khc-73 in *Drosophila* motoneurons enhances synaptic function, a phenotype resembling miR-310-313 deletion. Further evidence shows that genetic removal of Khc-73, by using Khc-73 RNAi or by a deficiency uncovering Khc-73 genomic region, is sufficient to restore normal...
synaptic function in miR-310-313 deletion. Taken together, our data present a novel pathway by which synaptic plasticity is regulated.
Résumé

La croissance et la plasticité synaptiques sont des éléments critiques pour le bon fonctionnement du cerveau, et participent aux processus de la mémoire et l'apprentissage. Le but de nos recherches est d’identifier et de caractériser les mécanismes moléculaires qui sous-tendent le règlement de la plasticité synaptique morphologique ainsi que fonctionnelle. À cette fin, nous avons élaboré un crible d'augmentation-de-fonction génétique dont le but était d'identifier de nouveaux régulateurs de croissance synaptique, en utilisant la jonction neuromusculaire (JNM) larvaire de la mouche drosophile (*Drosophila melanogaster*) comme synapse modèle. Grâce à ce crible, nous avons identifié un groupe de microARN (miARN) bien conservé au niveau évolutionnaire, miR-310-313, dont l'expression affecte la plasticité synaptique à la JNM de la drosophile. Nous avons produit une délétion de ce groupe de miARN par l'excision imprecise d'un élément génétique mobile (*P-element*). Nos résultats démontrent que la perte de miR-310-313 entraîne une augmentation significative du courant post-synaptique évoqué et du contenu quantique, une mesure de l'efficacité de la libération de neurotransmetteur présynaptique. Ce phénotype peut être rescapé en surexprimant miR-310-313 pendant le stade larvaire du système nerveux, suggérant une exigence d'expression génétique temporelle et spatiale spécifique pour ce groupe de miARN. Les miARN répriment l'expression de leurs cibles par appariement de bases avec le 3'UTR de leur mARN. Des algorithmes quantificatifs ont prédit Khc-73, un membre de la superfamille des protéines Kinesin (KIFs), comme une des cibles les plus plausibles pour miR-310-313. Nous émettons l'hypothèse que le groupe
miR-310-313 régule la fonction synaptique en réprimant l’expression de Khc-73. Dans le soutien de notre hypothèse, nous démontrons que la surexpression de Khc-73 dans les neurones moteurs de la drosophile améliore la fonction synaptique, un phenotype ressembling à la délétion de miR-310-313. De plus, la répression génétique de Khc-73, en utilisant soit Khc-73-RNAi ou une délétion couvrant la région génomique qui contient le gène Khc-73, est suffisante pour restituer la fonction synaptique normale en l'absence de miR-310-313. Ensemble, nos données présentent un nouveau sentier génétique par lequel la plasticité synaptique est régulée.
CHAPTER I : INTRODUCTION
1.1 Synaptic plasticity

Neurons communicate with one another via synapses, specialized structures that are the site of neurotransmitter release. Once synapses form, they undergo continuous expansion, retraction and remodelling throughout all stages of development. Synaptic structure and function are dynamically tuned to ensure stable function in neuronal circuits in response to intracellular and extracellular cues. This ability of synaptic connections to continuously undergo modification, also known as synaptic plasticity, is thought to underlie higher brain functions such as learning and memory. Despite increasing evidence of the importance of changes in synaptic morphology and function, we still know relatively little about the molecular building blocks that govern synaptic growth and plasticity.

This project was designed to identify genes that participate in the regulation of synaptic plasticity. For these studies, I used the powerful genetic and molecular tools available in *Drosophila* larval neuromuscular junction.

1.2 *Drosophila* larval Neuromuscular Junction

*Anatomy and accessibility:* Fruit flies possess the powerful tools for temporal and tissue specific expression of transgenes. This advantage together with the ease by which mutant flies can be generated, as well as their short generation cycle make fruit flies an ideal model system for identifying and characterizing novel genes. The glutamatergic larval NMJ synapses share many presynaptic and postsynaptic molecules and structures with most excitatory synapses in
mammalian central nervous system. Discoveries made using *Drosophila* larval neuromuscular junction can be readily applied to vertebrate systems (Collins and DiAntonio 2007). In particular, the neuromuscular junction (NMJ) synapses of *Drosophila* larva have been used effectively to study the role of synaptic molecules. The larval abdominal segments contain repeated pattern of 30 muscles that are innervated by about 40 motor neurons (Gramates and Budnik 1999). This innervation pattern is stereotypic across each segment, in particular segments 2-7, making each NMJ readily identifiable. The corresponding NMJ can then be studied from one single fly to another, facilitating more consistent comparison among experimental samples. In addition, the fly NMJ is accessible to immunohistochemistry, electrophysiology, electron microscopy and live imaging, allowing the study of its functional properties as well as its morphological ultrastructure (Keshishian, Broadie et al. 1996).

*Homeostatic synaptic growth: Drosophila* larval NMJ shows structural and functional plasticity. After synaptogenesis in embryonic stage, as the larva grows from a first to a third instar larva over a period of about five to seven days, the surface area of the postsynaptic muscle increases as much as a 100-fold. During this period, the presynaptic nerve terminal grows in parallel to maintain synaptic efficacy, resulting in increases in the number of boutons and the number of active zones per bouton. Several studies have revealed the presence of orthograde and retrograde mechanisms that control this coordinated synaptic growth (Haghighi, McCabe et al. 2003; Collins, Wairkar et al. 2006; de Bivort, Guo et al. 2009; Matthias Fischer 2009).
**Gal4-UAS system**: Studying synaptic growth and plasticity at the larval NMJ benefits from many genetic and molecular tools available in *Drosophila*. Perhaps one the biggest advantages for studying the function of genes in *Drosophila* is the availability of the Gal4-UAS system (Brand and Perrimon, 1993). This system is based on transgenes that contain binding sites for the yeast transcriptional activator GAL4 in their promoter (Upstream Activation Sequence, UAS) region. Crossing flies carrying such transgenes with Gal4 expressing flies will allow for tissue- or cell-specific expression of the transgene.

In summary, *Drosophila* larval neuromuscular junction has served as an excellent model to study synaptic growth and modifications (Young Ho Koh 2000; Keshishian and Kim 2004; Collins and DiAntonio 2007).

**1.3 Objectives and hypothesis**

Aiming to identify novel regulators of synaptic plasticity, I conducted a gain-of-function forward genetic screen, using the *Drosophila* larval NMJ as my research system. From this screen, I identified a miRNA cluster, miR-310-313, which regulates synaptic function. MiR-310-313 targets *Drosophila* Khc-73, a member of kinesin superfamily proteins (KIFs). I hypothesize that miR-310-313 regulates synaptic function by downregulating the expression of Khc-73. In the following parts of the introduction I will present a detailed overview of miRNA. In chapter III, I will explain in-depth the evidence supporting my hypothesis.
1.4 Overview of miRNA

MiRNAs are small, non-coding RNAs that are present in diverse multicellular organisms from worms to mammals. They are key regulators of important developmental processes such as developmental timing, cell fate determination and apoptosis (Ambros 2004). Mature miRNAs are 21-22 nucleotides long that upon binding target mRNAs can lead to translational repression or degradation of the transcript. MiRNAs interact with the 3’ untranslated regions (3’-UTR) of target mRNAs by base-pairing (Lee, Feinbaum et al. 1993; Wightman, Ha et al. 1993; Reinhart and Slack 2000).

The first miRNA, lin-4, was discovered in 1993 through genetic studies of cell lineages in *C. elegans* (Lee, Feinbaum et al. 1993). *lin-4* does not code for a protein but instead produces a pair of small RNAs. Worms that lack lin-4 repeat developmental events specific to the first larval stage (L1) at later stages of development, a phenotype that could be suppressed by mutation in its target, *lin-14* mRNA. Interestingly, *lin-4* nucleotide sequence was found to be complementary to multiple sites in the 3’UTR of *lin-14* (Lee et al, 1993, Wightman et al, 1993). These studies formed the foundation of miRNA dependent gene regulation as a mechanism that was later discovered to be conserved from worms to humans (Lai 2005; Bushati and Cohen 2007).

Since the discovery of the founding members of miRNAs in *C. elegans*, many laboratories have identified and characterized numerous miRNAs in different species (Bartel 2004; Lim, Lau et al. 2005; Flynt and Lai 2008; Bartel 2009). Computational methods have been developed to identify the targets of
miRNAs, providing insight into miRNA functions based on the characterized roles of their targets (Lewis, Shih et al. 2003). Though miRNAs constitute one of the most prevalent classes of gene regulation in higher eukaryotes, for the vast majority of miRNA, the phenotypic consequences of loss of miRNA are not known. In many cases, several miRNAs target the same transcript; therefore, loss of one miRNA can be compensated by the others (Lai 2003). In addition, often miRNAs are involved in fine-tuning gene expression in a spatial and temporal specific manner, making it difficult to assess the consequence of their loss.

1.4.1 Biogenesis of miRNA

Most miRNA genes are found in regions distant from annotated genes. These miRNAs are transcribed from their own promoters. Some miRNAs are located within introns of pre-mRNAs. Transcriptions of these miRNAs are likely to be controlled by the promoters of the host mRNAs. Other miRNAs genes are clustered in the genome. These clustered miRNAs are often functionally related and co-transcribed as a single transcript (Bartel 2004).

Most miRNA genes are transcribed by RNA polymerase II to produce a stemloop intermediate which contains primary miRNA (pri-miRNA). This pri-miRNA is capped at its 5’ end and is polyadenylated at its 3’ end. It is then cleaved by the RNase III endonuclease Drosha and the double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha to generate a ~70-nt hairpin precursor miRNA (pre-miRNA). This pre-miRNA is then exported into the cytoplasm by Exportin-5. In cytoplasm, it is further cleaved by
another RNase III endonuclease, Dicer, to produce a ~21-nt miRNA:miRNA* duplex. The miRNA strand then incorporates in an Argonaute-containing RNA-induced silencing complex (RISC), whereas the miRNA* strand is degraded (Bushati and Cohen 2007).

1.4.2 Mechanism of miRNA function
Once bound to RISC, the mature miRNA directs the RISC complex to its target mRNA via base-pair recognition (Bushati and Cohen, 2007). The 5’ end of a miRNA contains the “seed region” which is critical for base-pairing with the 3’UTR of its target mRNAs. The seed region corresponds to the first 2nd to 8th residues of the miRNA and is most conserved among homologous metazoan miRNAs (Bartel 2004). In the case of perfect base-pairing, which is mostly the case in plants, target mRNAs are degraded. Most animal miRNAs, however, imperfectly base-pair with their targets, this results in translational repression instead. The target mRNAs might be destabilized by deadenylation and decapping in this case, but the precise mechanism of translational repression is not clear (Bushati and Cohen 2007).

1.4.3 miRNA expression pattern
miRNAs show time-specific and cell-specific expression patterns. For example, lin-4 acts early in C. elegans larval stage to ensure specific events take place during the first larval stage (L1) (Lee, Feinbaum et al. 1993). miR-1, a conserved miRNA, is primarily expressed in mammalian heart tissues (Lagos-Quintana, Rauhut et al. 2002) and miR-124 is a highly brain-specific
miRNA (Lagos-Quintana, Rauhut et al. 2002). Therefore at each cell type there is a distinct miRNA expression profile at each stage of development.

It has been suggested that many miRNAs show a non-overlapping expression pattern with their targets, both temporally and spatially. This mutually exclusive strategy might be the result of switching off the expression of targets in miRNA-expressing cells, thereby helping to maintain and determine cell types by repressing unwanted transcripts (Bushati and Cohen 2007). In contrast, some miRNAs co-express with their targets to fine-tune their expressions. In this case, residual level of target expression has a specific function. MiRNAs therefore function to ensure the expression of its target stays optimal (Bushati and Cohen 2007).

1.4.4 miRNAs function in nervous system

Microarray analyses have revealed the abundance of miRNAs in the nervous system in different species (Lagos-Quintana, Rauhut et al. 2002; Krichevsky, King et al. 2003; Kim, Krichevsky et al. 2004). Brain-specific miRNAs were shown to play various roles in different aspects of nervous system development. MiRNAs are also suggested to be involved in regulating higher brain functions such as memory (Gao 2008; Corbin, Olsson-Carter et al. 2009).

MiRNAs are required for forming neurons and establishing cell identity: In Drosophila, miR-9a, a brain-specific miRNA, targets senseless to ensure sensory organ precursors (SOPs) specification. mir-9a mutant embryos and
adult flies show extra sensory neurons as a result of an increased production of sensory organ precursors (SOPs) (Zordan, Cisotto et al. 2006). In *C. elegans*, Lsy-6 is required for asymmetric specification of chemosensory neurons (Chang, Johnston et al. 2004). Lsy-6 is expressed in ASEL (ASE left) neurons. Loss of Lsy-6 results in loss of Gcy-7, a ASEL-specific chemoreceptor, as well as an ectopic expression of ASER-specific chemoreceptor Gcy-5 in ASEL neurons, resulting in two ASER neurons (Johnston and Hobert 2003).

**MiRNAs are required for neuron survival:** Mutant mir-8 flies have elevated Atrophin protein in the nervous system, which in turn leads to increased neuronal apoptosis in CNS and defects in motor activities (Karres, Hilgers et al. 2007). *Bantum* mutant flies fail to suppress neurodegeneration resulted from PolyQ toxicity (Bilen, Liu et al. 2006).

**MiRNAs are required for maintaining the differentiated identity of mature neurons:** In nonneuronal cells and progenitor cells, mammalian miR-124 expression is suppressed by RE1 silencing transcription factor (REST). REST is, however, absent in mature neurons, allowing miR-124 to suppress nonneuronal transcripts (Conaco, Otto et al. 2006).

**MiRNAs are required for regulating dendritic dynamics:** miR-134, a brain-specific miRNA, localizes at mammalian dendritic synapses. Schratt *et al* have shown that overexpression of miR-134 leads to reduced spine volume, whereas reduction of endogenous miR-134 by introducing a 2’-O-methylated antisense oligonucleotide results in an opposite phenotype. miR-134 affects
spine morphology by inhibiting Lim Kinase 1, which is known to affect actin filament dynamics (Schratt, Tuebing et al. 2006).

miRNAs participate in higher brain functions: The mammalian homolog of CaMKII is synthesized at synapses and required for memory formation. In Drosophila, transport of CaMKII mRNA and protein synthesis also exhibit memory specific features. The 3’UTR of CaMKII mRNA is necessary for mRNA localization in synaptic punctae, where CaMKII mRNAs localize with Armitage, a RISC component. The 3’ UTR of CaMKII mRNA contains the binding motifs for miR-280 and miR-289, indicating their potential involvement in the synaptic expression of CaMKII (Ashraf, McLoon et al. 2006).

Taken together the above studies from Drosophila, C. elegans and mammalian models, it is evident that miRNA are key regulators in developing and mature nervous system. Despite so, very little is known about miRNAs participation in NMJ growth.

1.4.5 miRNAs at the neuromuscular junction

At present, there are only two findings that indicate miRNA involvements at the neuromuscular junction. In Drosophila, the let-7 cluster, which expresses both pre and post-synaptically, affects NMJ size and abdominal muscle phenotypes (Sokol, Xu et al. 2008). In C. elegans, Simon et al demonstrated that in mir-1 mutant worms, the expressions of two nicotinic acetylcholine receptor subunits, UNC-29 and UNC-63 were increased, thereby reducing
synaptic function at NMJs. miR-1 also controls a retrograde signal from muscle to presynaptic terminus by targeting a muscle-specific transcription factor, MEF-2. This retrograde signal alters presynaptic acetylcholine secretion, thereby altering synaptic function (Simon, Madison et al. 2008). Despite these two findings have given us insights into the importance of miRNAs in NMJ morphology and function, nonetheless, a requirement for miRNAs in presynaptic neurons in regulation of neurotransmitter release has not been directly demonstrated. The finding presented in this thesis therefore represents the first evidence of the roles of presynaptic miRNAs in regulating presynaptic release at the Drosophila larval NMJ.

In Chapter II of this thesis, I will describe how I conducted the gain-of-function genetic screen, thereby identifying miR-310-313 as a novel regulator of synaptic plasticity. I will also discuss the method I used to generate a mir-310-313 deletion mutant, as we refer to it as KT40, which allowed me to further characterize the in vivo function of this miRNA cluster. In chapter III, I will explain my reasoning for choosing Khc-73 as the most important target of miR-310-313 function for its role in modulating synaptic function.
CHAPTER II : EXPERIMENTAL PROCEDURES
2.1 Fly strains and genetics

Flies were cultured on standard medium at 25°C. The following fly stocks were used: Wild type strains w¹ and w¹¹¹8 (Bloomington Stock Center), UAS-HA:Khc-73 FL and UAS-Khc-73 hairpin dsRNA (Siegrist and Doe 2005), UAS-mCD8-GFP (Lee and Luo 1999), UAS-mir-310-313 (provided by Dr. E. Lai), Df(2R)Exel6070, Df(2R)Exel7164 and Df(2R)Exel6285 (Parks, Cook et al. 2004). Gene Search fly collection was generously provided by D. van Meyel (Mindorff, O'Keefe et al. 2007). Neuronal Gal4 drivers: OK6-Gal4 (Aberle, Haghighi et al. 2002) Elav-Gal4 (Luo, Liao et al. 1994) and BG380-Gal4 (Budnik 1996). Gene Switch strain elavGS-Gal4 was a gift from Dr. Keishishian. The following strains were provided by the Drosophila Genetic Resource Center (DGRC), Kyoto Institute of Technology, Kyoto Stock Center: P(GawB)NP2713 (DGRC#113019), P(GawB)NP4255 (DGRC#113419), P(GawB)NP5240(DGRC#113645),P(GawB)NP5914(DGRC #113798).

2.2 Larval Dissections

Small populations of flies (about 20/vial) were cultured on standard medium at a constant 25°C. Wandering third instar larvae were collected, immobilized onto a dissection pad using insect pins (Austerlitz). Immobilized larvae were then covered in ice-cold hemolymph-like HL3 saline (Stewart, Atwood et al. 1994), and cut open along their dorsal surface with Vannas spring scissors (Fine Science Tools). Gut and fat body were removed with Dumont #5 forceps (F.S.T.), and the larvae were fixed for 10 minutes in 4% paraformaldehyde in
phosphate buffered saline (PBS). Fixed preparations were washed extensively in PBT (PBS and 0.1% Triton X-100) prior to immunostaining.

2.3 Immunohistochemistry

The fixed third instar larvae were blocked in PBTN (PBT plus 5% normal goat serum) for 30 minutes, followed by overnight incubation with primary antibodies at 4°C. The larvae were subsequently incubated with fluorescence-conjugated secondary antibodies at room temperature for 2 hours. Primary antibodies were used in the following concentrations: Rabbit anti-GFP (1:500) (A6455, Molecular Probes), rabbit anti-DGluRIII 1:2000 (Marrus, Portman et al. 2004), mouse anti-Brp (Nc82) 1:250 (Developmental Studies Hybridoma Bank, U.S.A.), rabbit anti-Synaptotagmin 1:2000 (Littleton, Bellen et al. 1993), mouse anti-Dlg (C. Goodman Lab), rabbit anti-Dvglut 1:5000 (Daniels, Collins et al. 2004), rat anti-HA (Roche) and Cy5-conjugated goat anti-Hrp 1:250 (Jackson ImmunoResearch Laboratories Inc.). Secondary antibodies were used in the following concentrations: Alexa 488-conjugated goat anti rabbit 1:500 (Molecular Probes), Cy3-conjugated goat anti-mouse 1:500 (Amersham Bioscience) and Rhodamine goat anti-rat 1:500 (Jackson ImmunoResearch Laboratories Inc.).

2.4 Microscopy

Synapses and ventral nerve cords were imaged using a Zeiss LSM3 confocal laser scanning microscope. Settings were optimized for detection without
saturation of the signal. Images were obtained at room temperature using a Zeiss Plan-Apochromat 63X/1.4 oil immersion objective (Carl Zeiss, Inc.). Z-stacks were collected using AIM software (Zeiss). Images were compiled and cropped in Adobe Photoshop CS2.

2.5 Quantification of fluorescence intensity
Maximum projection images of NMJs were used for fluorescence quantification. For each synaptic marker, integrated fluorescence signal per synaptic area was normalized to that of Hrp using MetaMorph software (Molecular Devices).

2.6 Bouton and branch point counts
Synaptic boutons and branch points visualized with antibodies against Dlg and HRP were counted manually as previously described (Wan, DiAntonio et al. 2000). The counts were performed with a 63X/1.4 oil immersion objective using Zeiss imager Z1 (Carl Zeiss, Inc.). All quantifications used the NMJs on muscle 4 or muscles 6 and 7 in abdominal segment 3. All bouton counts were normalized to the total muscle surface area. Average normalized bouton counts of different genotypes were compared to control. Statistical significance between samples was determined using a two-tailed distribution Student’s t-test.
2.7 Electrophysiology

All electrophysiological experiments were performed by Dr. Kazuya Tsurudome, postdoctoral fellow in Dr. Haghighi’s laboratory. Wandering third instar larvae were dissected in cold HL3 solution following standard protocol (Feng, Ueda et al. 2004). Spontaneous and evoked potentials were measured as previously described (Haghighi, McCabe et al. 2003). The amplitude and the frequency of mEJPs were calculated from continuous recordings in the absence of stimulation (60–100 s). Quantal content was estimated by dividing the mean EJP (at least 40 events) by the mean mEJP (at least 100 events). All recordings were performed on muscle 6, abdominal segment 3, at room temperature in HL3 solution containing 0.5 mM Ca$^{++}$.

2.8 Gain-of-function forward genetic screen

The Gene Search (GS) library was generated by Dr. van Meyel (Mindorff, O’Keefe et al. 2007). Each Gene Search line contains a double-headed UAS transgene which allows misexpression of an endogenous gene in either direction (Toba, Ohsako et al. 1999). Dr. van Meyel’s GS library was pre-screened for lethality caused by GS-directed misexpression in the embryonic nervous system. Their potential importance in early brain functions made us wonder whether they would also play a role during the period of rapid synaptic growth in larval development. This pre-screened GS library was crossed to motorneuronal driver OK6-Gal4 (Aberle, Haghighi et al. 2002) and screened for abnormal resulting NMJ morphologies. To increase the efficiency of my screen, I used a OK6-Gal4 line that expressed GFP-fused post-synaptic
shaker potassium channels. This way the resulting NMJs could be visualized directly without performing immunohistochemistry. The results reported in this thesis were based on the NMJs of at least four dissected larvae for each of the GS lines screened.

2.9 Geneswitch experiments
The Geneswitch expression system is based on the RU486 (mifepristone)-dependent GAL4 protein and its target Upstream Activating Sequence (UAS) (Osterwalder, Yoon et al. 2001). In this thesis, we made use of a pan-neuronal Geneswitch driver elavGS-GAL4 (Osterwalder, Yoon et al. 2001), to investigate the effect of expressing the UAS-transgene in neurons during larval stage. Driver and UAS lines were cultured on standard medium at 25°C. Since RU-486 was not present, the expression of the UAS-transgene was not activated. Once the progeny embryos develop into first instar larvae, these first instar larvae were transferred to the medium containing the gene-switch activator RU486, where they continued to grow at 25°C to third instar larvae. These third instar larvae were subsequently selected and dissected for studying their structural and functional phenotypes. Concentrations of RU486 used were: 21.5 ug/mL.

2.10 Generation of mir-310-313 deletion
The mir-310-313 deletion KT40 was obtained by inducing the mobilization of the P-element found in strain P{GSV1} GSd033. Virgin females of genotype
Δ2-3, Sb/Cyo were mated with P\{GSV1\}GSd033 homozygous male. Δ2-3, Sb/P\{GSV1\}GSd033 female progeny were selected, crossed to Sco/Cyo balancer males. Single white eye male progenies (indicative of P-element loss) were isolated and one hundred individual stocks were established. Genomic DNA of this 100 excision lines were then extracted and screened by PCR with forward primer and reverse primer : 5’-GAAATCGCCACTCAACTTGG-3’ and 5’-AATGCAGCAGCTGTTATGGA-3’, which respectively correspond to a region 5’ and 3’ to the miR-310-313 genetic locus. PCR products from putative deletions were subsequently sequenced. KT40 contains an 1159 bp deletion removing all four predicted miR-310-313 transcripts, while leaving flanking genes intact. KT 2 is a precise excision of P-element GSd033 which was verified by sequencing the PCR product from forward primer 5’-AAACTGGCATTCCGTCTTA-3’ and reverse primer 5’-CTGGCAAGTCTGTGACGAAG-3’.
CHAPTER III : RESULTS
NOTE: All the electrophysiology experiments presented in this chapter were performed by Dr. Kazuya Tsurudome, a post-doctoral fellow in my lab. The corresponding results and figures were also provided by Dr. Trurudome.

3.1 Identification of miR-310-313

Aiming to identify novel genes that are involved in regulating synaptic growth, I conducted a gain-of-function forward genetic screen using the Gene Search (GS) library generated by Mindorff et al (2007). Each of these GS lines contains a vector that has UAS at both P-element ends, allowing bi-directional overexpression of the flanking endogenous genes in Gal4 expressing tissues (Toba, Ohsako et al. 1999).

3.1.1 A range of NMJ phenotype was observed

To study the potential NMJ-specific roles of the endogenous genes being overexpressed by the GS library, we used a motoneuronal driver OK6-Gal4 (Aberle, Haghighi et al. 2002) in our screen. Of the 100 GS lines that I screened, most of them when overexpressed in motoneurons resulted in normal NMJ size and structure when compared to control (genotype: OK6-Gal4/+). NMJ size was estimated by the proportion of the muscle area covered by the NMJ to the total surface muscle area. Overexpression of five GS lines led to reduced synaptic size and that of ten others resulted in NMJ expansion (Table 1). For each GS line, at least four third instar larvae were dissected and their NMJs were compared to ensure phenotypic consistency.
Table 1

<table>
<thead>
<tr>
<th>GS lines that lead to NMJ</th>
<th>Closest endogenous gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSD 033</td>
<td>Mir-313</td>
</tr>
<tr>
<td>GSD 219</td>
<td>bl</td>
</tr>
<tr>
<td>GSD 227</td>
<td>Btk29A</td>
</tr>
<tr>
<td>GSD 247</td>
<td>Mir-313</td>
</tr>
<tr>
<td>GSD 306</td>
<td>grp</td>
</tr>
<tr>
<td>GSD 321</td>
<td>CG11779</td>
</tr>
<tr>
<td>GSD 441</td>
<td>Mir-313</td>
</tr>
<tr>
<td>GSD 445</td>
<td>grp</td>
</tr>
<tr>
<td>GSD 482</td>
<td>ken</td>
</tr>
<tr>
<td>GSD 489</td>
<td>Paps (antisense)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GS lines that lead to NMJ</th>
<th>Closest endogenous gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSD 217</td>
<td>CG32223</td>
</tr>
<tr>
<td>GSD 226</td>
<td>cbt</td>
</tr>
<tr>
<td>GSD 408</td>
<td>esg</td>
</tr>
<tr>
<td>GSD 415</td>
<td>CG8370/ATPCL</td>
</tr>
<tr>
<td>GSD 435</td>
<td>CG9894</td>
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</tbody>
</table>
across different larvae. Table 1 lists the closest endogenous genes flanking the fifteen GS P-elements that led to abnormal NMJ when overexpressed in motorneurons.

### 3.1.2 Three GS lines were selected for further characterization

Since the purpose of this genetic screen was to quickly identify genes that could play a role in regulating synaptic growth, we only selected the GS lines that resulted in obvious NMJ phenotypes for further characterization. Of the GS lines that led to abnormal NMJs in Table 1, overexpression of GSd 033, GSd 247 and GSd 441 in motoneurons exhibited significantly increased numbers of bouton and branch point. Individual bouton appeared to be smaller. Representative muscle 4 NMJ of GSd 033 overexpression is shown in Figure 1B, as compared to control in Figure 1A.

### 3.1.3 The three GS lines could overexpress miR-310-313

Since individual presynaptic overexpression of GSd 033, GSd 247 and GSd 441 led to a 3-fold increase in number of boutons (Figure 1D), we were very interested in identifying the closest endogenous genes that flank these three GS lines. These endogenous genes might play essential roles in the nervous system in maintaining normal synaptic growth. Unexpectedly, inversed PCR and sequencing data revealed that the GS P-elements contained in GSd 033, GSd 247 and GSd 441 were inserted to the same genomic region: about one kilo-basepair upstream of a miRNA cluster, miR-310-313 (Mindorff, O'Keefe et al. 2007), making this cluster the most probable endogenous gene being
Figure 1  Overexpression of miR-310-313 in motorneurons leads to NMJ expansion. (A-C) Representative third instar larval NMJs at muscle 4 as marked by presynaptic HRP and postsynaptic Dlg. Compared to control (A), overexpression of GSd 033 in pre-synaptic motorneurons leads to excess synaptic boutons and synaptic branches (B). Individual bouton size is noticeably reduced. Overexpressions of GSd 247 and GSd 441 result in similar morphology (data not shown). Overexpression of transgenic UAS-mir-310-313 (C) leads to qualitative similar changes in synaptic morphology. (D) Bar graph representing the quantification for the normalized synaptic bouton number per muscle surface area (MSA) for the genotypes shown in A to C. In this and in other quantification graphs, average and standard error are plotted. Genotypes are as follows: (A) OK6-GAL4/+, (B) OK6-GAL4/GSd 033, (C) OK6-GAL4; UAS-miR-310-313. *** represents p<0.001. Scale bar = 10µm
Figure 2 P-element insertions upstream of the miR-310-313 genomic locus. MiR-310-313 cluster consists of four miRNA members: mir-310, mir-311, mir-312 and mir-313. This miRNA cluster is the closest endogenous gene flanking the GS P-element contained in GSD 033 (red), which is located about one kilo-basepair upstream of the mir-310-313 genomic locus. The GS P-elements of GSD 247 and GSD 441 also locate in close proximity to that of GSD 033 (not shown). The four NP lines used as mir-310-313 enhancer traps are also found upstream of mir-310-313. The genomic region deleted in KT40 is marked.
overexpressed by the three GS lines (Figure 2). However, as gene search P-elements possess the ability to overexpress flanking genes in both directions, it was important to confirm that it was the gain-of-function of the miR-310-313 cluster that led to NMJ expansion. To that end, I crossed the UAS-mir-310-313 transgenic line generated by our collaborator Dr. E. Lai (Sloan-Kettering Institute) to the same motorneuronal driver that I used for the screen, OK6-Gal4 (Aberle, Haghighi et al. 2002). This resulted in a 1.5-fold increase in the number of bouton per muscle surface area (Figure 1C and 1D), qualitatively resembling the phenotypes caused by overexpressing the three GS lines.

3.1.4 Where does miR-310-313 express?
Prior to further characterizing miR-310-313, it was essential to investigate its expression pattern to ensure its relevance in the regulation of synaptic growth. As presynaptic overexpression of miR-310-313 led to abnormal NMJ, I expected this miRNA cluster to be expressed in the nervous system. MiR-310-313 cluster consists of four members: miR-310, miR-311, miR-312 and miR-313 (Leaman, Chen et al. 2005). Expression patterns of clustered miRNAs are often similar (Ruby, Stark et al. 2007). Various laboratories have conducted Northern blot and in situ hybridization analyses to study their expression profiles. The four members demonstrate enriched expression throughout embryonic stage (Aravin, Lagos-Quintana et al. 2003; Leaman, Chen et al. 2005; Lu, Fu et al. 2008). Their expression during larval stage, however, was not detectable with the molecular techniques used.
3.1.4.1 MiR-310-313 expresses in motorneurons during larval stage

To investigate whether miR-310-313 expresses during larval stage, I used miR-310-313 enhancer traps, P-elements driving expression of Gal4 in tissues where miR-310-313 is expressed, to drive a transgenic GFP expressing line (UAS-mCD8-GFP). Figure 3A shows the GFP signal reflecting the expression pattern of miR-310-313 cluster in third instar larva. GFP fluorescence was enriched in ventral nerve cord where it could localize with evenskipped, a marker for motorneuronal cell bodies (Ly, Yao et al. 2008), suggesting miR-310-313 could localize in motorneurons. By using multiple copies of miR-310-313 Gal4 and UAS-mCD8-GFP, we captured GFP signal along motor axons and at NMJ, overlapping with HRP, which stained all neuronal membrane (Figure 3B and 3C). This result strongly suggests that miR-310-313 cluster is normally expressed in motor neurons during larval stages.

3.2 Characterization of miR-310-313 cluster

Having established that miR-310-313 could express in motorneurons during larval stage, we wanted to further characterize this miRNA cluster. Despite the gain-of-function of miR-310-313 yields striking NMJ phenotypes, a significant increase in number of boutons and branches, this approach does not provide evidence for the endogenous role of this miRNA cluster. To examine whether miR-310-313 is required in vivo for normal synaptic growth, I turned
Figure 3 MiR-310-313 can localize in motorneurons. P\{GawB\} inserts in the genomic locus of *mir-310-313* used to express UAS-mCD8-GFP in third instar larvae. (A) Confocal image of two motorneuron cell bodies as marked by *even skipped* (*eve*). GFP expresses in some motorneurons. (B and C) Representative third instar larval NMJs at muscle 4 as marked by presynaptic HRP (red). GFP signal overlaps with HRP along motor axons and at NMJ. Genotypes are as follows: (A) P(GawB)NP2713/+; UAS-mCD8-GFP/+ , (B) P(GawB)NP2713; UAS-mCD8-GFP and (C) P (GawB) NP4255; UAS-mCD8-GFP. All the other NP lines used demonstrate the same phenotypes. Scale bar =10µm
to the loss-of-function analysis. For that, I generated a miR-310-313 deletion line by imprecisely excising the P-element contained in GSd 033. I screened for imprecise excision lines as described in Chapter II. Briefly, genomic DNA from each excision line was extracted and amplified with PCR primers that flank the miR-310-313 genomic locus. The PCR products were then analysed on an electrophoresis gel to determine their molecular sizes. Deletion lines were isolated based on their lower bands than the control band. Figure 4 shows an image of an agarose gel demonstrating the band indicating the smaller size of KT40 PCR product. Potential deletion lines were then sequenced to determine the precise genomic region deleted. One of the deletion lines deletes miR-310-313 cluster entirely without disrupting any neighbouring genes. I refer to this deletion line as KT40. The KT40 stock is homozygous viable and fertile. I used KT40 homozygous stock, as well as the heterozygotes KT40/Df(2R)Exel6070 and KT40/Df (2R)Exel7164 for all miR-310-313 loss-of-function analyses in this thesis.

3.2.1 MiR-310-313 is not required for normal synaptic structure
Since overexpressing miR-310-313 led to significantly expanded NMJ and increased number of bouton, I predicted that removal of this cluster would result in opposite phenotypes: smaller NMJs and decreased number of bouton. To my surprise, gross NMJ structure of KT40 third instar larvae was hardly distinguished from control. As shown in Figure 5, not only did the number of bouton per muscle surface area remain normal, number of branch point also appeared to be unchanged from control in KT40/deficiency heterozygotes.
Figure 4 MiR-310-313 deletion was identified with its lower band. Genomic DNA from each excision line was extracted and amplified with PCR primers that flank the miR-310-313 genomic locus. This pair of primer generates a 2-kb band in precise excision lines. The PCR products were then analysed on an electrophoresis gel to determine their molecular sizes. Deletion lines were isolated based on their lower bands. The first lane corresponds to a 1-kb plus ladder.
Figure 5
Figure 5 Loss of miR-310-313 does not alter gross NMJ structure.
(Top) Muscle 4 NMJs co-stained with anti-Hrp and anti-Dlg in control and heterozygote Df(2R)Exel6070/KT40 mutant larva. (Bottom) Bar graph representing the quantifications for the normalized synaptic bouton number per muscle surface area (MSA) and the normalized number of branch point for the control, heterozygote KT40/Df1 (Df(2R)Exel6070) mutant larva, as well as heterozygote KT40/Df2 (Df(2R)Exel7164) mutant larva. n≥20 for all genotypes. No statistical significance was found using Student’s t-test (P>0.1). Scale bar = 10µm
Individual bouton size appeared to be normal as well. These findings suggest that miR-310-313 cluster is not required for normal synaptic growth.

### 3.2.2 Loss of miR-310-313 results in more active zones

Although the gross synaptic structure was normal in the KT40 background, I wondered if we could identify any subtle abnormalities in KT40 synapses. We decided to examine whether various synaptic markers were distributed normally at KT40 NMJs. One way to achieve this was to immunohistochemically stain NMJs with the antibodies against the synaptic proteins of interest, and quantified the corresponding fluorescence intensities. The synaptic proteins that I examined in KT40 background included Bruchpilot (Brp), an active-zone protein which affects neurotransmitter release (Kittel, Wichmann et al. 2006); Synaptotagmin, a synaptic vesicle-associated protein (Littleton, Bellen et al. 1993), Disc-large, a PSD-95 homology that targets and clusters shaker potassium channels at synaptic terminals (Tejedor, Bokhari et al. 1997; Zito, Fetter et al. 1997) and Vglut, a vesicular glutamate transporter. As shown in Figure 6, Brp level was significantly elevated in KT40 background while levels of the other synaptic proteins quantified remained unchanged. I also examined the post-synaptic integrity in KT40 by quantifying the intensity of post-synaptic glutamate receptors (GluRIII). There was no significant difference between control and loss of miR-310-313. These findings suggest that mir-310-313 might regulate the pre-synaptic density/number of active zone, thereby altering pre-synaptic neurotransmitter release.
Figure 6

A
Figure 6 Loss of miR-310-313 cluster leads to an increase in active zone associated protein, Bruchpilot. (A) Representative third instar larval NMJs at muscle 4 from KT40/+ or KT40/ Df(2R)Exel6070 as marked by presynaptic HRP (red). Synaptic proteins marked by green from top to bottom are: Disc Large (Dlg), Drosophila Vesicular glutamate transporter (Vglut), Synaptotagmin (Syt). Loss of mir-310-313 does not affect the intensities of these synaptic proteins. (B) NMJs at muscle 4 from KT40/+ or KT40/ Df(2R)Exel6070 larvae. NMJs are co-stained with anti-GluRIII (red) and anti-Brp (green) antibodies. Brp intensity is noticeably increased in KT40/Df background. (C) Quantification for fluorescence signals associated with anti-Dlg, anti-GluRIII, anti-VGLUT anti-Syt and anti-Brp versus that of anti-Hrp at muscle 4 NMJs for KT/+ or KT40/ Df(2R)Exel6070 . Significant difference was found only for Brp levels, Student t-test (n≥18 for each genotype).
3.2.3 miR-310-313 is required for normal synaptic function

Bruchpilot (Brp) localizes at presynaptic active zones to an electron dense structure called T-bar, where synaptic vesicles accumulate and neurotransmitter release takes place. In bruchpilot mutant, T-bars are completely missing from NMJ. Presynaptic clustering of calcium channels becomes abnormal and release of vesicle is adversely affected (Kittel, Wichmann et al. 2006). As higher numbers of T-bars correlate with enhancement in neurotransmitter release at NMJ (Wojtowicz, Marin et al. 1994; Haghighi, McCabe et al. 2003), I expected that this miRNA cluster could regulate synaptic release, and loss of this miRNA cluster might lead to enhanced functional phenotypes.

To test the above prediction, we have conducted electrophysiology analysis on muscle 6 of KT40 wandering third instar larvae. Note again that all electrophysiology experiments described in the thesis were conducted by Dr. Trurudome. As well all electrophysiology figures were provided by Dr. Trurudome. In support of our prediction that KT40 NMJs would have enhanced synaptic functions, recordings of KT40 third instar larvae showed a significant increase in excitatory junctional potentials (EJPs) and excitatory junctional currents (EJCs), while the miniature excitatory potentials and currents (mEJPs and mEJCs) remained unchanged compared to control larvae. This resulted in a significant increase in quantal content (QC), which represents the ratio between EJP and mEJP. These functional defects were suppressed by restoring miR-310-313 in pre-synaptic neurons (Figure 7A and 7B). These findings, in addition to the increase in Brp intensity at NMJ,
suggest that miR-310-313 is required for normal synaptic function and it likely acts through pre-synaptic mechanisms, such as neurotransmitter release.

To confirm that the background from which KT40 was generated did not contribute to the enhanced functional phenotypes, we also recorded from a precise excision line, KT2. As shown in Figure 7C, KT2 did not show any functional abnormalities, suggesting it was the loss of miR-310-313 that underlay the enhanced synaptic functions.

3.2.4 Restoration of miR-310-313 only in motorneurons is sufficient to suppress the deletion phenotype

Loss of miR-310-313 resulted in significant enhancement of the evoked synaptic activities, suggesting miR-310-313 is a negative regulator of synaptic function. Where is this miRNA cluster then required to serve this function? Based on the pre-synaptic expression pattern of miR-310-313, I reasoned that miR-310-313 acted presynaptically. Providing the miR-310-313 cluster in motorneurons was therefore expected to rescue the synaptic functional phenotypes in KT40. As shown in Figure 7D, overexpressing the transgenic UAS-mir-310-313 only in motorneurons was sufficient to suppress the increases in EJC and QC in KT40, suggesting this miRNA cluster is in fact required presynaptically to control synaptic release.
Figure 7 MiR-310-313 is a negative regulator of synaptic function.

(A) Representative EJP, EJC and mEJC traces recorded at muscle 6 of third instar larvae. EJP and EJC traces are 10 superimposed traces at 0.5Hz stimulation. mEJCs are sample traces of continuous recording in the absence of stimulation. Genotypes are as follows: Df(2R)Exel6070/+, Df(2R)Exel6070/KT40 and Df(2R)Exel6070/KT40; Elav-Gal4/UAS.mir.310.313 (Elav rescue). (B) Quantification of the functional phenotypes shown in (A). Average mEJC amplitudes are similar in all three genotypes. EJC amplitude and quantal content are increased in KT40/KT40, which are suppressed by pre-synaptic expression of UAS-mir-310-313 using Elav-Gal4. (C) The precise excision line KT2 does not show abnormal synaptic functions. Restoring mir-310-313 only in motoneurons using BG380-Gal4 (D) and during larval stage using Elav-GS-Gal4 (E) also suppress KT40 phenotypes. * represents p<0.05, ** represents p<0.01, *** represents p<0.001.
3.2.5 Restoration of miR-310-313 only during larval stage is sufficient to suppress the deletion phenotypes

When is this miRNA cluster required to regulate synaptic function? We are interested in understanding whether mir-310-313 is required during larval stage, a developmental period of rapid synaptic growth. To address the temporal requirement of miR-310-313 cluster, I took advantage of an inducible Gal4 driver as described by Osterwalder et al (Osterwalder, Yoon et al. 2001) and in Chapter II of this thesis. This so-called geneswitch system allowed me to activate transcription of UAS-transgenes during any developmental stage in a tissue-specific fashion. We activated transgenic mir-310-313 expression presynaptically only starting from the first larval stage (L1) onwards and performed electrophysiological analyses of the corresponding third instar (L3) larvae. As shown in Figure 7E, providing miR-310-313 only during larval stage was sufficient to rescue synaptic function in KT40 background, suggesting this miRNA is required during larval stage to modulate synaptic activity.

3.3 Khc-73: a candidate miR-310-313 target

Having established that miR-310-313 acts to regulate normal synaptic transmission, the next obvious question to ask was: what does miR-310-313 target in this regulation?

MiRNAs recognize hundreds of mRNA targets with their seed regions (Bushati and Cohen 2007; Bartel 2009). However, not every miRNA-target
interaction would result in significant phenotypes. Which target is the key target in modulating synaptic function? Several laboratories have attempted to predict the targets of miR-310-313 with various computational algorithms (Stark, Brennecke et al. 2003; Rajewsky and Socci 2004; Stark, Brennecke et al. 2005; Bartel 2009). Among them, Khc-73 is one of the highest scoring candidates (www.targetscan.org). Khc-73 is a member of the kinesin superfamily proteins (KIFs), which are molecular motors that transport cellular organelles along microtubules. In situ hybridization performed by Li et al (1997) has demonstrated the enrichment of Khc-73 in central and peripheral nervous system during late embryonic stage (Li, Liu et al. 1997). I considered Khc-73 the most plausible candidate because of its relevant expression pattern as well as the various nervous system functions performed by fellow KIFs.

One of the well-characterized roles of KIFs in the nervous system is their ability to transport towards the terminals synaptic vesicles containing synaptic proteins which are required for normal synaptic transmission. KIF17, a dendrite-specific motor protein, for example, could modulate synaptic transmission by transporting NMDA receptor-containing vesicles to dendritic synapses in mammalian neurons (Setou, Nakagawa et al. 2000). Similarly, KIF1A, a brain-specific KIF, is enriched in axons and carries vesicles containing vesicle proteins such as synaptophysin, synaptotagmin and Rab-3A (Okada, Yamazaki et al. 1995; Klopfenstein, Tomishige et al. 2002).
In *Drosophila*, KIF3 is expressed in subsets of neurons in central and peripheral nervous system. It is required for anterograde transport of choline acetyltransferase (ChAT). *KIF3* mutant larvae have uncoordinated movement (Ray, Perez et al. 1999; Terada and Hirokawa 2000). Moreover, cargos including synaptic membrane proteins and mitochondria are jammed in the axons of *kinesin-I(khc)* mutant flies. This results in abnormal synaptic terminals and adversely affected neurotransmitter release, causing paralysis in mutant larvae (Hurd and Saxton 1996). More recently, Pack-chung et al. (2007) have identified a novel KIF member in *Drosophila, imac*. The *imac* mutant shows normal axonal outgrowth and targeting. However, synaptic boutons fail to form at the target muscles. Although some axonal transport of synaptic terminal protein remain functional in *imac* mutant, some synaptic proteins are not transported normally (Pack-Chung, Kurshan et al. 2007).

Altogether, KIFs have been highlighted as key microtubule-dependent motors in the nervous system. I expect Khc-73, like its family members, to be a part to carry cargoes that are crucial for the proper functions of the nervous system. Prior to further characterizing Khc-73 function though, I asked whether Khc-73 normally expresses in larval nervous system.

### 3.3.1 Does Khc-73 express during larval stage?

To date, there is no information on Khc-73 distribution during larval development. As there is no Khc-73 specific antibody available to directly detect the endogenous protein, and our attempt to generate one was
unsuccessful, we relied on the Khc-73 transgenic line generated by Siegrist et al. (2005) to examine its subcellular localization during larval development (Siegrist and Doe 2005). We verified that this transgenic line contains the entire wildtype \textit{khc-73} transcript fused to a HA-tag (\textit{UAS-HA:Khc-73 FL}). I overexpressed this transgenic construct with motoneuronal driver \textit{BG-380} (Budnik et al 1996) and studied the HA distribution. As shown in Figure 8, HA signal was detected in motoneuronal cell bodies along the midline of ventral nerve cord and along motor axons coming out from the cell bodies. Despite this result did not reflect the \textit{in vivo} localization of Khc-73, it nonetheless suggested that Khc-73 could localize along motor axons, potentially transporting important cargoes to regulate synaptic function during larval stage.

\textbf{3.3.2 Khc-73 is a target of miR-310-313}

Based on our data showing epitope-tagged Khc-73 localization to motor axons, we set out to test whether Khc-73 is an important target of miR-310-313. Our collaborator had cloned the 3’UTR of \textit{khc-73} mRNA downstream of a luciferase reporter and studied the level of Khc-73 expression in S2 cultured cells. In the presence of miR-310-313, luciferase level was reduced by about 50\% (Figure 9), suggesting miR-310-313 can regulate Khc-73. The control luciferase reporter without the 3’UTR, on the other hand, did not response to miR-310-313. This suggests to us that miR-310-313 interacts with the 3’UTR of \textit{khc-73} transcript, and represses its expression.
Figure 8  HA-tagged Khc-73 can localize in motor neuronal cell bodies and motor axons. Representative third instar larval ventral nerve cord as marked by presynaptic HRP (blue). HA-tagged Khc-73 is expressed in motor neuron cell bodies along the midline of ventral nerve cord. HA signal is also found along motor axons coming out from the cell bodies, overlapping with Hrp signal.
**Figure 9 Khc-73 is a target of the miR-310-313 cluster.** Quantification for luciferase activity for khc-73-3’UTR reporter in the presence or absence of the miR-310-313 cluster. The expression of luciferase reporter is repressed two-fold in the presence of miR-310-313. P=2.48x10^{-8} Student’s t-test.
3.3.3 Overexpression of Khc-73 phenocopies miR-310-313 deletion

Since miR-310-313 could target Khc-73 as shown in the *in vitro* luciferase assay, if Khc-73 was the main target of miR-310-313 in regulating synaptic function, I expected that an elevated Khc-73 protein level in wildtype background should lead to abnormal functional phenotypes that mimic KT40, whereas removal of Khc-73 should lead to opposite functional effects. As shown in Figure 10, the first part of my expectation was fulfilled. Similar to KT40, overexpressing Khc-73 in motorneurons resulted in a significant increase in synaptic release, as reflected by the significant increases in EJC and QC.

Since *khc-73* mutant does not exist to date and flies possessing deficiency uncovering the *khc-73* genomic region are embryonic lethal, we reduced Khc-73 level by using a transgenic RNA interference (RNAi) approach (Siegrist and Doe 2005). Specifically knocking down *khc-73* transcript levels in motorneurons, however, did not lead to significant changes in synaptic function as we anticipated (Figure 10). Nonetheless, I expected that the extent to which Khc-73 was reduced by RNAi might still be enough to reverse KT40 functional phenotypes.

3.3.4 Reduction of Khc-73 pre-synaptically is sufficient to restore normal synaptic function

As shown in Figure 10, lowering Khc-73 expression pre-synaptically by using Khc-73 RNAi was sufficient to restore normal synaptic function. In support of
Figure 10 Presynaptic overexpression of Khc-73 phenocopies loss of miR-310-313. Removal of Khc-73 suppresses KT40 phenotypes. (Top) Representative EJP, EJC and mEJC traces recorded at muscle 6 of third instar larvae. EJP and EJC traces are 10 superimposed traces. mEJCs are sample traces of continuous recording in the absence of stimulation. Genotypes are as follows: control (BG380-Gal4/+), Khc overexpression (BG380-Gal4/++; UAS.khc.73/+) and khc-RNAi overexpression (BG380-Gal4/++; UAS-khc73-RNAi) (Bottom, left) Quantification of the functional phenotypes shown in the top panels. Average mEJC amplitudes are similar in all three genotypes. EJC amplitude and quantal content are increased in overexpression of Khc-73, which mimicked KT40 functional phenotypes. Reducing Khc-73 by khc73-RNAi did not lead to any significant functional abnormalities. Despite so, removal of khc-73 by RNAi is sufficient to restore normal function in KT40 background (Bottom, middle). (Bottom right) Likewise, removing one allele of khc-73 in KT40 also rescues synaptic functions. Dfkhc-73 corresponds to Df(2R)Exel6285 which uncovers khc-73 genomic locus. * represents p<0.05, ** represents p<0.01, *** represents p<0.001.
this result, removing one allele of \textit{khc-73} in KT40 background was also sufficient to rescue synaptic function (Figure 10). These two genetic interactions provide strong evidence that Khc-73 is an \textit{in vivo} target of miR-310-313 and it is the key target of miR-310-313 in regulation of synaptic function.
CHAPTER IV : DISCUSSION
4.1 miRNAs as negative regulators of synaptic release: a surprise?

In this thesis, I have presented evidence that miR-310-313 cluster is required for maintaining normal synaptic release. Loss of this miRNA cluster leads to significant increases in post-synaptic evoked functional potentials as well as presynaptic release. Restoration of miR-310-313 only in presynaptic motorneurons is sufficient to rescue these defects in deletion mutants. More importantly, restoring this miRNA cluster only during larval stages is enough to resume normal synaptic release. Our findings represent the first in vivo evidence demonstrating the requirement for miRNAs in the regulation of synaptic strength in a spatiotemporal-specific manner. As KT40 flies are viable and fertile, what would be the physiological consequences of the loss of miR-310-313? It would be interesting to further examine if there are any locomotion defects or enhancement in KT40 flies.

miRNAs have been known for their roles in early nervous system development. Thus far there has been no direct finding reported on their roles on regulating synaptic function in Drosophila. Despite so, the idea that miRNAs can act as negative regulators of synaptic release does not come by surprise. In the past decade, numerous groups of researcher have proposed a number of mechanisms that are involved in regulating synaptic function. Given the fact that there are multitudes of synaptic proteins that participate in these regulatory pathways, it would not be surprising that the expression levels of these genes are tightly regulated at multiple levels. While research focus had
been put on transcriptional regulation of neuronal genes, more recently a large body of evidence has begun to emerge suggesting that post-transcriptional regulations of transcript are also important. Our findings in this thesis have highlighted the significance of miRNA-mediated gene repression for normal synaptic function. As our preliminary data have shown, motorneuronal overexpression of Dicer, which is required for producing all mature miRNAs, leads to a decrease in quantal content. We propose that, in addition to miR-310-313, more miRNAs may act as negative regulators of synaptic release. These miRNAs may involve in downregulating other synaptic proteins which participate in the regulation of neurotransmitter release, thereby influencing synaptic function.

4.2 How does Khc-73 regulate synaptic strength?

In this thesis, we have also shown that miR-310-313 targets Khc-73, a motor protein that belongs to the *Drosophila* Kinesin superfamily (KIF). Overexpression of Khc-73 in motor neurons phenocopies miR-310-313 deletion phenotypes. Furthermore, reducing khc-73 level is enough to rescue *mir-310-313* mutant functional abnormalities. Our findings present a novel function of Khc-73 protein during larval stages.

It has been known that Khc-73 is closely related to *C.elegans* unc-104, mouse Kif13B and human GAKIN protein. They are anterograde transporters, directed towards the plus end of microtubules (Jamain, Quach et al. 2001;
The human homolog of Khc-73, GAKIN, contains a central MAGUK binding stalk (MBS), which binds to the GK domain of human Disc Large (hDlg) protein in T Lymphocytes. It is suggested that this interaction may serve as the mechanism by which hDlg and potentially the associated protein complexes are trafficked intracellularly (Hanada, Lin et al. 2000). Interestingly *Drosophila* Khc-73 also possesses this MBS domain in its middle stalk region. Khc-73 has been shown to interact with the PSD-95 homologue Discs large (Dlg) with this MBS domain (Siegrist and Doe 2005). This *in vivo* interaction is required for inducing cortical polarity in *Drosophila* neuroblasts (Siegrist and Doe 2005). This early interaction with Dlg had made us wonder whether Khc-73 also interacts with Dlg during larval stage in maintaining normal synaptic transmission.

Dlg has been shown to regulate the localization of K-channels (Tejedor, Bokhari et al. 1997). If Khc-73 does transport Dlg to the terminal, one could envision a scenario where Dlg could affect the K-channel density at presynaptic terminals, which could lead to changes in neurotransmitter release by altering the action potential duration. Yet, we found no change in Dlg level in *KT40* mutant larvae. This result suggests that Khc-73 might not carry Dlg during larval stage to regulate synaptic function. In addition, our electrophysiological analysis does not support a contribution by K-Channel current alterations in *KT40* mutant larvae. On the other hand, our data suggest that the expression of active zone protein, Bruchpilot, increases significantly in the absence of miR-310-313. Our preliminary data suggest that presynaptic
overexpression of Khc-73 also leads to increase in Brp (data not shown), mimicking KT40 mutant. Although we did not provide evidence for the direct interaction of Brp and Khc-73, our findings suggest that miR-310-313 regulates the expression of Khc-73, which could in turn affect the transport of Brp to synapses, either directly or by intermediate adaptors.

Another possible way by which Khc-73 affects synaptic function is through its C-terminal domain. Like its human counterpart GAKIN, Khc-73 also has a cytoskeleton associated protein (CAP)-Gly-rich domain at its C-terminal region, which putatively binds to microtubules. This domain is also present in some cytoskeleton and dynein-associated proteins such as restin, which serves to connect endocytic vesicles and microtubules (Li, Liu et al. 1997; Siegrist and Doe 2005; Hirokawa and Noda 2008). This microtubule binding domain might transport tubulin oligomers and microtubules along microtubule track in an anterograde manner. It remains to be investigated whether a potential interaction between Khc-73 and the microtubule network contributes to the regulation of synaptic release.

4.3 One target accounts for all KT40 phenotypes

We have established that loss of miR-310-313 could be rescued by repressing only one of its targets, Khc-73. Why Khc-73 but not the other potential targets? Are there any factors that determine which miRNA-target relationship is important for the phenotypic consequence of interest?
As miRNAs only require 7 nucleotides to recognize their target mRNAs, it is predicted that each miRNA can target hundreds of transcripts. Not every predicted miRNA-target interaction, however, would yield striking phenotypic consequences, where one target accounts for all noticeable phenotypes. It is therefore challenging to identify the key targets when studying miRNAs. For this reason, in spite of the fact that more than 140 miRNA genes have been identified in the *Drosophila* genome (Bartel 2009), only eleven miRNA-target relationships have been well-characterized, as they are reported in the latest review (Smibert and Lai 2008). In fact, two factors could predict the importance of a target for a specific miRNA. First, the number of conserved miRNA recognition sites present in the 3’UTR, which is believed to be directly related to the extent to which the target activity is attenuated. Second, the phenotypic importance resulted from the miRNA mediated-attenuation of the target (Flynt and Lai 2008).

The 3’UTR of *lin-14* mRNA, for example, contains multiple recognition sites for the founding member of miRNA, *lin-4*. This is also the case for other well-characterized miRNA-target relationships: *let-7:lin-41, let-7:hbl-1* and *lsy-6:cog-1* (Bartel 2009). In search for the key target of miR-310-313, we observed that there are three conserved mir-310-313 recognition sites in the 3’UTR of *khc-73* mRNA, including an 8-mer site (www.targetscan.org), which was reported to be the most effective type of recognition site (Bartel 2009). This boosted our confidence in further exploring the interaction.
between \textit{khc-73} and \textit{mir-310-313}, and our findings did in fact suggest that Khc-73 was the key target of miR-310-313 for regulating synaptic function.

Despite the many recognition sites found in the 3’UTR of \textit{khc-73} mRNA, our data indicate that miR-310-313 might adjust Khc-73 level only to a small extent. Partial removal of Khc-73, either by using RNAi approach or eliminating one copy of the \textit{khc-73} gene, was sufficient to restore synaptic function in KT40 background. This is similar to the case of \textit{Drosophila mir-279}, where absence of mir-279 leads to ectopic expression of CO$_2$-sensing neurons. Loss of one copy of its key target \textit{nerfin} was sufficient to revert the mir-279-null phenotypes (Flynt and Lai 2008). This suggests that the importance of a target does not solely depend on how much its level is reduced; a rather small miRNA-mediated reduction could nonetheless lead to striking phenotypic consequences.

**4.4 Concluding remarks**

In this thesis, we have established that miR-310-313 is required presynaptically during \textit{Drosophila} larval stage for normal synaptic release and Khc-73 is the key target for this regulatory role of miR-310-313. It is likely that Khc-73 affects the transport of active zone protein, Bruchpilot, to the terminal, thereby influencing the pre-synaptic release of neurotransmitter. Our data suggest that the fluorescence intensity of Brp in miR-310-313 mutant background is elevated when compared to control, indicating an increase in the number or density of T-bars per active zone. Future ultrastructural analyses
will have to perform to verify this prediction. We believe our findings in this thesis will open a new research path for exploring the role of other miRNAs in tuning synaptic plasticity.
CHAPTER V : REFERENCES


