Syncrip Regulates mRNA Localisation and Translation at the *Drosophila* Neuromuscular Junction

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Abstract

Evidence in different systems suggests that local translation is involved in synaptic plasticity in both neuron and muscle, but the mechanism by which this occurs is still poorly understood. The mRNA-binding protein Syncrip is conserved from fly to mammals and is thought to be involved in localized translation in both oocytes and neurons. Previous work has shown that Syncrip associates with mRNAs encoding key synaptic proteins at the *Drosophila* larval neuromuscular junction. Here I show that Syncrip is necessary for the structure and function of the neuromuscular junction. First, the loss of Syncrip leads to overgrowth of the neuromuscular junction. Second, Syncrip is required for proper expression of the Ca^{2+}-sensor Synaptotagmin1 at the presynapse, and loss of Syncrip causes a decrease in vesicle release probability. Third, while it was not possible to measure mRNA distribution in neurons, Syncrip mutants, like other perturbations in synaptic plasticity, correlate with changes in mRNA localization in muscle. Fourth, the overexpression and loss of Syncrip function suggest that the nuclear and nucleolar trafficking of the eukaryotic translation initiation factor eIF4E may be important to regulating synaptic morphology. These data suggest that Syncrip is involved in mRNA localization and translation in synaptic plasticity.

Chapter 1: Introduction

Synaptic plasticity is a general term with multiple meanings. The synapse is a stable, and specialized connection between two cells through which information can be communicated. A neuron can form multiple synapses, with both neurons and non-neuron cells, such as muscle cells. In large multi-neuron organs, such as the central nervous system, complex circuits of communication can arise across multiple neurons. It is these circuits that facilitate higher order brain functions, such as learning and memory. The establishment of neuronal circuits, however, is not sufficient alone for memory and learning. In addition, the flow of information through a circuit must be changeable, or plastic, throughout the life of the neurons. The unit of communication, at which plastic changes occur, is the synapse, and changes fall into four broad processes; 1) new synapses are grown or 2) synapses are removed, thereby changing the connections within a circuit, alternatively 3) the strength of existing synapses are increased 4) or decreased, thereby changing the efficacy of specific connections. It is these dynamic changes that constitute synaptic plasticity. While many processes contribute to synaptic plasticity, it is thought that translation is necessary for changing gene expression at a synapse.

There is substantial evidence that, at least in part, the translation events that govern plasticity occur in specific cytoplasmic domains, often local to the synapse. Local translation is thought to facilitate swift, and synapse-specific changes in gene expression at great distances from the nucleus. Thus, if translation occurs at synapses, then mRNA must be localized to synapses, even if only transiently. As will be discussed, there is much evidence that this is the case in many different systems. The third instar larva is a highly mobile, and rapidly growing phase of the *Drosophila melanogaster* life cycle. The larval

bodywall muscle allows the larva to crawl, and increases over 40-fold in size in five days. To maintain contraction efficacy, the neurons that innervate the larval muscle must expand in parallel. To this end, the larval neuromuscular junction (NMJ) is a highly plastic, but relatively simple synapse. Coupled with the range of genetic tools available to the fruit fly researcher, the *Drosophila* larval NMJ is a popular and powerful model for addressing key questions in neuroscience. In this study the localization of mRNAs encoding synaptic proteins was investigated in both neuron and muscle at the *Drosophila* larval NMJ. It is hypothesized here that mRNA localization contributes to synaptic plasticity. This was interrogated by assaying the distributions of key mRNAs in mutants in which key processes that constitute synaptic plasticity are perturbed. Moreover, the conserved mRNA-binding protein Syncrip, which is known to bind a number of mRNAs encoding key synaptic proteins, was an experimental focus. Syncrip is required for mRNA localization and translation in the oocyte, and it is hypothesized here that, at least in part, Syncrip regulates NMJ structure and function through mRNA localization and local translation. Synaptic plasticity is central to memory and learning, and while these higher order processes may not occur at neuromuscular junctions, the same plastic changes are shared between NMJ synapses, and those in the central nervous system. Indeed, many of the molecular components that constitute the NMJ synapse, and even the primary neurotransmitter, are conserved between the larval NMJ and the vertebrate brain. To introduce this thesis, first mRNA localization, and the mechanisms behind this phenomenon, will be discussed. Then key examples will be used to illustrate what is known about mRNA localization in neurons and muscle. Finally, the model system of choice, the larval NMJ will considered, and Syncrip, a protein of interest to this study, will be introduced.

Aims of this chapter:

- **To introduce mRNA localization as a conserved mode of post-transcriptional regulation.**

- **To survey the different mechanisms of mRNA localization through select examples.**
- **To discuss the evidence for mRNA localization and local translation in neurons and muscles in different systems.**
- **To present the larval neuromuscular junction as a simple system in which to study synaptic plasticity.**
- **To introduce the conserved mRNA-binding protein Syncrip, and discuss its role in mRNA metabolism.**

mRNA localization is a conserved mechanism for targeting gene expression

The subcellular localization of messenger RNA (mRNA) is an important and conserved mechanism for regulating changes in gene expression at specific cellular locations. Almost thirty years ago *in situ* hybridization in *Ascidia* embryos (*Styela plicata*) correlated the asymmetric distribution of *β-actin* mRNA with tissue differentiation (Jeffery *et al.* 1983). Jeffery *et al.* not only provided the first example of mRNA localization, but also illustrated its biological significance, supporting previous work that postulated mRNA to act as a cytoplasmic determinant of cell fate (Kalthoff. 1979). Soon after, it was observed that while bulk mRNA was distributed evenly throughout chicken embryonic myoblasts and fibroblasts, specific mRNAs encoding cytoskeletal proteins exhibited non-homogenous distributions (Lawrence and Singer 1986). This, and other examples soon after, reinforced the concept of mRNA localization as a mechanism for regulating gene expression (Recently reviewed in Holt and Bullock. 2009; Meignin and Davis, 2010).

More recently vast leaps have been made in the field, characterizing mRNA localization in a range of organisms, from fungi and plants, to amphibians and mammals (Reviewed in Meignin and Davis. 2010; Zarnack and Feldbrügge. 2007; Okita and Choi. 2002). In

particular, large-scale approaches have confirmed mRNA localization as a ubiquitous mode of regulation. Recently, a high-throughput fluorescent *in situ* hybridization (*FISH*) screen in *Drosophila* embryos examined the localization pattern of ~25% of the genome (Lécuyer *et al.* 2007). The study revealed that over 70% (1644) of mRNAs were classified as asymmetrically distributed, revealing 35 different patterns of distribution within a single cell. In addition, genome-wide screens in *Xenopus* and human cell extracts have revealed specific subsets of RNAs that are enriched on mitotic microtubules (Blower *et al.* 2007). Also, a screen in cultured mouse embryonic fibroblasts isolated actively growing cell protrusions, and characterized the mRNAs localized within, revealing 50 new localized transcripts (Mili *et al.* 2008). While many advances have been made in developing systems, mRNA localization has also been studied in differentiated somatic cells (Huttelmaier *et al.* 2005), including muscle (Adereth *et al.* 2005; Ganesan *et al.* 2012), and most notably neurons (Reviewed in Kiebler and Bassell. 2006). In addition to amassing a list of asymmetrically localized mRNAs, much work has been done in the field to elucidate the different mechanisms by which mRNAs are spatially restricted.

Key mechanisms of mRNA localization

i) Selective transcription in multi-nuclear syncitia

Syncitia such as the *Drosophila* blastoderm embryo and most muscle fibres, contain multiple nuclei distributed throughout a single cell. One of the simplest forms of mRNA localization is to restrict transcription to specific nuclei, with particular spatial arrangements. This mechanism of mRNA localization has been observed in both the *Drosophila* blastoderm embryo and muscle fibres. For example, in vertebrate muscle syncitia, mRNAs encoding the δ-subunit and ε-subunit acetylcholine receptor (AChR) are transcribed in nuclei proximal to the site of AChR protein expression, the NMJ. Local transcription is restricted to specific nuclei by neurotransmission and the expression of specific signaling molecules, and leads to mRNA accumulation at the NMJ postsynapse

(Figure 1.1 A) (Burszjtajn *et al.* 1989; Rotundo 1990; Schaeffer *et al.* 2001; Fromm and Rhode. 2004; reviewed in Chakkalakal and Jasmin; 2003).

Figure 1.1. Different mechanisms of mRNA localization

The *Drosophila* blastoderm embryo is another well-studied syncitium, where multi-layered control of transcription across multiple nuclei leads to an intricate pattern of mRNA localization (Schroeder *et al.* 2004; Hou *et al.* 2009). These mRNA patterns then contribute to embryonic patterning. The spatial restriction of mRNAs in the *Drosophila* embryo is not solely restricted in space by local transcription however. Local transcription can be combined with perhaps the most prevalent mode of mRNA localization, active transport by molecular motors.

ii) Active transport by molecular motors

Transport of mRNA along microtubules by molecular motors is thought to be the most common mechanism for localizing mRNA. The cytoplasmic destination for an mRNA is usually the product of *cis*-acting structural signaling elements in the mRNA that are frequently, but not always, found in the untranslated regions (UTRs). These localization signals then recruit a changing cast of RNA-binding proteins (RBPs) from the nucleus and cytoplasm that dictate the translational status, stability, and through association with motor complexes, cytoplasmic destination of the mRNA. Intriguingly, there is strong evidence that the mRNA can signal to dictate the type of motor protein that it is carried by, the speed of transport, and the choice of microtubules that the motors move along (Van De Bor *et al.* 2005; Zimyanin *et al.* 2008; Krauss *et al.* 2009). By recruiting different mRBPs, motors, and by modulating motor protein properties, mRNAs can influence their own destination. mRNA transport by each of the major molecular motor classes has been shown in different systems; mRNAs are either transported along microtubules by Dynein or Kinesin, or along actin by Myosin (Bullock. 2007).

Motor-based transport is the most rapid mechanism for sorting mRNAs, and can traverse long distances. This is an especially advantageous mechanism in development, where large cells must establish polarity within a strict temporal program. For example, in the

Drosophila blastoderm embryo, *fushi tarazu, wingless,* and other pair rule transcripts localize to the apical cytoplasm by dynein-dependent active transport (Figure 1.1 B). The mRNA signals that target *wg* mRNA to the apical cytoplasm are conserved across *Drosophiladae* family and are thought to form 3D structures that recruit mRNPs and dynein (Wilkie *et al.* 1999; Davis and Ish-Horowicz; dos Santos *et al.* 2008; Wilkie and Davis. 2001). Further, in the *Drosophila* oocyte, the axes-specifying mRNAs *gurken, bicoid,* and *oskar* are all directed to distinct localization patterns by molecular motors (Figure 1.1 E). *bicoid* mRNA is localized to the anterior of the stage 9 oocyte along microtubules, while *gurken* is restricted to the dorso-anterior corner. Both *gurken* and *bicoid* are transported to their subcellular domains by dynein motor complex along microtubules (Schnorrer *et al.* 2000; Delanoue *et al.* 2007). In contrast, localization of *oskar* mRNA to the posterior pole proceeds by Kinesin heavy chain 1 motor complex, through a biased random walk (Palacios and St Johnston 2002; Zimyanin *et al.* 2008). Similarly to the oocyte and embryo, active transport is likely to be highly prevalent in restricting protein expression in large somatic cells, such as neurons (see below; reviewed in Kiebler and Bassell. 2006) and possibly muscle (see below). While more prevalent in larger cell types, mRNA localization also occurs in smaller cells. For example, in budding yeast *ASH1* mRNA localizes almost entirely from the mother cell to the bud tip to specify the daughter cell mating-type (Bertrand *et al.* 1992). This localization is striking and requires the recruitment of She2P, a nucleocytoplasmic trafficking protein. She2P is the primary *ASH1* binding protein, and interacts with the motor machinery She1p, a type V myosin motor, through a bridging molecule She3P (Figure 1.1 F) (Bookwalter *et al.* 2009).

iii) Degradation and stabilization

Protecting mRNAs from degradation in some regions of the cell, but not others, will lead to a subcellular enrichment of specific mRNAs. In the *Drosophila* embryo, selective degradation restricts a number of mRNAs to the posterior pole plasm, including *nanos*

mRNA (Figure 1.1 C). *nanos* mRNA is distributed throughout the embryo, and only 4% of *nanos* mRNA is localized and protected from degradation at the pole plasm (Bergstein and Gavis. 1999). The translational regulator Smaug interacts with 3'UTR of *nanos* mRNA and recruits the CCR4/POP2/Not deadenylase complex to deadenylate *nanos* mRNA and target it for destruction. At the pole plasm however, Smaug is prevented from destabilizing *nanos* mRNA by Oskar protein (Zaessinger *et al.* 2006). Thus, through selective destabilization, *nanos* mRNA and translation is restricted to the pole plasm. Similarly, in the zebrafish embryo pole plasm, mRNA stability is spatially regulated to sort transcripts to specific regions. *vasa* mRNA is stabilized in the primordial germ cells by *cis*-acting mRNA sequences, while *vasa* mRNA that is partitioned elsewhere (in somatic cells) is rapidly degraded (Wolke *et al.* 2002). Further, in the sea slug *Aplysia*, mRNAs in sensory neuron dendrites appear to be selectively stabilized by synapse formation between neurons (Hu *et al.* 2002). As a result, it appears that selective degradation of mRNAs is a conserved mechanism for mRNA localization in both developing and somatic cells.

iv) Diffusion and anchoring.

While selective degradation removes mRNA from subcellular regions to restrict localization, an alternative mechanism is to entrap diffusing mRNAs at the site of localization. Continual entrapment and anchoring can be achieved by a static molecular 'snare' or 'tether', and will progressively localize the free diffusing mRNA almost exclusively to one region. For example, in late *Drosophila* oogenesis several mRNAs were hypothesised to be restricted by this mechanism, including *nanos,* and later confirmed by live cell imaging of *nanos* mRNAs (Lantz *et al.* 1999; Forest and Gavis. 2003). The diffusion of *nanos* mRNA throughout the oocyte is enhanced by cytoplasmic flow caused by streaming, and the subsequent trapping of *nanos* at the pole plasm is facilitated by actin (Figure 1.1 D) (Forest and Gavis. 2003).

Though the different mechanisms introduced here are discussed separately, it is important to note that export of mRNA from the nucleus, to cytoplasmic localization, translation, and then degradation, is a multi-step process. Any combination of mechanisms can be employed to regulate mRNA localization. One cell type that is thought to be particularly dependent on mRNA localization to function, and may employ a number of different mechanisms of distribution, is the neuron. To this end, mRNA localization in neurons is a prominent focus of research. While examples of neuron-localizing mRNAs are known, less is known about the mechanisms that distribute mRNAs in neurons compared to other systems. Nevertheless, exciting advances are being made in determining how mRNAs are localized within the elaborate and specialized appendages of the neuron.

mRNA localization is implicated in neuronal growth, repair, and synaptic plasticity

Like many of the cell types in which mRNA localization has been found to be prevalent, neurons are large and highly polarized. Moreover, neurons exhibit a non-determinant growth pattern and can change shape, through growing and pruning synapses, as differentiated and somatic cells. The ability to change structure is thought to be dependent on translation within specific domains of the neuron, such as dendrites, axons, and synapses (Figure 1.2) (Reviewed in Wang *et al.* 2010). The evidence for localized mRNA translation in neurons is compelling. Here a brief introduction to this evidence, and the role of mRNA localization in neurobiology, will be given with select examples.

Figure 1.2. Putative sites of local translation in neurons

i) mRNA localization in neurons appears to be a conserved mode of regulation.

Neurons pose a number of challenges to the spatial regulation of gene expression. First, neurons produce highly polarized elaborations with non-determined branch structures. Second, neuronal appendages can grow to great lengths (in humans, the sciatic nerve axon is frequently over 1 metre). Third, a mature neuron can form multiple synapses with other cells (up to 10,000 in mammalian central nervous system) and each synapse can demand distinct patterns of gene expression. As a result, if every change in gene expression at a synapse was dependent upon *de novo* transcription, translation, and protein trafficking to the synapse, it is likely that synaptic plasticity would not occur on a physiologically relevant timescale. An alternative mechanism is to prelocalize mRNAs and ribosomes to dendrites, axons, and synapses, and to regulate translation locally in a synapse-specific manner. This model for local translation was first proposed almost 30 years ago, when polyribosomes were observed in the dendrites of rat dentate gyrus neurons by electron microscopy (Steward and Levy 1982). The authors wrote, “it is difficult to conceive of a situation which is intuitively more appealing as a mechanism for protein synthesis-dependent maintenance or modification of a synapse”. While this study was initially met with skepticism, the importance of mRNA localization in neurons is now widely accepted (reviewed in Wang *et al.* 2010), and has been directly observed in a number of systems (Wang *et al.* 2009; Lyles *et al.* 2006; Campbell and Holt 2001; Leung *et al.* 2006). Indeed local translation and mRNA metabolism in neuronal appendages has emerged as a leading research focus owing to its importance in plasticity and neuronal pathologies (see below). Consistent with the idea that local translation facilitates changes in gene expression in specific regions of the neuron, such as axonal compartments or individual synapses, multiple studies have revealed proteins involved in mRNA localization and translation to be central to neuronal function (Kraut *et al.* 2001; Sau *et al.* 2011;Reviewed in Jung *et al.* 2010). Furthermore, large, unbiased screens have also

revealed hundreds of neuron-localized mRNAs in mollusk and mammalian systems (Eberwine *et al.* 2002; Moccia *et al.* 2003; Poon *et al.*, 2006, Suzuki *et al.* 2006). Of these studies, some key examples illustrate current thought of the role of mRNA localization in the neuron.

ii) Axon-localized mRNA facilitates growth and repair.

Axon-localized mRNAs have been correlated with axonal growth and pathfinding, and many cytoskeletal proteins, and their cognate mRNAs, are found in growing axons from multiple systems (Olink-Coux and Hollenbeck, 1996; Bassell *et al.*, 1998; Eng *et al.*, 1999; Zheng *et al.*, 2001; Lee and Hollenbeck, 2003; Moccia *et al.*, 2003; Willis *et al.*, 2005). These studies have revealed a number of axonal-localized mRNAs, by both *FISH* and non-biased biochemical screens, most of which encode proteins involved in the cytoskeleton or axon regeneration. Confirmation that axon-localized mRNAs were translated in growing neurons (named growth cones) came from studies in the living *Xenopus* embryo (Figure 1.2 D) (Leung and Holt. 2008). cDNA encoding β-actin, an axon-localized mRNA, was tagged with a photoconvertible fluorophore named Kaede. Micro-injection of β-actin-Kaede in *Xenopus* blastomeres lead to specific expression in retinal axonal growth cones. Photoconversion of β-actin-Kaede revealed *de novo* translation of β-actin-Kaede in the axon growth cone. This powerful study confirmed previous work that correlated *β-actin* mRNA translation in axon growth cones with morphogen-lead pathfinding (Leung *et al.* 2006). In the mature axon, local translation has also been observed in response to injury in a number of studies (Figure 1.2 E) (reviewed in Willis and Twiss 2006). In a fascinating study, axons in the peripheral nervous system of live rats were crushed, and the levels of the neuropeptide calcitonin were measured in the axon (Toth *et al.* 2009). While the authors had previously shown that calcitonin accumulated in injured axons, but decreased in the cell body (Arvidsson *et al.* 1990), further work showed that local administration of small interfering RNA against either

calcitonin, or its receptor, at the site of injury inhibited axon regeneration. Further, in the mollusk *Aplysia californica*, crushing of pedal neurons *in vivo* led to the long-term enrichment of *Aplysia β-thymosin* mRNA in injured axons, which encodes a homologue of the wound-healing factor β-thymosin. The axonal-localization of *β-thymosin* mRNA was correlated with a particular phase of wound healing, many days after injury. Importantly, this result was recapitulated both in cultured *Aplysia* neurons, and *in vivo* (in live *Aplysia*). In addition, more recent work has furthered previous observations, in which *β-actin*, but not *γ-actin* mRNA, was observed to localize to cultured mammalian axons (Bassell *et al.* 1998; Zheng et al 2001; Tiruchinapalli *et al.* 2003), a process dependent upon the 3'UTR of *β-actin* mRNA (Kislauskis *et al.* 1994). Willis *et al.* further these observations by reproduction *in vivo* (Willis *et al.* 2011). *β-actin*, but not *γ-actin* mRNA, is localized to mature axons, and injury increased *β-actin* enrichment only. Moreover, injury leads to an increase in β-actin protein in axons. These studies clearly demonstrate that at least one function of mRNA localization and local translation in neurons is to repair axonal damage.

It is not clear, however, whether translation occurs in mature uninjured axons. Localized translation has been shown to occur mostly in growing axons, injured mature axons, or dendrites. It is of note that while dendritic ribosomes have been readily observed in a number of systems, axonal ribosomes proved elusive in ultrastructural studies (Lasek *et al.* 1973). Coupled with the postulates that axonal proteins could arrive from slow transport from the cell body, or even from adjacent glial cells by an unknown mechanism, lead to the initial rejection of the idea of axonal protein synthesis (Gainer *et al.* 1977). Indeed, early studies that showed axons to contain the necessary components for translation, including tRNA and substantial levels of ribosomes, were met with contention (Black and Lasek 1977; Guiditta *et al.* 1980; reviewed in Piper and Holt 2004). While more recent advances have since confirmed that axons in a range of different systems are competent for translation, it is still not clear whether this occurs constitutively in axons, or only in

response to growth or injury. An interesting study in adult rats showed that incubation of freshly dissected sciatic nerve segments with radiolabeled amino acids produced *de novo* protein (Hanz *et al.* 2003). Similarly, translation-dependent labeling of axonal preparations from other invertebrates (crayfish, snail, and mollusk) support the notion that maintenance of translation machinery in axons is a conserved phenomenon (Sheller and Bittner 1992; Davis *et al.* 1992; Van Minnen *et al.* 1997; though see Martin *et al.* 2000 for a discussion into why invertebrate axons are not always good models for vertebrate axons). A final study of note has recently confirmed both the presence of ribosomes in mature axons, and a fascinating novel mechanism by which ribosomes are transferred to injured axons from their associated glial cells in mice (Figure 1.2 F) (Court *et al.* 2008; Eyman *et al.* 2007; Alvarez 2001). The local supply of ribosomes to axons is a fascinating mechanism for regulating local translation, and could potentially explain why detection of ribosomes in some axonal systems has proved elusive for so long. While these studies support the notion that uninjured axons contain both mRNA and full translation machinery, and are thus competent for translation, these data do not show that translation occurs in uninjured axons *in vivo*. Nevertheless, the observation that mature uninjured axons are competent for translation, and contain axon-localized mRNAs, suggests that at minimum, somatic axons are primed ready for translation, if not constitutively active.

iii) mRNA localization and synaptic plasticity.

While the localization of mRNA to broad subcellular compartments, such as axons, can facilitate growth and repair, a finer degree of local translation is required for the synapse-specific changes in gene expression that constitute plasticity. To this end, mRNA is localized to synapses, allowing individual synapses to translate new proteins in a stimuli-specific manner. While a great many dendrite-localized mRNAs are known, examples will be focused on where the role of the localized mRNA is known to contribute to, or at least correlate with, changes in synaptic plasticity (Figure 1.2 A) (For general reviews see

Martin *et al.* 2000; Sutton and Schuman 2006; Bramham and Wells 2007; Wang *et al.* 2010). A key study that showed the requirement for local translation in neurons was performed on rat hippocampal slices. Transmission across synapses in the hippocampus was shown to increase in amplitude upon incubation with the neurotrophic factors neurotrophin-3 and brain-derived neurotrophic factor. This was shown to occur independently of the cell bodies (removed by dissection). Moreover, application of two different inhibitors of translation was sufficient to prevent the plastic changes that increased synaptic transmission (Kang and Schuman, 1996). While this study did not show which mRNAs were localized to the axons or dendrites in the rat hippocampus, it did illustrate the requirement for local translation away from the cell body for synaptic plasticity. In a more recent study, live-cell imaging of photoconvertible proteins in *Aplysia* synapses has demonstrated that mRNA localizes to synapses, and is translated locally to induce plastic changes (Wang *et al.* 2009). Moreover, upon repetitive stimulation of the cultured sensory and motor neurons, translation was spatially restricted to stimulated synapses only. This study showed that mRNA is localized to synapses *in vivo*, and in translation occurs in a synapse- and stimulus-specific manner.

In mammals, one of the best-studied dendrite-localized mRNAs is *Arc,* which encodes Activity-Regulated Cytoskeleton-Associated Protein. *Arc* mRNA, is transcribed in response to neuronal activity, and localized to recently-activated post-synaptic regions, where it serves as a template for activity-dependent translation (Link *et al.* 1995; Steward *et al.* 1998; Moga *et al.* 2004; Rodriguez *et al.* 2005; Reviewed in Bramham *et al.* 2010). It has been shown that the local accumulation of *Arc* mRNA and protein in dendrites and synapses correlates with the maintenance, but not establishment, of long-term memory (Guzowski *et al.* 2000). Indeed, mice lacking Arc exhibit similar deficiencies in long-term, but not short-term, memory (Plath *et al.* 2006). The wealth of studies focused on *Arc* mRNA place mRNA localization and local translation as central to synaptic plasticity in mammals, and thus to memory formation also. Indeed, plastic changes in synapse

efficacy have long been associated with memory formation, but have never been directly shown to be linked in mammals. This association was elegantly demonstrated, however, by a study in adult *Drosophila* (Ashraf *et al.* 2008). Olfactory conditioning is a powerful technique in flies, and combined with the tractable genetics of *Drosophila*, offers unique molecular insights into the basis of memory. As with other species, long term memory formation upon conditioning is dependent upon translation in flies (Tully *et al.* 1994). Ashraf *et al.* showed through live imaging of *CaMKII* mRNA in adult brains (see Chapter 4 for an explanation of the MS2 system) that transport of *CaMKII* mRNA to dendritic processes correlated with memory formation, and that synaptic protein synthesis was necessary for memory formation.

While the above studies provide evidence for mRNA localization in synaptic plasticity, in almost all cases mRNA localization is correlated with synaptic plasticity in dendrites, rather than the axons. While dendrites have been studied to a great extent, there are only a handful of cases in which mRNA has been shown to localize to a mature presynapse at the axon terminal (Figure 1.2 F). The best-studied presynaptic mRNA is *sensorin*, which localizes to the presynapse formed between *Aplysia* sensory and motor neurons in culture. The formation of synapses between sensory and motor neurons appears to stabilize *sensorin* mRNA at the presynapse (Schacher *et al.* 1999; Lyles *et al.* 2006). Serotonin-induced plasticity results in Sensorin translation in individual neurites, and protein reporters under the control of *sensorin* 5'UTR and 3'UTRs are translated at the presynapse in response to serotonin (Liu *et al.* 2003; Wang *et al.* 2009). Further, it has been shown that presynaptic translation is required for vesicle recycling in both developing and mature rat hippocampal cultures (Sebeo *et al.* 2009). While there is strong evidence for presynaptic translation, most research in the mature neuron has been focused on the postsynaptic translation. To this end, presynaptic translation is observed in only a handful of cases outside of development, and is considered a relatively contentious issue (Reviewed in Akins *et al.* 2009).

iv) Mechanisms of mRNA localization in neurons

While large-scale screens in developmental systems (such as the oocyte and embryo) have provided key insights into the mechanisms of mRNA localization, far less is known about how neuronal mRNAs reach their destination. Nevertheless, a number of key studies have revealed insights into the mechanisms behind mRNA localization in neurons.

In a key study, fluorescently-labeled *myelin basic protein (MBP)* mRNA was microinjected into cultured oligodendrocytes (Ainger *et al.* 1993). The *mbp* mRNA formed granules that were rapidly transported along microtubules at speeds comparable with motor complexes (~0.2μms). This work first characterized mRNA dynamics in neurons, and described the bidirectional movement of mRNA transport particles of various sizes, likely by motors. Consistent with these observations, a following study bulk-labeled mRNA in live cells used the membrane-permeable SYTO14 dye to visualize the dynamic movements of endogenous RNA in cultured neurons. Labeled RNA was distributed non-randomly in discrete foci of various size, and a small subpopulation exhibited movement with speeds similar to those previously observed (~0.1μms) (Ainger et al 1993). Moreover, the transport particles exhibited bidirectional movement and trafficking was dependent upon microtubules. Further support for the role of motor proteins in neuronal mRNA trafficking came from biochemical extraction of native mRNPs. It was found that conventional kinesin heavy chain KIFb associates with *CaMKII* and *Arc* mRNAs, both of which are known to localize to dendrites. It was found that lowering conventional kinesin heavy chain (KIF5b) expression disrupted mRNA localization, while overexpression of KIF5b lead to enrichment of mRNA into the most distal dendrites (Kanai *et al.* 2004). Indeed, in a separate study, it was shown that antisense knockdown of KIF5b lead to a decrease in the translocation of micro-injected mRNA into dendrites (Ainger et al 1993). While mRNA trafficking in neurons is far from understood, it appears that motor protein complexes represent the prevalent mode of transport. While the use of motor proteins to distribute

mRNAs across the great distances of neuronal processes is intuitive, little research has been done on the diffusion of macromolecules in neurons. While macromolecules are known to diffuse more slowly in neuronal processes than in free water (Popov and Poo. 1992), neurons can last a lifetime and the diffusion of mRNA and other large molecules should be considered as an inevitable form of distribution.

As in other systems, the destination of a neuron-localized mRNA appears to be a dictated by *cis*-acting localization signals. For example, *Arc* mRNA contains two dendritic targeting elements in the mRNA 3'UTR that are necessary for dendritic localization of transcribed (but not injected) *Arc* mRNA in hippocampal neurons (Kobayashi *et al.* 2005). Similarly, the recruitment of *CaMKII* mRNA to dendrites in the adult fly central nervous system during memory formation is dependent on RNA elements in the 3'UTR, which are sufficient for synaptic localization (Ashraf *et al.* 2008). Interestingly, a recent study has indentified two different neuron-localization signals within the same mRNA (Meer *et al.* 2012). In cultured *Aplysia* sensory and motoneurons, the export of *sensorin* mRNA from the cell body to the neurite, and then localization to the synapse, are controlled by two distinct signals. The 3'UTR of *sensorin* mRNA is sufficient for export to distal processes, while the 5'UTR is required for concentration of mRNA at synapses. The authors refined their search for the 5'UTR localization element to a 66-nt *cis*-acting signal. Similarly, in a different study, microinjection of different mRNAs into oligodendrocytes lead to the discovery of the conserved A2 response element necessary to localize multiple mRNAs to dendrites (Shan *et al.* 2003). Examples where the the *cis*-acting mRNA localization signals are known for neuron-localizing mRNAs however, are rare. Finally, in some cases the mechanism of localization for a transcript can be conserved between different tissues. As a result, insight into how a mRNA may localize in a neuron may be gained from studies in more amenable cell types. For example, *inscuteable* mRNA was shown to localize apically in *Drosophila* neuroblasts, though the mechanism was unknown. Microinjection of *inscuteable* mRNA into the *Drosophila* blastoderm embryo however showed that

inscuteable mRNA localized apically in this cell type also (Hughes et al. 2004). Microinjection studies in mutant embryos revealed that *inscuteable* mRNA is trafficked by dynein in the blastoderm embryo, and gave the researchers insight into how mRNA localization occurred in neuroblasts.

While the above studies mark significant progress in understanding how mRNA is trafficked in neurons, the regulation of motor proteins, and the *cis*-acting mRNA sequences involved, are still poorly understood in comparison to examples in developing systems. Nevertheless, mRNA localization is central to fundamental processes, such as memory, learning, injury response and growth, and as a result is a key research focus. The study of mRNA localization in muscle, however is not pursued by as many research groups. Nevertheless there are strong links between mRNA metabolism and muscle function and disease.

mRNA localization in muscle and disease.

mRNA localization is an active field of research with a great depth of history. While mRNA localization in development and neurobiology are a particularly strong point of interest, little is known however about mRNA localization in muscle. There are relatively few examples of asymmetric localized mRNAs, and only a few documented interrogations of mechanism. Moreover, most studies are restricted to cultured developing cells, which are largely grown in the absence of innervation and contraction, two processes that are central to muscle biology. The lack of research into mRNA localization in muscle is surprising for a number of reasons. First, muscles are large and form highly polarized contacts with other cells, two properties that have long been popular in choosing models for studying mRNA localization, including oocytes, embryos and neurons. Second, mRNA biology is known to be central to muscle function. mRNA *FISH* methods in muscle have been published for decades, and have shown mRNAs to localize to key subcellular

structures, and to change distribution throughout development (Figure 1.3). Despite these data, relatively few studies have exploited these techniques to further understanding. While mRNA *FISH* screens have provided powerful insight into the extent of mRNA localization, and the governing mechanisms, in multiple tissues, no such studies have been performed in muscle (Lecuyer *et al.* 2008; Wilkie *et al.* 2001b). Third, myopathies represent a key research focus in medicine. While myopathies are not common (Muscular dystrophy affects ~1/17,000, inflammatory myopathies ~1/1000), they greatly affect the lives of patients, and represent a significant burden on health services. Indeed, the cost to health services for myopathies exceeds that of more prevalent serious diseases, such as rheumatoid arthritis and systemic sclerosis (Bernatsky *et al.* 2011). One major reason for the cost of patient care is myopathies are degenerative and currently without cure. While muscular dystrophy can be fatal, particularly in children or early adolescents, most myopathies will affect a patient's quality of life for many years and require persistent treatment. As over 14% of myopathies are inherited, including the most common forms of Muscular Dystrophy (MD), gene-association studies are prevalent. Research into one congenital form of MD, Myotonic Dystrophy has placed mRNA as central to disease via a novel, and so far unique, disease mechanism never observed in disease models. Genetic lesions associated with Myotonic Dystrophy type 1 were mapped to a CTG repeat expansion in a gene Dystrophia Myotonic Protein Kinase (DMPK) (Brook *et al.* 1992; Tanjea *et al.* 1995). These expanded CTG repeats in the gene, lead to CUG repeats in the *dmpk* mRNA and restrict the mRNA to the nucleus. Accumulation of *dmpk* mRNA in muscle nuclei sequesters essential splicing factors and causes Myotonic Dystrophy. Subsequent to these data, Myotonic Dystrophy type 2 was also shown to be caused by expansive repeats of a similar sequence, CCUG, in a different gene (Liquori *et al.* 2001; Margolis *et al.* 2006). This research shows the profound role mRNA localization plays in muscle disease via a novel mechanism, and may provide insight into the role of mRNA toxicity in other non-muscle pathologies. Despite this major advance, the role of mRNA in muscle disease outside of Myotonic Dystrophy is not widely researched. Here a brief

introduction into past and current research on muscle mRNA localization is presented (for reference see Figure 1.3).

Figure 1.3. Proposed sites for local translation in muscle

As with many different tissues, the localization of mRNA is necessary for muscle specification in a range of developing organisms. In *Ascidians*, localization of maternal mRNAs is necessary for tissue specification. A number of mRNAs first localize to the vegetal pole of the egg, and then to a region of cortical cytoplasm. Many of these mRNAs then proceed to segregate asymmetrically into different daughter cells during subsequent divisions, and end up in two posterior cells of the gastrulating embryo. Of these mRNAs, *macho-1* is both necessary and sufficient for muscle specification. *macho-1* encodes a Zinc Finger Nuclease and is competent for specifying muscle-fate when injected into non-muscle embryonic cells (Nishida and Swahada 2001).

Following the specification of muscle fate, asymmetric mRNA localization has been shown to occur within embryonic myoblasts and muscle cells. In myoblasts from different vertebrate models, two dominant mRNA localization patterns have been observed; restriction to the myoplasm periphery, and to the perinuclear space. Original studies in chicken embryonic myoblasts showed that although polyadenylated RNA was homogenously distributed throughout the cell, actin mRNA was concentrated to myoblast peripheries (Figure 1.3 D) and *tubulin* and *vimentin* mRNAs (both or which encode cytoskeletal proteins) were most concentrated at the perinuclear region (Figure 1.3 C) (Lawrence and Singer 1988). Further, mRNAs encoding two cytoplasmic isoforms of Creatine Kinase (CKM and CKB), an enzyme central to energy maintenance in contraction, were observed to have these two different localization patterns in mouse myoblasts. *ckb* mRNA was found at the cell periphery, while *ckm* mRNA exhibited a striking perinuclear localization (Wilson *et al.* 1995). The distributions of both mRNAs were dependent upon sequences in the mRNA 3'UTR. Moreover, the localization elements present in *ckb* and *ckm* 3'UTRs specified localization in muscle only; transfection of mRNAs into non-muscle cells did not lead to mRNA localization. This suggests that muscle-specific mRNA localization pathways operate. In addition to *creatine kinase*

mRNAs, the mRNAs encoding different isoforms of Actin exhibit different preferences for the nucleus periphery and myoplasm periphery. mRNA encoding β-actin localizes to the myoblast cell periphery and the perinucleus, while α- and γ-*actin* mRNAs are largely perinuclear and excluded from the cell periphery (Hill and Gunning. 1993). More recent studies in cultured mouse myoblasts have identified the *cis*-acting element that specifies a perinuclear distribution of mRNA encoding Slow Troponin C (sTnC) (Reddy *et al.* 2005). The authors show that a 40-nt sequence in the *sTnC* mRNA is both necessary and sufficient to direct perinuclear mRNA distribution. The perinuclear distribution of mRNA in muscle, and the synthesis of proteins in the same region, is consistent with the role of nuclei as organizing centres in muscle. The perinuclear region in muscle has been shown to be enriched with endoplasmic reticulum and translationally active ribosomes and may be a common destination for mRNAs.

In embryonic muscle cells derived from myoblasts, similar mRNA distributions are observed at the perinuclear region and the cell periphery, however, mRNAs have also been shown to localize adjacent to contractile structures. In 14-day embryonic chick pectoral muscle, electron microscopy revealed that mRNA encoding Myosin Heavy Chain, a principle contractile component, was localized adjacent, but not colocalized, to contractile ultrastructures around the periphery of the muscle cell (Figure 1.3 B) (Pomeroy *et al.* 1991). *mhc* mRNA and polysomes were shown to orientate into filaments parallel to the longitudinal axis of the muscle. The authors postulate that translation proximal to myofilaments allows nascent proteins to assemble rapidly into contractile structures, but that assembly is not likely cotranslational. In contrast, subsequent studies suggested that mRNA localized to myofilaments are evidence of nascent proteins being directly assembled into contractile complexes as they are translated (Fulton and L'ecuyer. 1993). *vimentin*, *desmin*, and *vinculin* mRNAs encode cytoskeletal components that localize to the costamere in the chick embryonic muscle, which connects the contractile machinery, the sarcomere, to the cytoskeleton (Fulton and L'ecuyer. 1993). Interestingly, the

corresponding proteins localize to the same region at an earlier developmental stage, suggesting that protein and mRNA are both actively localized within the muscle. Even more interesting was the observation that *vimentin* mRNA localized to the perinuclear region in myoblasts, but to the costamere in embryonic muscle cells (Lawrence and Singer 1988; Fulton and L'ecuyer. 1993). There are known examples of the same mRNAs changing localization pattern throughout development. For example, the oocyte-localizing transcript *gurken* is first restricted to the posterior, then dorsal-anterior regions of the *Drosophila* oocyte. Following the localization of *vimentin* mRNA throughout muscle development showed a dynamic localization pattern that changed with development (Cripe *et al.* 1993). Consistent with the idea that muscle mRNAs redistribute during development, mRNAs encoding Utrophin, a protein considered as a therapeutic complement to mutant *dystrophin* in muscular dystrophy, redistribute during development to associate with cytoskeletal-bound polysomes (Gramolini *et al.* 2001). While *utrophin* mRNA associates with free cytoplasmic polysomes in non-differentiated cells, translation is restricted to the cytoskeleton in differentiated muscle cells.

Following embryonic development, the asymmetric localization of mRNA continues to occur in mature muscle. One study in which the distribution of *Myosin Heavy Chain (MHC)* was observed in adult and embryo rabbits, showed that *mhc* mRNA relocated from a 'patchy' distribution throughout the myotube in the embryo, to the contractile machinery in the adult (Morris and Fulton 1994). Moreover, the authors examined the localization of *mhc* mRNA in animal myotubes following injury by stretching. They reported that in regenerating adult muscle *mhc* mRNA formed a 'patchy' distribution similar to that observed in embryonic muscle. Despite the observation that mRNA localization is regulated in muscle disease, development, and injury, and that specific sequences of mRNA are necessary and sufficient for proper localization, the mechanisms by which mRNAs are distributed are largely unknown. One clear exception to this is the mechanism by which mRNAs are localized to synapses in vertebrate muscle fibres. In multinuclear

myofibers the d- and e-subunits of the Acetylcholine receptor (AChR) are selectively transcribed only in nuclei proximal to the NMJ. Localized expression of *aChR* mRNAs are dependent upon the neuron-derived growth factors and electrical signaling (Figure 1.3 A; Figure 1.1 A) (Brenner *et al.* 1990; Simon *et al.* 1992; Fromm and Rhode 2004; reviewed in Schaeffer *et al.* 2001; and Chakkalakal and Jasmin. 2002).

The studies presented here show that mRNA localization in muscle changes with development, disease, wound regeneration, and synaptic plasticity. This suggests a key role for mRNA localization in these processes. Moreover, these data suggest that translation can be locally restricted to key subcellular compartments, and in particular, the perinuclear region, the myoplasm periphery, the cytoskeleton, the postsynapse, and the contractile machinery. Muscle cells are large, and local translation may establish the polar organization of macromolecules necessary to contract with appropriate directionality. Despite these data, there are very few recent studies on mRNA localization in muscle, and fewer still research groups dedicated to this field.

The larval neuromuscular junction is a powerful model for studying synaptic plasticity.

The *Drosophila* larval neuromuscular junction (NMJ) has become a powerful and popular model for addressing key questions in neuroscience. The NMJ was first employed as a model system by Jan and Jan (Jan and Jan 1976, Jan *et al.* 1977, 1978) and then by Magazanik and Vyskocil (Antonov *et al.* 1979; Vyskochil and Magazanik. 1978; Magazanik and Vyskochil. 1979). These groups first described the physiology and pharmacology properties of the larval NMJ. Further, they provided the first genetic interrogation of synaptic transmission. Subsequent to these findings, the NMJ was adopted by other groups who took advantage of the simple synapse to study the effect of ion mutations on neurotransmission (Wu *et al.* 1978, 1983; Ganetzky and Wu 1982, 1983). Later, the NMJ was used as an accessible model for developing synapses by Kershishian

(Anderson *et al.* 1988; Halpern *et al.* 1991; Johansen *et al.* 1989). A key study into mutants of NMJ proteins revealed that the development of the NMJ was regulated by electrical activity, and thus, was regulated in a comparable manner to more complex systems (Budnik *et al.* 1990). Since then the larval NMJ was proved a useful tool for the genetic dissection of key fields in neuroscience, including synaptic plasticity, axon pathfinding, neuron trafficking, and cell signaling, and is a mainstay in the neuroscience journals and conferences (selected references: Budnik *et al.* 1990; Van Vactor *et al.* 1993; Kraut *et al.* 2001; Ataman *et al.* 2006; Yan Wong *et al.* 2012). Indeed a recent review summarizes how decades of *Drosophila* neuroscience, with many experiments performed on the larval NMJ, have contributed to a greater understanding of vertebrate neuroscience (Bellen *et al.* 2010).

The success of the NMJ as an experimental tool stems from a few key properties. First, the fillet (or flat) preparation of a larva is rapid (less than 5 minutes) and allows excellent accessibility to the body wall muscles and the neurons that innervate them, for both imaging and for electrophysiology. Second, the highly stereotyped pattern of bodywall muscle and motoneurons allows swift recognition of specific muscles in specific hemisegments. The larva contains 30 muscles innervated by approximately 36 motoneurons per hemisegment repeated in each abdominal segment. The muscles are large multinucleate single myofibres (ventral longitudinal muscles can be 40μm x 400μm) with distinct shape and orientations relative to the cuticle. The motoneurons extend from the larval central nervous system (CNS) in long axon bundles before branching off to innervate each hemisegment. Different motoneurons innervate different muscles in a highly specific and characteristic pattern. As a result, specific muscles, and even specific synapses, can be observed from animal-to-animal, thereby reducing experimental noise when observing large numbers of cells. Third, the larval NMJ holds a privileged place in neuroscience as a system where individual synapses can be counted and correlated with electrophysiological properties. This property is owing to the relative simplicity of the NMJ,

coupled with the fact that synapses are housed in large, readily discernable varicosities named boutons on the surface of muscle (Figure 1.4; see below). Boutons can be counted as an approximate and robust reflection of synapse number, and where necessary, the synapses themselves can be readily immunolabeled both pre and postsynaptically within a bouton. Finally, the most exploited property of the larval neuromuscular junction is that it is a continuously changing synapse, and that the size, structure, and efficacy, of this plastic synapse will respond to a number of stimuli. These properties underpin much of the research that employs the larval NMJ, including this study, and warrant further introduction. First however, the basic physiology of the larval NMJ will be discussed.

Figure 1.4. The basic physiology of the *Drosophila* third instar larval neuromuscular junction

i) Basic physiology of the larval NMJ

The larval bodywall consists of 7 repetitive abdominal segments (A1-A7) (See Figure 1.4 for reference throughout this section). Each segment is divided into two hemisegments, which are symmetrical about the ventral midline of the larva. Each hemisegment contains 30 uniquely-identifiable skeletal muscle fibres, repeated in each abdominal segment, with some variation in thoracic segments and the terminal segment (Figure 1.4 B). Each multinucleated muscle is large and readily identifiable owing to its distinct shape, size, and orientation. Within each segment approximately 36 motoneurons innervate the muscles at NMJs (Langraf and Thor 2006). Most muscles are innervated by two neurons, though muscle 4 is innervated by only one. Each motoneuron originates from the ventral ganglion of the larval central nervous system, and branches off from central axon bundles to innervate specific muscles with high fidelity and a characteristic NMJ branching pattern. An NMJ is composed of varicosities, or synaptic boutons, that penetrate into the surface of the muscle (Figure 1.4 D). Each bouton houses multiple synapses that innervate the muscle.

NMJs fall into three broad categories. The great majority of muscles are innervated by the first class, type I boutons, which release glutamate as the primary excitatory neurotransmitter (Jan and Jan 1976; Johansen *et al.* 1989). In addition to numerous large clear vesicles of glutamate (30-40nm in diameter), the spherical type I boutons contain mitochondria and endosomes (Atwood *et al.* 1993; Jia *et al.* 1993). Type I boutons are further classified into two types; the type I big (Ib) boutons and type I small (Is), which originate from distinct neurons. Type Ib boutons range in size from approximately 2.5-5μm in diameter, while type 1s boutons vary from approximately 1.5-3μm (Figure 1.4 D). In addition to their difference in size, another discerning feature exists between type Ib and type Is boutons. Type1b boutons are juxtaposed to a complex elaboration of membranous

folds in the membrane called the subsynaptic reticulum (SSR). The SSR is a key component of the synapse ultrastructure, and houses the postsynaptic glutamate receptors, as well as multiple ion channels, translational regulators, scaffolding molecules, and cell-adhesion complexes that maintain the synapse (Figure 1.4 E) (Budnik *et al.* 1990). The SSR apposing the type Is boutons is far less dense and elaborate than that of type 1b boutons. As a result, immunofluorescence of a SSR marker, such as the molecular scaffolding protein Discs Large, readily discerns type Ib and type Is boutons (See Chapter 3, Figure 3.1). Ultrastructural studies of type I boutons have correlated vesicle accumulation at presynaptic membrane with electron-dense structures named T-bars, owing to their resemblance to the capital letter T (Atwood *et al.* 1993; Jia *et al.* 1993). T-bars are the functional unit of the presynaptic site of release, named the active zone (AZ), and are thought to aid vesicle clustering, tethering, and release (Jia *et al.* 1993; Prokop *et al.* 1999; Verstreken and Bellen 2002; Kittel *et al.* 2006). Each bouton contains multiple active zones, precisely juxtaposed to clusters of glutamate receptors in the muscle SSR. Axon potentials arriving at a type I bouton leads to release of glutamate from presynaptic vesicles at active zones, which traverses the 15-20nm distance between the presynaptic and postsynaptic membranes, before binding to muscle glutamate receptors. The receipt of glutamate at the muscle triggers an increase in membrane permeability to Ca^{2+} and Na^{+} cations, both through the glutamate receptors themselves, and other voltage-gated channels. The downstream signaling effects of this change in electrochemistry induce contraction, before ATPase-dependent ion pumps restore the muscle to a resting state.

The second class of NMJ, type II, has much longer branches than type I NMJs that run the entire length of the muscle, but have smaller boutons (~1μm diameter), and completely lack any SSR (Johansen *et al.* 1989). Unlike type I motoneurons, which tend to innervate one muscle only, type II motoneurons innervate all but eight body wall muscles in the larva (Monastirioti *et al.* 1995). Type II boutons contain a mixture of both the glutamate and octopamine neurotransmitter vesicles and exhibit no clear presynaptic

specialization or active zones when examined by electron microscopy (Johansen *et al.* 1989; Monastirioti *et al.* 1995; Jia *et al.* 1993). Like type II boutons, the third type of boutons, type III, are small and multiple (referred to as elliptical boutons) but with smaller branches. Type III boutons release peptide neurotransmitters (including insulin-like protein and inter-leukins) and innervate different muscles in different segments (Cantera and Nassell 1992; Gorzyca *et al.* 1993). Compared to the clear presynaptic specializations that mark active zones in type I boutons, type II and type III vesicles release sites are poorly characterized. Indeed, the exact roles of type II and type III boutons are not clear, as mutational studies have revealed type I boutons as overwhelmingly dominant in regulating rapid contraction. Instead, it is thought that type II and type II NMJs perform a modulatory role, refining but not dictating contraction of muscle. Much work, including this study, focuses on the NMJ innervating muscles 6 and 7 of abdominal segments A2 and A3. Muscles 6 and 7 have a number of properties that lend themselves to study. First, muscles 6 and 7 are large longitudinal muscles that are particularly easy to locate. Second, in the fillet prep, muscles 6 and 7 lay on top of other muscles, and are the muscles nearest to the coverslip or microscope objective. As a result, muscles 6 and 7 have long been favoured for imaging and electrophysiology owing to their accessibility. Finally, while many NMJs have a highly characteristic shape, the NMJ on muscles 6 and 7 is one of the few junctions in which the neuron lies relatively flat on the muscles. As a result, it is easy to image in one optical plane.

ii) The larval NMJ is a constantly changing synapse that responds to various stimuli

While the wiring of the muscles is completed in embryogenesis, the rapid growth of the larva over five days, coupled with the dynamic movement of a larva in crawling, climbing, and eating, mean that the NMJ has to constantly grow to maintain contraction efficacy. As a result the NMJ continuously grows to new synapses, and new boutons and branches in which to house them, throughout the larval stage. Indeed the number of boutons

innervating a muscle will increase ten-fold during larval growth (Gorczyca *et al.* 1993; Keshishian *et al.* 1993). This process has been observed live, making the plastic growth of the larval NMJ a highly attractive model for studying a key aspect of synaptic plasticity, the assembly of new synapses (Zito *et al.* 1999). A key hallmark in the history of larval NMJ biology came from the observation that the growth of synapses is highly plastic and can respond to a variety of stimuli with changes in neuron morphology and synapse structure, number and efficacy (Budnik *et al.* 1990; Broadie, and Bate. 1993; Jarecki and Keshishian, 1995). This provided a quantitative insight into how the synapse changes in response to perturbations in plasticity. Crucially, mutagenic studies allowed the genetic dissection of the key components of plasticity, namely synaptic transmission, signaling and secondary messengers, and translation. For example, mutants that affect synapse excitability lead to compensatory changes in NMJ structure. The K^+ channels Shaker and Ether-a-gogo repolarize the membrane potential of muscle and neuron following synaptic transmission. Loss-of-function mutations in *Shaker* and *Ether-a-gogo* therefore result in persistent transmission and hyperexcitability, and subsequently expansion of the synaptic boutons and branches in which to house them (Budnik *et al.* 1990; Schuster *et al.* 1996). Conversely, mutations in Na^+ channels that depolarize (rather than repolarize) the neuron during an action potential, such as *Para*, lead to hypoexcitability and synapse undergrowth. Although the number of boutons observed in a *Para* mutant is similar to that of wildtype, they are smaller and house fewer synapses (Lnenicka *et al.* 2003). Similarly, mutations affecting the presynaptic Ca^{2+} channel Cacophony, or transgenic constructs that perturb the activity of Ca^{2+}/Calmodulin-dependent Kinase II (CaMKII) reveal the importance of Ca^{2+} as a secondary messenger in the signaling pathways that dictate NMJ structure and efficacy (Kawasaki *et al.* 2000; Rieckhof *et al.* 2003; Frank *et al.* 2006).

Further study into the translational regulators that are both enriched at the NMJ postsynapse, and regulate the expression of synaptic proteins, confirmed the hypothesis that translation was central to the plastic growth of the NMJ. The translational regulators

Nanos and Pumilio are both enriched at the NMJ SSR, and are required for proper NMJ growth and neurotransmission. Indeed, it has even reported mRNA encoding glutamate receptors, and polysomes, are enriched at the postsynapse (Sigrist *et al.* 2000; Speese *et al.* 2012; though see Chapter 5 for full discussion). As a result, mutagenic studies in the larva have revealed the genetic basis of synaptic plasticity, by directly observing changes in synapse number (through microscopy) and efficacy (through electrophysiology). These studies have been furthered by more recent techniques in which the activity of the NMJ can be conditionally perturbed, using light-inactivation of synaptic proteins, temperature-induced action potentials, and even changes in rearing conditions. This has allowed finer dissection of the processes behind plastic synapse growth, and maintains the larval NMJ as a key tool for approaching key questions in neuroscience.

mRNA-binding proteins (RBPs) are central to neuronal function and disease

Consistent with the idea that local translation facilitates changes in gene expression in specific regions of the neuron, such as axonal compartments or individual synapses, multiple studies have revealed proteins involved in mRNA localization and translation to be central to neuronal function (Kraut *et al.* 2001; Sau *et al.* 2011;reviewed in Jung *et al.* 2010). Following from this came two key observations that place mRNA-binding proteins (RBPs) as central to neuronal biology; 1) there is a predilection for mutations in RBPs to correlate with neuropathologies in humans (Reviewed in Wang *et al.* 2007), and 2) in experimental models, mutation in RBPs perturbed key neuronal processes, including synaptogenesis and plasticity (reviewed in Kiebler and Bassell. 2006; Sinnamon and Czaplinski 2011). One prominent example of RBPs in neuropathology, both in human patients and experimental systems, is shown by mutations in SMN (Survival of Motor Neuron Protein) causing SMA (Spinal Muscular Atrophy) (Lefebvre *et al.* 1995 and 1997). SMN is a RBP with roles in splicing (Liu and Dreyfuss 1996; Massenet *et al.* 2002), stress granule formation (Liu *et al.* 1997), and local translation (Leung *et al.* 2006; Sharma *et al.*

2005; Al-Ramahi *et al.* 2007). SMN is expressed ubiquitously, however in SMN-deficient patients only motoneurons degenerate, reflecting the importance of RBPs in maintaining neuronal function (Lefebvre *et al.* 1995). Another example of RBPs in neuronal disease come from the human RBP trio TDP-43 (TAR DNA-binding protein), Angiogenin, and FUS (Fused in Sarcoma) that are all strongly correlated with amyotrophic lateral sclerosis (ALS). Mutations in all three cases have been correlated with both familial and sporadic ALS, with TDP-43 mutations being by far the most common (Chen-Plotkin *et al.* 2010; Greenway *et al.* 2006; Kabashi *et al.* 2008; Talbot 2009.). More recently, alterations in the expression of the heterogeneous nuclear ribonucleoprotein (hnRNP) Syncrip (**SYN**aptotagmin-binding **C**ytoplasmic **R**NA-**I**nteracting **P**rotein, or hnRNPQ and R in mammals), have been shown to correlate significantly with Alzheimer's disease. Microarray analysis on neurons from the entorhinal cortex (one of the first areas to be affected by Alzheimer's) containing the stereotypical Alzheimer's tangles were compared to healthy patients and expression was mapped across 26,097 genes; the top 24 significant changes in gene expression were considered, including Syncrip (Vani Guttula *et al.* 2012). Syncrip is implicated in mRNA localization and local translation, and is a focus of this study.

Syncrip, a conserved RBP is required for mRNA localization in oocytes, and may regulate NMJ structure.

In a previous study by McDermott *et al. Drosophila* CG17838 was identified as one of a number of proteins able to bind the RNA localization signal present in the oocyte-localized *gurken* mRNA (Figure 1.5). CG17838 was shown to be necessary for proper localization of *oskar* and *gurken* mRNA in the *Drosophila* oocyte, both of which are necessary for proper axes specification. As a result, egg-chambers deficient in CG17838 exhibited polarization phenotypes consistent with *gurken* and *oskar* mislocalization (McDermott *et al.* 2012). CG17838 was indentified as the *Drosophila* homolog of the (hnRNP) SYNCRIP/hnRNPQ and hnRNP R. The name SYNCRIP is derived from the two known

functions of SYNCRIP, i) binding Synaptotagmins, protein necessary for neurotransmitter release in neurons, ii) association with cytoplasmic RNA. *Drosophila* Syncrip (Syp) has similarity with the mammalian homologues; sharing three RNA-recognition motif (RRM) domains (with 52% - 68% amino acid identity, and 63% - 82% amino acid similarity) and two nuclear localization signals (NLS), and producing multiple annotated protein isoforms (A-H) (Figure 1.5 B) (McDermott *et al.* in preparation)**.** In mice there are two splice isoforms. Both isoforms contain an RGG box (a region rich in arginine and glycine residues that can bind RNA and protein), an N-terminal acidic region, and three RNA-recognition motifs (RRM domains), which can also bind mRNA and protein. *Drosophila* Syncrip isoforms lack the acidic domains and RRG box. Isoform 1 has a C-terminal expansion that is absent from isoform 2 while isoform 2 has a shorter, unique C-terminus. In humans, a third isoform exists, hnRNPQ2, that lacks a portion of the second RRM domain (Mourelatos *et al.* 2001.). As mouse isoform 2 was shown to bind to Synaptotagmins (Syts) (Mizutani *et al.* 2000), and shown to localize to the cytoplasm (Bannai *et al.* 2004), SYNCRIP refers to isoform 2 unless otherwise stated. Mammalian SYNCRIP is also highly homologous (82% identity) to hnRNP R (Mizutani *et al.* 2000). SYNCRIP and hnRNP R are both ubiquitously expressed across a number of mouse tissues, including skeletal muscle, the nervous system, and ovaries. While SYNCRIP is cytoplasmic, hnRNP R is mostly nuclear. It is thought that SYNCRIP is the cytoplasmic counterpart of hnRNP R (Mizutani *et al.* 2000). A number of functions have been reported for SYNCRIP, mostly involving mRNA metabolism. These data will be discussed here.

Figure 1.5. *Drosophila* Syncrip homologues

i) RNA editing

RNA editing is the enzymatic process by which chemical modifications are made to an RNA molecule that change the transcript sequence. As a result, the properties of the edited transcript, and the encoded protein, can be altered. Most common editing processes proceed through deamination of Cytosine and Adenosine bases, changing them to Uracil and Inosine respectively. Syncrip is implicated in the editing of Cytosine to Uracil in the mRNA encoding Apolopiprotein (apo) B. apoB is a lipoprotein central lipid transport and metabolism in the body. The *APOB* gene encodes two isoforms, the first expressed exclusively in the small intestine, the second in the liver. Synthesis of the small intestine isoform results from editing the transcript to introduce an alternative stop codon. In mouse, SYNCRIP isoform 1, also known as GRY-RBP, has been identified as a part of large multiprotein complex that mediates the editing of *apoB* mRNA (Blanc *et al.* 2001; Chen *et al.* 2007). SYNCRIP isoform 1 recruits the catalytic component of the editosome (Apobec-1), the Apobec-1 complementation factor, and *apoB* mRNA through direct interaction with each molecule. Through its direct interaction with mRNA and RBPs, SYNCRIP isoform 1 regulates the editing of mammalian *apoB* mRNA. mRNA editing however, is not the only mechanism by which SYNCRIP can regulate mRNA expression.

ii) mRNA stability

The expression levels of the proto-oncogene *c-fos*, a member of the immediate early gene family of transcription factors, are crucial to cell proliferation, differentiation, apoptosis, and transformation. Regulating the stability of *c-fos* mRNA is an observed mechanism for regulating C-Fos expression, and sequences both within the coding and untranslated region of the transcript recruit factors that control *c-fos* destruction (Schiavi *et al.* 1994). Interestingly, recruitment of deadenylation and decay machinery to *c-fos* mRNA is

dependent upon ribosome-binding to the transcript, and likely, translation. SYNCRIP was purified with Poly(A) binding protein in the complex that stabilizes *c-fos* mRNA by bridging association between the transcript poly(A) tail and instability sequences in the coding region (Grosset *et al.* 2000). Translation is thought to disrupt this association, and allow deadenylation and decay to occur. Interestingly, researchers have used C-Fos as a marker of neuron activity, as expression levels correlate with action potentials in mammals (Dragunoa and Faulll. 1989; VanElzakker *et al.* 2008; Day *et al.* 2008). Given Syncrip's postulated role in regulating translation in neurons (see below), it is tempting to speculate that Syncrip may mediate this effect through regulating *c-fos* mRNA stability. Syncrip has also been implicated in regulating the availability of another key transcription factor, *c-myc*. As with *c-fos*, the stability of *c-myc* mRNA is regulated by ribosome-dependent exosome complexes (Bergstrom *et al.* 2006; Sparanese and Lee 2007). Rare codons in the *c-myc* transcript are thought to induce ribosome pausing during translation, allowing cleavage to occur (Lemm and Ross. 2002). *c-myc* mRNA is stabilized by a complex containing SYNCRIP that sequester *c-myc* mRNA into translationally silent mRNPs (Weidensdorfer *et al.* 2009). In addition to regulating the stability of mRNAs from proto-oncogenes, Syncrip has also been shown to be necessary for rapid viral replication and translation.

iii) Viral RNA replication and translation

Syncrip has been shown to bind both strands of the double-stranded mouse hepatitis virus (MHV) 5'UTR along with PABP and a host of other RBPs to directly regulate the rate of MHV genome synthesis (Choi *et al.* 2004). SYNCRIP has been shown to colocalize with newly synthesized Hepatitis virus RNA and knockdown of SYNCRIP suppresses Hepatitis RNA replication (Liu *et al.* 2009). In addition, translation of hepatitis mRNA during viral proliferation was inhibited by knockdown SYNCRIP, indicating that Syncrip plays an important role in the expression of viral proteins in infection (Kim *et al.* 2004).

iv) RNA splicing

There are three lines of indirect evidence linking Syncrip to splicing. First, *Drosophila* CG17838 has been shown to be the only hnRNP to be consistently isolated in spliceosome complexes affinity purified using pre-mRNA (Herold *et al.* 2008). Further, mass spectrometry on mouse spliceosomes identified hnRNP Q, which is similar to SYNCRIP, as a spliceosome-associated protein (Neubauer *et al.* 1998). Moreover, hnRNP Q immunoprecipitates with pre-mRNA and RNA introns, and immunodepletion of hnRNP Q leads to a decrease in splicing efficiency (Mourelatos *et al.* 2001).

v) mRNA localization and translation control in mammalian neuronal processes

A number of key studies have placed Syncrip as central to mRNA regulation in mammalian neurons, and in dendrites particularly. In 2000 an unidentified 68kDa protein, pp68, was shown to be phosphorylated on a tyrosine residue in response to insulin and osmotic shock in adipocytes (Hresko and Muechler 2000). Pp68 was subsequently shown to be the tyrosine-phosphorylated form of Syncrip by mass spectrometry, and was found to be present in free polysomes extracted from adipocytes (Hresko and Muechler 2002). Biochemical experiments revealed that Syncrip could be phosphorylated *in vitro* by an insulin-receptor tyrosine kinase, but that binding of Syncrip to poly(A) mRNA inhibited this phosphorylation.

In parallel to these studies Mikoshiba and colleagues cloned Syncrip from mouse cDNA and identified its role in binding ubiquitous Synaptotagmin isoforms (Mizutani et al 2000). Subsequently, the Mikoshiba laboratory focused on Syncrip's role in neuronal mRNA metabolism. In sectioned rat hippocampal neurons, and cultured human neurons, Syncrip was shown to be mostly present in cell bodies, but also in a punctate pattern in proximal dendrites (Bannai *et al.* 2004). The cytoplasmic Syncrip foci were hypothesized to be dendritic mRNP granules. Consistent with this, Syncrip was shown to preferentially bind to

poly(A) mRNA over poly(U) mRNA and poly(A) DNA. Biochemical purification of Syncrip from a kidney cell line revealed its association with 111 proteins; 44 were ribosomal proteins; 26 were mitochondrial ribosomal proteins, which are encoded by nuclear DNA; 26 were RNA binding proteins, including 8 hnRNPs, 4 DEAD box helicases, and 4 splicing factors. These data complemented previous observations as placing Syncrip central to complexes that process and translate mRNA. Further, Bannai *et al.* showed that in cultured rat hippocampal cells, Syp-GFP mimicked the expression pattern observed in brain sections. Moreover, time-lapse analysis in live neurons that Syp-GFP granules were transported bidirectionally in dendrites at ~0.05μm/s. This transport was sensitive to pharmacological microtubule disruption and proposed to occur along microtubules. Finally, Syp-GFP was shown to colocalize with the RBP Staufen 1, and dendritic mRNA. mRNA staining however, was achieved with ethidium bromide, which does not distinguish between poly(A) mRNA and ribosomal RNA. To refine this approach, Syp-GFP was shown to colocalize with the 3'UTR of the *IP_3R1* mRNA, which encodes the inositol 1,4,5-trisphosphate receptor type 1. Staufen protein and *IP_3R1* mRNA had previously been shown to localize to the dendrites of central nervous system neurons (Furuichi *et al.* 1993; Kiebler *et al.* 1999). These data show that Syncrip associates, and is transported, with localized dendritic mRNAs and RBPs.

Subsequently, biochemical purification of mRNPs from the developing rat brain (Elvira *et al.* 2006) and cultured embryonic mouse hippocampal neurons (Kanai *et al.* 2004) revealed Syncrip presence in large mRNP complexes. Immunoprecipitation of conventional Kinesin (KIF5) tail revealed its association with Syncrip. In separate experiments, immunoprecipitation of KIF5 revealed mRNAs encoding *CaMKIIα* (*Ca^{2+}/Calmodulin dependent protein kinase*) and *Arc* in dynamic mRNP transport particles. As with *IP_3R1, CaMKIIα* and *Arc* mRNAs had previously been shown to localize to specific subcellular regions of the neuron. Indeed the neuronal localization of *CaMKIIα* (Burgin *et*

al. 1990; Blichenberg 2001; Mori *et al.* 2000; Ashraf *et al.* 2006) and *Arc* (Link *et al.* 1995; Lyford et al 1995; Steward *et al.* 1998) mRNAs represents two of the most studied examples known to date and their localization is known to be central to neuronal function (See Introduction). Taken together, the studies summarized here suggest that Syncrip could be in present in mRNPs that contain actively localized mRNAs and RBPs required for neuronal function, however it is important to recognize that this this has not been shown directly. The role that Syncrip plays, or the mechanism by which it functions, however, remain elusive.

Previous work shows that Syncrip associates with mRNAs encoding synaptic proteins, and is likely required for proper neuromuscular morphology.

Key experiments preceding this study have provided preliminary data on which this study is based. First, it was shown that Syncrip was expressed in larval muscle and neurons (Suzanne McDermott and Carine Meignin). Second, it was shown that RNAi-mediated knockdown of Syncrip in muscle or neuron resulted in an overgrowth of the larval neuromuscular junction (Carine Meignin). While this data indicates that Syncrip is necessary in both neuron and muscle for NMJ structure, there are a number of caveats to this experiment that require addressing. Firstly, knockdown of Syncrip protein (e.g. by Western blot) was not confirmed in these experiments. Second, animals expressing anti-Syncrip RNAi were compared to wildtype animals, and not the animals expressing the RNAi or Gal4 drivers alone. As a result it is not possible to soundly conclude that the observed change in NMJ morphology result from expression of anti-Syncrip RNAi, rather than a background mutation in the parental strains. In addition Suzanne McDermott and Carine Meignin then correlated loss-of-function *Syp* mutations with a change in NMJ structure relative to wildtype controls. Time constraints however prevented the comparison of *Syp* mutant NMJs with a rescue control, in which Syncrip is expressed in the *Syp* mutants. As a result it was not possible to conclude that mutations in the *Syncrip*

gene, as opposed to elsewhere in the genome, were responsible for changes in NMJ structure. Using a genomic construct that was created by Carine Meignin and Karolina Varga (Davis Group), a key aim of this study was to test if Syncrip is necessary for proper NMJ structure, but also to examine the mechanism by which Syncrip functions.

Consistent with the notion that Syncrip regulates NMJ structure, immunofluorescence revealed that Syncrip is expressed throughout muscle, but enriched in nuclei and at the NMJ postsynapse (Carine Meignin and Suzanne McDermott). It was hypothesized that that Syncrip may contribute to regulating translation of synaptic proteins local to the NMJ (Suzanne McDermott and Carine Meignin). Consistent with this hypothesis, immunoprecipitation of Syncrip followed by Reverse Transcription Real Time PCR revealed that Syncrip associates with *dlg, shaker, fasII,* and *eIF4E* mRNA, but not *rp49* mRNA, in whole larvae (Suzanne McDermott). Further analysis of Syncrip-associated mRNAs by Deep Sequencing revealed that Syncrip associates with multiple mRNAs, with *dlg, shaker* being highly significant targets (Suzanne McDermott, Russell Hamitlon, Discussed in chapter 4, See table 4.3).

In addition to its role in mRNA metabolism, Syncrip is known to directly bind Synaptotagmin 1 in mice, a key vesicle release protein (See Chapter 3). Immunoprecipitation of Syncrip from larvae revealed that Syncrip and Synaptotagmin 1 associate in the same complexes, potentially through direct binding.

If further experimentation reveals that Syncrip is necessary for NMJ morphology, then these preliminary data reveal two mechanisms by which Syncrip may function. First, through associating with mRNAs encoding key synaptic proteins and regulating their translation, potentially at the NMJ postsynapse. Second, Syncrip associates with Synaptotagmin, and may be required either for Synaptotagmin function or expression.

If Syncrip conserves function between the oocyte and NMJ, then it is likely that changes in mRNA localization and translation facilitate synaptic plasticity, and that Syncrip regulates these processes. To this end, the role of Syncrip in synapse structure and function was investigated.

Central hypotheses

i) mRNA localization in neuron and muscle contribute to synaptic plasticity at the *Drosophila* neuromuscular junction.

ii) Syncrip, a conserved mRNA-binding protein, regulates the translation of key synaptic proteins, at least in part, through mRNA localization in neuron and muscle.

Key questions

i) Is Syncrip necessary for NMJ structure and function?

ii) Where are mRNAs encoding key presynaptic proteins distributed within motoneurons?

iii) Where are mRNAs encoding key postsynaptic proteins distributed within muscle?

iv) Does mRNA localization at the NMJ respond to perturbations in synaptic plasticity?

v) Is Syncrip necessary for mRNA localization at the NMJ?

vi) How does Syncrip regulate translation of mRNAs encoding key synaptic transcripts?

Chapter 2: Materials and Methods

Molecular biology

i) Buffers and reagents

All solutions and buffers used were prepared as described in 'Molecular Cloning-A Laboratory Manual' (Sambrook *et al.*, 1989), unless otherwise stated. Phosphate buffered saline (PBS), Tris-EDTA (TE), Tris-acetate-EDTA (TAE), and Luria Betani (LB) media, were prepared and autoclaved by the Department of Biochemistry media kitchens. The media kitchens also prepared LB-agar plates with antibiotics. The composition of these buffers is given below:

Buffer	**Recipe**
10 x PBS, pH 7.4	1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4
TE	10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0)
10 x TAE	0.4 M Tris base, 10 mM EDTA (pH 8.0), 1.2% (v/v) glacial acetic acid
LB bacterial media	For 1 litre: 10 g Bacto tryptone (Difco), 5 g Bacto yeast extract, 5 g NaCl
LB-Agar	For 1 litre: 16 g Bacto tryptone (Difco), 10 g Bacto yeast extract, 5 g NaCl
2xTY bacterial media	For 1 litre: 10 g Bacto tryptone (Difco), 10 g Bacto yeast extract, 5 g NaCl

ii) Polymerase chain reaction (PCR)

Standard PCR reactions were performed in 50 μl using 25 - 50 ng plasmid template DNA, 1 x PCR buffer containing 15 mM $MgCl_2$ (Roche), 0.2 mM each dNTP (Roche), 0.25 μM each primer, 2.5 Units Taq DNA polymerase (Roche). The standard cycling program consisted of 5 min, 94 °C, followed by 30 cycles of 20 s, 94 °C; 30 s, annealing 45-65°C (dependent on the T_m of the primers used) and elongation 0.5 – 2 min (approximately 1 minute per kb of the PCR product), 72°C. After a final 10 min extension at 72°C, reactions were held indefinitely at 4°C. Products were verified by agarose gel electrophoresis and if required, gel purified using the QIAquick gel extraction kit (Qiagen).
For a full list of primers used in this study see Appendix 2.1.

iii) Making competent XL-1 Blue Cells for amplifying DNA plasmids

To generate a large stock of transformation-competent XL-1 Blue cells, commercial XL1-Blue cells were cultures were grown overnight in 5ml LB at 37°C. The liquid culture was then diluted 1/500 into a total volume of 500ml LB plus 12.5 μg/ml tetraclycline, 15mM MgCl2**.** Cells were then grown at 37°C until OD_{600} = 0.3 – 0.4. Cells were pellets at 3000rpm for 20 minutes at 4°C. Competence media was then prepared: RbCl, 50 mM $MnCl_2$, 30 mM Potassium acetate, 10 mM $CaCl_2$, 15% glycerol, filter sterilized and balanced to pH=5.8. Pellets of bacteria were resuspended in 250μl aliquots of competence media and snap frozen in liquid nitrogen. XL-1 Blue cells were then stored at -80°C and thawed on ice when needed. Aliquots were never refrozen or reused once thawed.

iv) Amplification and purification of plasmid DNA

For standard plasmid DNA amplification (see protocol below for handling DNA encoding MS2-loops), competent XL-1 Blue cells were transformed with plasmids using heat-shock.

XL-1 Blue cells were made competent 100 μl cells were incubated with 1-50 ng of plasmid DNA or 20% of a ligation reaction for 30 min on ice. The cells were then heat-shocked at 42˚C for 45 s. After a further 2 min on ice, prewarmed 900 μl LB media was then added and the cells allowed to recover for 1 h at 37˚C with gentle shaking. Cells were plated onto LB-agar plates containing the appropriate antibiotic for plasmid selection (100 μg/ml ampicillin and kanamycin; 50 μg/ml chloramphenicol) and grown overnight at 37˚C. A single transformed colony was then chosen and grown in either 5 ml (Miniprep) or 50ml (Midiprep) LB containing the appropriate antibiotic for 12-16 h at 37˚C. Depending on the downstream application and the amount of plasmid required the plasmid was then either isolated using the QIAprep Spin Miniprep Kit (Qiagen 27106), or grown further and isolated using the QIAfilter Plasmid Midi (Qiagen 12143). All kits were used according to the manufacturer's protocol.

v) DNA Sequencing

Careful and systematic sequencing of DNA is central to successful molecular cloning and transgenesis. DNA sequencing was performed by Source Bioscience Sanger Sequencing service within Oxford University. Sequencing was performed by SpeedREAD technology. 1μg of plasmid DNA was required in a total volume of 10μl (100ng/μl). 100ng of primer DNA was required in a total volume of 10μl (10ng/μl).

vi) Gel electrophoresis

Samples were mixed with 1 x gel loading buffer (Bioline) and loaded onto 1% (unless otherwise stated) agarose gels (1 g agarose (agarose MP, Roche), 100 ml 1 x TAE) containing 0.5 μg/ml ethidium bromide. Electrophoresis was carried out in 1 x TAE running buffer at a constant voltage of 95 V, and nucleic acids were visualized using a UV transilluminator. *It is important to not overload lanes with DNA. For a ~3mm gel lane, no*

more than 200ng of DNA should be loaded. This is particularly important for gel purification of DNA following restriction enzyme cutting.

vii) Gel extraction of DNA

All DNA used for cloning was gel extracted before ligation following manufacturer's protocols (QIAquick Gel extraction Kit: 28704). Gel slices were cut as thin as possible to minimize agarose contamination.

Molecular Cloning

i) Standard molecular cloning of pUASTattb6xMS2

First, to produce a 33bp sequence that encoded three novel 8-cutter restriction sites (Pme1, Pac1, and Asc1) two PCR primers with overlapping complementarity were used (see primers).

8-cutter sequence:

CTCGAG	Xho1
TTAATTAA	Pac1
GGCGCGCC	Asc1
GTTTAAAC	Pme1
GGTACC	Kpn1

NNNCTCGAGATTCTTAATTAAGCTGGGCGCGCCGTATGTTTAAACTGCAGGTACCNNN

The resulting sequence was flanked by Xho1 and Kpn1 sites and was incubated with thee restriction enzymes, then purified by polyacrylamide gel electrophoresis (agarose gel electrophoresis is not appropriate for such small DNA fragments). pUASTattb was incubated with Not1 and Kpn1, then purified by agarose gel electrophoresis. The 8-cutter sequence and linearized pUASTattb were then ligated and transformed into XL-1 Blue

cells. 20 bacterial colonies were grown by miniprep. Incorporation of the 8-cutter sequence was selected for by sensitivity to Pme1.

The DNA sequence encoding the 6XMS2 loops was obtained from Jayan Nair and Maria Leptin (EMBL, Heidelberg) and amplified by PCR to produce a 240nt sequence, flanked by Pac1 and Pme1 restriction enzyme sites.

6xMS2 sequence (MS2 loop sequence is underlined):

TTAATTAA Pac1
GTTTAAAC Pme1

NNNTTAATTAAAGGAAACATGAGGATCACCCATGTCTGCAGGTCGACTCTAGAAAACATGAGGATCACCCATGTCTGCAGTATTCCCGGGTTCATTAGATCCTAAGGTACCTAATTGCCTAGAAAACATGAGGATCACCCATGTCTGCAGGTCGACTCTAGAAAACATGAGGATCACCCATGTCTGCAGTATTCCCGGGTTCATTAGATCCTAAGGTACCTAATTGCCTAGAAAACATGAGGATCACCCATGTCTGCAGGTCGACTCCAGAAAACATGAGGATCACCCATGTCTGCAGTATTCCCGGTTTAAACNNN

The 6xMS2 DNA, and pUASTattbMCS, were then digested by Pac1 and Pme1 and ligated. *E. coli* containing the ligated plasmid were selected for via miniprep and gel electrophoresis.

ii) Protocol for molecular cloning with MS2 loops

DNA encoding the repetitive MS2 sequences is highly recombinogenic. As a result it was frequently found that MS2 loops were recombined out of plasmids in *E. coli*. To circumvent this problem, a number of critical improvements were made to the molecular cloning protocol:

1. Bacteria in which recombination was heavily suppressed were used. In particular SURE2 (Stop Unwanted Rearrangement Events) competent cells were useful (Stratagene: 200152). SURE2 genotype:*e14-(McrA-) Δ(mcrCB-hsdSMR-*

mrr)171 endA1 gyrA96 thi-1 supE44 relA1 lac recB recJ sbcC umuC::Tn5 (Kanr) uvrC [F´ proAB lacIqZΔM15 Tn10 (Tetr) Amy Camr.

2. Recombination was suppressed by growing cells at 29°C.

3. Cells were grown at very low density in large volumes to produce enough DNA for midiprep. Typically a single colony, or 50μl of a 5ml culture, would be grown in 300ml media for 18 hours.

4. Growing cells were never permitted to exceed past exponential phase (OD_{600}>300).

5. Cells were grown in 2xTY broth rather than LB broth.

6. At every stage of molecular cloning DNA sequencing was necessary to ensure that all six MS2 loops were still encoded by the plasmid.

7. To subclone cDNA sequences into pUASTattB6xMS2 it was found that using non-ligation based methods was far more successful. To this end a cloning kit was employed (Clontech Infusion HD: 639648; see below).

iii) Clontech Infusion HD cloning kit

Classical restriction enzyme cutting and ligating appeared hindered by the presence of the MS2 loops. Instead, the Clontech Infusion HD cloning kit was used to introduce cDNAs to the pUASTattB6xMS2 vector, as per the manufacturer's protocol (Clontech Infusion HD: 639648; protocol available online: http://www.clontech.com/GB/Products/Cloning_and_Competent_Cells/Cloning_Kits/Cloning_Kits-HD). Briefly, the cDNA sequences of interest were PCR amplified with 15 bp flanking sequences that were complementary to the pUASTattB6xMS2 multicloning site flanking the Xho1 restriction enzyme site (the clontech infusion site in-house primer design software was used; see primers). The pUASTattb6xMS2 plasmid was then linearized by Xho1 and purified by gel electrophoresis. Incubation of the cDNA sequence and the linearized pUASTattb6xMS2 plasmid with the infusion enzyme then mediated cloning without the use of classical

ligation enzymes (Figure 2.1). While this protocol produced few colonies (typically fewer than 10 for an entire ligation reaction), over 50% were found to contain the desired product. For unknown reasons, the Clontech Infusion HD cloning kit was highly successful for introducing cDNAs into pUASTattb6xMS2, while classical sub-cloning was not.

Figure 2.1. Molecular cloning using Clontech Infusion

Transgenesis

Transformation of *Drosophila* was outsourced to Genetic Services (Massachusetts USA). PhiC31-mediated site-specific recombination introduced all transgenes in this study to attp2 landing site on chromosome 3. All stocks were balanced over *TM6B*.

In vitro transcription of fluorescently-labeled RNA

For standard transcription, 5-10 μg plasmid DNA was digested using the appropriate restriction enzyme (New England Biolabs) according to manufacturer's instructions. Digests were visualized by TAE-agarose electrophoresis and purified using the QIAquick PCR purification kit (Qiagen), or by phenol/chloroform extraction. Fluorescently labelled and capped sense RNAs for injection into living oocytes were *in vitro* transcribed in 50 μl reactions. 1-2 μg linearized template DNA (*gurken* cDNA was a gift from T. Schüpbach) was added to 1 x transcription buffer (40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM DTT and 2 mM spermidine) (Roche), 0.4 mM rATP, 0.4 mM rCTP, 0.36 mM UTP (Roche), 0.04 mM AlexaFluor 546- or 488-UTP (Molecular Probes), 0.12 mM rGTP, 0.3 mM 7mG(5')ppp(5')G cap analogue (Ambion), 40 U RNasin RNase inhibitor (Promega) and 20 U T7, T3 or SP6 RNA polymerase (Roche), according to the promoter present in the template. The transcription reaction was incubated for 3 h at 37˚C, after which the template DNA was digested with 2 U RQ1 RNase-free DNase (Promega) for 15 min at 37˚C. Unincorporated nucleotides were removed using Sephadex G-50 RNA spin columns (Roche) according to the manufacturer's instructions. RNA was then precipitated by the addition of 1/5 volume 5 M NH_4OAc pH 5.2 and 2.5 volumes 100% EtOH. Following incubation at -20˚C overnight/on dry ice for 1 h, the RNA was pelleted by centrifugation at 14000 rpm for 20 min at 4˚C. The pellet was washed with 75% EtOH and

resuspended in 5-10 μl DEPC-treated water depending on the size of the pellet. Final RNA concentration was typically 250-500 ng/μl.

Drosophila protocols

Strains were raised on standard cornmeal-agar medium at 18˚C or 25˚C. Oregon-R was used as the wild-type strain. For a full list of Drosophila strains used see Appendix 2.2.

i) Dissection of Drosophila ovaries for mRNA FISH

Flies were transferred to freshly yeasted bottles less than 12 hours after eclosion. The ovaries of 2-3 day old females were dissected in 1 x PBS and immediately transferred to 3.7% formaldehyde in 1 x PBS + 0.1% Tween20 (PBST) for 20 min. After fixation, ovaries were washed three times for 5 min in PBST. Following *in situ* hybridization , ovaries were stained with 1 μg/ml 4', 6-diamidino-2-phenylindole (DAPI) to label nuclei, and mounted in Vectashield (Vector laboratories).

ii) Dissection of Drosophila third-instar larvae

Third instar larvae were size-matched and dissected according to standard protocols in HL-3 buffer on sylguard plates (Ramachandran and Budnik 2010; Verstreken *et al.* 2008). For live imaging HL-3 was used with low calcium levels (1.5mM). For quantifying NMJ structure HL-3 was used without calcium and with 1mM EGTA to minimize spontaneous contraction during fixation.

Genetics

The genomic rescue construct was generate with a fosmid (FlyFos024580; http://flybase.org/cgi-bin/gbrowse/dmel/?name=3R:16603660..16635842) covering all

syncrip isoforms except A and H by Carine Meignin. The construct was inserted at the attP40 of the chromosome 2 (insertion line - Bloomington 25709).

For *Syncrip* mutants an insertion in the syp gene, syp^{e00286}, which is predicted to affect all isoforms, was tested as a homozygote and trans-heterozygote in combination with the deficiency Df(3R)BSC124 (hereon referred to as syp^{Df124}). All lines developed to pupae, with $syp^{e00286}/syp^{e00286}$, syp^{e00286}/syp^{Df124},and $syp^{Rescue}/syp^{Rescue}$ all exhibiting developmental delays relative to wildtype of approximately one, one, and two days respectively. $syp^{e00286}/syp^{e00286}$, syp^{e00286}/syp^{Df124} animals however failed to pupate with almost 100% penetrance (Suzanne McDermott).

Immunofluorescence

i) Postsynaptic immunofluorescence.

For standard postsynaptic immunofluorescence third instar larvae were dissected in HL-3 buffer and fixed in fresh ice cold paraformaldehyde (4% in PBTx) for 20 minutes. No blocking agent was used. Blocking with BSA can increase background fluorescence in muscle. Blocking with NGS and Glycine was tested but did not improve imaging. The postsynaptic antibodies tested in this study produced highly specific labeling with minimal background without blocking. Following washing, primary antibodies were incubated with the sample overnight at 4^{o}C, washed, then incubated with secondary antibodies for 2 hours at room temperature. To ensure that test and control animals were exposed to the exact same concentration of antibody, wildtype and mutant larvae were incubated in the same tubes. Larvae were distinguished by distinctive cuts made to the corners of wildtype larvae during dissection.

ii) Presynaptic immunofluorescence.

For standard postsynaptic immunofluorescence third instar larvae were dissected in HL-3 buffer and fixed in fresh ice-cold paraformaldehyde (4% in PBTx) for 20 minutes. For presynaptic staining with the antibodies tested here a blocking step was necessary to reduce background signal. Best results were achieved using 0.1% NGS in PBTx for 4 hours at room temperature before applying the primary antibody overnight at 4^{o}C. Following washing, primary antibodies were incubated with the sample overnight at 4^{o}C, washed, then incubated with secondary antibodies for 2 horus at room temperature. To ensure that test and control animals were exposed to the exact same concentration of antibody, wildtype and mutant larvae were incubated in the same tubes. Larvae were distinguished by distinctive cuts made to the corners of wildtype larvae during dissection.

For a full list of antibodies used in this study see Appendix 2.3.

mRNA fluorescence in situ hybridization (FISH).

Sense and anti-sense RNA probes were synthesised for RNA *in situ* hybridization in 10 μl reactions. 1 μg linearized template DNA was added to 1 x transcription buffer (Roche), 1 x DIG labelling mix and 20 U T7, T3 or SP6 RNA polymerase (Roche). The DIG RNA labelling mix (Roche; 10 x) contains 10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP and 3.5 mM DIG-11-UTP, pH 7.5. The transcription reaction was incubated for 2 h at 37˚C, after which template DNA was digested with 2 U RQ1 RNase-free DNase (Promega) for 5 - 10 minutes at 37˚C. The RNA was visualized by TAE-agarose gel electrophoresis and used without any further purification for *in situ* hybridization. RNA probes were diluted in different buffers for different mRNA *FISH* protocols (see below).

i) Karr et al. 2008 mRNA FISH protocol.

The below protocol is based on that published in Karr *et al.* 2008, and again in more detail in Ganesan *et al.* 2012. This protocol contains details not available in the above

publications, obtained either through personal communication with the corresponding author (Dave Featherstone), or developed in this study.

A. Partial alkaline hydrolysis of RNA probe

Following *in vitro* transcription of DIG-labeled mRNA probe, 10μl 2XCarbonate Buffer was added (Carbonate Buffer: for 10ml, 127.2 mg Soduim carbonate 67.2 mg Sodium bicarbonate, add water to 10ml. Aliquot and freeze). The probe was subsequently incubated at 60^{o}C for 15 minutes for fragmentation. The incubation time can be varied and is proportional to how much fragmentation takes place. 15 minutes works well for probes that are ~3kb long (cDNA) and end up being 50- 300bp in size. Probes were aliquoted, and digested for 5 minutes, 10 minutes, 15 minutes, and 20 minutes, in parallel, and the digest that gave an average probe length of ~150nt was then selected. 50μl Stop Solution was added, and mixed well, to halt the reaction (Stop Solution; For 10ml, 164.1 mg sodium acetate add water to 10 ml PhD to 6.0 with acetic acid. Aliqiuot and store at -20^{o}C).

B. Lithium chloride precipitation of RNA

Following alkaline hydrolysis of the RNA probe, 10μl 4M $LiCl_2$ was added to the RNA followed by 250μl ice-cold ethanol. Following thorough mixing (by inversion not vortexing) the sample was incubated at - 20^{o}C for 2 hours. To precipitate the RNA pellet, the sample was spun at 4^{o}C, for 20 mins, at 13000g. Ethanol supernatant was then carefully decanted and the pellet was washed with 50μl cold 70% ethanol. The sample is then spun again at 4^{o}C, for 20 mins, at 13000g. The ethanol is then carefully removed with a pipette. *The pellet may be loose at this point.* The sample is then left to air dry. Care was taken not to over-dry the RNA. Following air drying, 50μl of freshly prepared resuspension buffer was added [Resuspension Buffer; for 1ml, 500ul Tris EDTA pH7.5 (10mM Tris HCL , 1mM EDTA) 500μl Deionized formamide 5μl Tween 20] and left for 10 minutes at room

temperature to rehydrate the pellet. The mixture was then pipetted up and down to aid rehydration. Mixing by vortex then allows full resuspension. The RNA can then be aiquoted and stored at -20°C.

C. Preincubation of primary and secondary antibodies

Ovaries were removed from female *Drosophila* adults that have been fattened on yeast for at least 24 hours at 25°C, and then fixed in 200μl of 4% (PFA)/PBS for 20 minutes at room temperature. The ovaries were divided up into eppendorf tubes, each containing 8 ovaries. 100μl of heptane was added to the surface of the fixation reaction and, using a 200μl standard Gilson pipette, the ovaries were pipettted up and down vigorously until the heptane was mixed into the solution and the ovaries were broken down into individual egg chambers. The ovaries were then rinsed in PBTx and washed for 3x 20 minutes in PBTx. The ovaries were then dehydrated in 100% methanol kept at -20°C. Ovaries were stored at this stage at -20°C. Ovaries were then reyhydrated in 80% Methanol, 50% Methanol, 20% Methanol and PBT in 20 minute increments at room temperature, followed by rinsing and washing in PBTx. The primary secondary antibodies used for mRNA *FISH* were then incubated separately in different eppendorf tubes at a known concentration. The antibody/ovary mixtures were then incubated on a rotator, in the dark, and 4°C overnight. The supernatant was removed from the ovaries and used immediately for immunofluorescence. Excess antibody can be stored at 4°C for further use, though this is not advisable for period longer than a few weeks. Preincubation of primary and secondary antibodies lowered the background signal in controls. Ovaries were removed from the antibody mixture following incubation to discourage the growth of fungi etc.

D. Hybridization protocol

Wandering third instar larvae were dissected in HL-3 buffer (Verstreken *et al.*, 2008), on sylgaurd plates, washed with ice-cold HL-3, then fixed for 15 minutes with fresh ice-cold

4% Paraformaldehyde (PFA)/PBS. *PFA was freshly prepared that day*. The larvae were then transferred larvae to tubes containing at least 100μl ice cold 4% PFA/PBS for a further 45 minutes on ice. Tubes with flat bottom were used to prevent the folding of larvae (HydroLogix tubes, 2.0ml, 3459IW). Larvae were then rinsed three times in PBTx (0.05%TritonX-100/PBS), and washed for 5minutes in P.Tx with shaking at 4°C. Larvae were then washed in PBTw with shaking at 4°C. Following washing, larvae were incubated with prehybridization buffer at room temperature on shaker for at least 1 hour (preferably 4 hours). Prehybridization buffer was freshly prepared each time.

To make 2 ml prehybridization buffer: 1ml- Deionized formamide (Sigma); 400μl-20XSaline Sodium Citrate buffer (Sigma or Biochemistry media kitchen); 40μl-50X Denhardt's Solution (Invitrogen); 50μl-tRNA (yeast tRNA from Invitrogen, 10mg/ml stock solution); 50μl sheared salmon sperm DNA (10mg/ml stock Ambion, before adding, heat at 100°C for 10 minutes and chill on ice); 2μl Heparin (50mg/ml, made stock and store at 4°C); 10μl- Tween-20; 448μl-DEPC water.

During prehybridization, RNA probes were thawed on ice and hybridization buffer was prepared. Hybridization buffer was freshly prepared each time. Hybridization buffer is the same as prehybridization buffer, only with 5% Dextran Sulfate. Before the RNA probes were added, they were denatured at 80^{o}C for 2 mins, then mixed with hybridization buffer at a concentration of 1ng/μl. Riboprobe concentration was quantified relative to resuspension buffer on a NanoDrop. The larvae were then incubated in probe solution overnight at 55°C with gentle shaking (100μl minimum). Following hybridization, the larvae were washed in freshly prepared wash buffer (50% Deionized formamide, 2x Saline Sodium Citrate buffer, 0.5% tween-20 in DEPC water). Approximately 10ml of wash buffer was needed for each tube. Each tube was washed in 1ml of pre-warmed freshly prepared wash buffer in the 55°C shaker incubator, for 8-10 washes, each lasting approximately one hour. The larvae were then washed overnight in pre-warmed wash buffer at 55°C.

Following washing, the larvae were then rinsed twice in PBTw at room temperature, and then left to equilibraite in PBTw for 30 minutes at room temperature. Larvae were then incubated in primary antibody (1:500 preincubated mouse anti-DIG; see antibody list) in 0.1%BSA/PBTw overnight at 4°C with gentle shaking. Following antibody incubation, larvae were rinsed in PBTw and then washed 6 times in PBTw at room temperature. During the washing steps Prolong Gold mounting medium was removed from -20^{o}C freezer and left to thaw. Larvae were then incubated in secondary antibody at room temperature for 2 hours (1:250 Donkey anti-mouse Alexa 594; 1:250 anti-HRP-Alexa488; see antibody list) and then washed 6 times for 10-20 minutes in PBTw before being mounted and left to cure overnight. Slides were then sealed shut with nail varnish and imaged within two weeks are preparation.

E. Notes

1. Dissection and washing was performed with RNAse-free technique throughout.
2. No more than 4 larvae were added to a tube. Motoraxon integrity was best maintained with one larva per tube.
3. Dextran Sulfate was best pipetted with a cut Gilson tip with increased the aperture.
4. Buffer was never applied with speed or force into the tubes.
5. Buffer was never applied buffer directly onto the larvae, but down the side of the tube instead.
6. Planning was necessary to ensure that the primary and secondary antibodies were preincubated with ovaries the night before they were used to label larvae.
7. To ensure sound experimental homogeny it was essential that different lines were grown, fixed, and labeled in parallel. With mutant animals that exhibited developmental delays it was necessary to size-match larvae with the controls.

ii) Carine Meignin Protocol.

Wandering third instar larvae were dissected in HL-3-without calcium and fixed for 20 minutes in 4% paraformaldehyde (Thermo Scientific) at room temperature. Larvae were then dehydrated in 100% methanol and stroed kept at -20°C. Larvae were rehydrated in 20 minutes steps in 80%, 60%, 40%, 20% methanol in PBTw (PBTw: 1 # PBS + 0.2% Tween 20) . Larvae were then post-fixed in 4% paraformaldehyde for 20 minutes, washed for 5 minutes in PBTw, then treated with proteinase K (3 µg/ml) for 10 min at room temperature followed by incubation on ice for 1h. The Proteinase K activity was then stopped by washing for two mintues in glycine (2 mg/ml). Larvae were then post-fixed a third time, in 4% paraformaldehyde for 20 minutes. Prehybridization buffer was then prepared (50% formamide; 5 x SSC; 100 µg/ml *E. coli* tRNA; 50 µg/ml heparin; 0.1% Tween 20; adjusted to pH=6.5 with HCl) and larvae were prehybridized for at least 1 hour (4 hourse is preferable) at 56°C in hybridization buffer. RNA probe were diluted 1/100 in hybridization buffer and denatured at 80°C for 3 minutes and then chilled on ice for 5 min. Probes were then added to the larvae and incubated overnight at 56°C in a heat block. Larvae were then washed for 6x 20 minutes with PBTw at room temperature on a rotating wheel. Larvae were then incubated with primary antibody overnight at 4°C on a rotating wheel. The larvae were then washed for 6x 20 minutes with PBTw at room temperature on a rotating wheel before being incubated with secondary antibodies for 2 hours at room temperature (Donkey anti-sheep fluorescent antibody – invitrogen; and goat fluorescent anti-HRP antibody – Jackson antibodies). Samples were then washed and mounted.

iii) Sigrist et al. mRNA FISH protocol.

Following *in vitro* transcription, the transcription reaction was split into two equal volumes. One was digested one for 5 min at 60°C and the other for 15 Min at 60°C. 2 µl was kept for later gel analysis to determine which time produced the probe of the correct length ~200bp. 2.3 µl 3M (pH 6.0) Sodium acetate was then added to the mixture. 50 µl 100% ethanol was added and the sample was spun 15 M at 4°C 14000 rpm. The RNA pellet

was then washed with 80% Ethanol and spun again for 5 min at 4°C 14000 rpm. The supernatant was discarded and the pellet was air dried before being dissolved in 50 µl of freshly made resuspension buffer (resuspension buffer: 50% Formamide. deionisated. 50% 1x TE. pH 7.5. 0.1% Tween 20)

Wandering third-instar larvae were dissected in HL-3 and fixed for 10 min with 4% PFA and again 50 min in 4% PFA on ice. Larvae were then washed 2x with PBTw while prehybridization buffer was prepared (prehybridisation buffer: 5ml deionized Formamide, 2ml 20x SSC; 0.2 ml 50x Dehnhardts (Sigma), 0.25 ml tRNA (Roche), 0.25 ml salmon sperm DNA (heated to 100°C 2x. cooled on ice) (Sigma), 0.01 ml Heparin 50mg/ml (Sigma), 0.05 ml 20% Tween 20, 1.24 ml water). Larvae were then prehybridized in 50% hybridization buffer and 50% water for one hour at room temperature (500µl was sufficient). For hybridization RNA probes were diluted in prehybridization buffer with 25% Dextran sulfate and incubated overnight at 55°C. with gentle shaking. Larvae were then washed in freshly prepared and preheated wash buffer for 8x 1 hour washes (wash buffer: 25 ml deionized formamide, 5 ml 20x SSC, 20 ml water, 0.25 ml 20% Tween 20). Then larvae were then washed in PBTw at room temperature for 3x 10 min and incubated with anti-DIG-AP-FAB-Fragments (Roche) 1:2000 in 5% Goat serum/PBTw at 4°C on a wheel over night. Following incubation with the primary antibody, larvae were washed 9x 10 min in PBTw at room temperature. Larvae were then incubated in freshly-made AP-buffer for 5 min (AP-buffer: 0.1 M NaCl. 0.1M Tris-HCl. pH 9.5. 5 mM $MgCl_2$. 0.1% Tween 20). Larvae were then transferred to a 24-well plate and then washed with BM Purple AP substrate (0.2 ml) (Roche) and then detected in BM Purple solution. The reaction was the stopped with 3 short washes in PBTw and then mounted in 80% Glycerol at 4°C.

iv) Stellaris oligo mRNA FISH protocol: oocytes

Oocytes were dissected, fixed, and stored as above. Following rehydration, the ovaries were washed 3x 20 minutes in PBTx. Hybe **(-)** was made fresh (Hybe **(-)** 2mM vanadyl-

ribonucleoside complex; 0.02% BSA by volume; 2x SSC; 10% de-ionized formamide by volume; MilliQ Add DEPC. pH = 7.2). Oocytes were washed in 50% PBT and 50% hybe **(-)** for ≥ 10 minutes, and then washed in in hybe **(-)** for ≥ 10 minutes. Hybe **(+)** was then made by adding 10% Dextran Sulfate concentration (Stock solution in 50%) to hybe **(-)**. Oocytes were the prehybridized in hybe **(+)** for ≥ one hour. Probe was added to hybe **(+)** at 25nM final concentration in the hybridization mix. It was found that for *dlg* and *grk* signal was obtained between 250nM – 2.5nM hybdrization concentration, though artefacts were observed, particularly at the oocyte posterior, at the higher side of this range. 1:10 dilution aliquots were made from the oligo 25μM stock and then diluted 1:100 for each hybridization reaction. Oocytes were left to hybridize for 3 – 24 hours (overnight usual fine) at 37^{o}C with gentle shaking in the dark. Oocytes were then washed in freshly prepared wash buffer (wash buffer; 15 % deionized formamide by volume; 2x SSC by concentration; MilliQ) 3x at 30^{o}C for 10 minutes. Samples were then mounted in Prolong gold, cured overnight, and sealed with nail varnish before imaging.

Source of probe	Source concentration	Dilution in hybe **(+)** to achieve 25nM	Final concentration in hybridization mix.
Stellaris stock (brown tube)	25μM	1:1000	25nM
1:10 dilution	2.5μM	1:100	25nM
1:100 dilution	250nM	1:10	25nM

v) Stellaris oligo mRNA FISH protocol: third instar larvae

The same protocol and buffers were applied to larvae as to oocytes. Different fixation and preincubation techniques were employed however.

Wandering third instar larvae were dissected in HL-3 buffer (Verstreken *et al.*, 2008), on sylgaurd plates, washed with ice-cold HL-3, then fixed for 15 minutes with fresh ice-cold 4% Paraformaldehyde (PFA)/PBS. *PFA should be freshly prepared that day*. The larvae were then transfered larvae to tubes containing at least 100μl ice cold 4% PFA/PBS for a further 45 minutes on ice. Tubes with flat bottom were used to prevent the folding of larvae (HydroLogix tubes, 2.0ml, 3459IW). Larvae were then rinsed three times in PBTx (0.05%TritonX-100/PBS), and washed for 5minutes in P.Tx with shaking at 4°C. Larvae were then washed in PBTw with shaking at 4°C. Larvae were then washed 3x 20 minutes in PBTw. Hereon the mRNA FISH protocol is identical to the Stellaris oligo probes protocol for oocytes above.

vi) Affymetrix QuantiView mRNA FISH protocol.

mRNA FISH was performed according to the manufacturer's guidelines. The following variations were made to try and lessen damage to tissue integrity and signal variation in both oocyte and larvae, but with no success:

1. Omission of protease treatment.
2. Omission of detergent treatment.
3. Omission of protease treatment and detergent treatment.
4. Shortening of hybridization steps to 30 minutes.
5. Gentle changes between different temperatures.
6. Dilution of probe between 1/100 and 1/10,000.
7. The use of Triton-X to improve permeability.
8. The use of Tween-20 to improve permeability.
9. Fixing larvae for 20 minutes only. Tissue damage was even more pronounced in this test.

Imaging and image analysis

i) Fixed-sample imaging

Fixed-sample imaging was performed on a widefield DeltaVision microscope (Applied Precision, Olympus IX70 with a Photometrics EMCCD). All imaging of NMJ mRNA *FISH* samples was acquired at 100x (Olympus objective, 1.514 oil) and are maximum intensity projections of 25 z-stacks across 5μm depth. All images were deconvolved using softWoRx (Applied Precision) (Davis, 2000; Parton and Davis, 2006) in order to re-assign out-of-focus light to the point of origin.

All mRNA *FISH* data was imaged identically:
TRITC: 100% bulb intensity; 0.2 second exposure.
FITC: 100% bulb intensity; 0.1 second exposure.
DAPI: 100% bulb intensity, 0.05 second exposure.
Z-stack: 25 stacks, separated by 0.2μm, totaling 5μm depth. Centre point was focused on the NMJ.
Binning: x2
Magnification: 100x
Aperture closed to remove contaminating light.
Deconvolution settings: conservative
Projection: Max Intensity

ii) Live-cell imaging

Live-cell imaging was performed on a custom-built upright widefield DeltaVision microscope (Applied Precision, Olympus IX70 with a Roper CoolSnap HQ). All live cell imaging was acquired at 60x (Olympus objective, water immersion) and are maximum intensity projections of 25 z-stacks across 5μm depth. All images were deconvolved using softWoRx (Applied Precision) (Davis, 2000; Parton and Davis, 2006) in order to re-assign out-of-focus light to the point of origin.

All live-cell imaging data was imaged identically:

TRITC: 100% bulb intensity; 0.1 second exposure.

FITC: 100% bulb intensity; 0.4 second exposure.

Z-stack: 25 stacks, separated by 2μm, totaling 5μm depth. Centre point was focused on the NMJ.

Magnification: 60x

Binning: x2

Aperture closed to remove contaminating light.

Deconvolution settings: conservative

All samples were imaged within 20 minutes of dissection.

iii) Quantitative analysis of presynaptic fluorescence

To quantify presynaptic fluorescence at the NMJ imaging conditions were standardized. DeltaVision files (.dv) were then processed using FIJI Image J. A macro was developed by Graeme Ball to allow automated analysis. For a copy of the macro code see Appendix 2.4.

iv) Microinjection

Third instar larvae were dissected in HL-3 buffer lacking calcium. This reduced spontaneous muscle contraction. Larvae were pinned to sylguard plates that were then imaged from below (i.e. through the sylguard) using a inverted DeltaVision microscope (Applied Precision, Olympus IX70 with a Photometrics EMCCD). RNA was loaded into a femtotip microinjection needle (eppendorf: 930000035). The tip of the microinjection needle was broken against a glass coverslip using micromanipulators. This increased the

aperture of the needle slightly and decreased the chance of blockage. Muscle 6 was injected with RNA and dye. The larvae was then transferred to a custom-built upright widefield DeltaVision microscope and imaged live (Applied Precision, Olympus IX70 with a Roper CoolSnap HQ).

Electrophysiology

Wandering third instar larvae were dissected in ice-cold 0.25mM calcium HL-3 solution (containing 70 mM NaCl, 5 mM KCl, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM Trehalose, 5 mM HEPES, 115 mM Sucrose; pH 7.2; Stewart et al. 1994). Dissected larvae, then, rinsed three times with HL-3 with 0.5 mM Ca^{2+}, and then incubated for at least 3 min before recording. All intracellular recordings were made at muscle 6 of abdominal segment A3, by using with sharp glass electrodes filled with a 2:1 mixture of 2 M potassium acetate to 2 M potassium chloride (resistance of 32-40 MΩ). Both Excitatory Junction Potential (EJPs) and Miniature EJPs (mEJPs) were amplified with an Axonclamp 2B amplifier in bridge mode under the control of Clampex 8.2 (Axon Instruments Inc). All experiments were performed at room temperature (20^oC–22^oC). EJPs were evoked by directly stimulating segmental nerve innervating either hemisegment A3 through a glass capillary electrode (internal diameter, ~10 mm) at 0.2 Hz. The applied currents were 6mA $\pm$ 3 with fixed stimulus duration at 0.3ms which was 50% larger than that required to activate both 1b and 1s boutons on recording muscles. Twenty to thirty evoked EJPs were recorded and analyzed for each experiment. Miniature EJPs (mEJPs) events were collected for 5 minutes. Data were collected only when resting membrane potential below -62mV, however, those data were rejected if resting membrane potential were shifted more than $\pm$ 5 mV during the course of experiment. In addition, only one muscle per larvae was recorded in each individual experiment. For paired-pulses protocol, two evoked stimuli were delivered at a short inter-pulse interval of 50ms (DT), repetitively five times with rate of 0.008Hz (every 2 minutes). EJPs and paired-pulse stimulation were analyzed with

Clampfit 9.2 software (Axon Instruments). Spontaneous release was analyzed using the Mini Analysis Program (Synaptosoft Inc., Decatur, GA). Evoked EJP amplitude was corrected by using nonlinear summation (McLachlan EM and Martin AR., 1998; Feeney et al., 1998). The quantal content of evoked release was calculated from individual muscle by ratio of the averaged EJP and averaged mEJP amplitude. Statistical analyses of EJP and mEJPs between genotypes were made using Student's t test (SigmaPlot 10.0, Systat software Inc.)

Chapter 3: Syncrip is required for the morphology and function of motoneurons

Introduction

i) mRNA binding proteins are central to synaptic plasticity

Memory and learning are dependent upon changes in gene expression at synapses that lead to the growth, loss, strengthening, or weakening, of synaptic terminals. This phenomenon is named synaptic plasticity. The *Drosophila* neuromuscular junction (NMJ) is a relatively simple and genetically tractable model for studying the basic molecular mechanisms underlying synaptic plasticity. Coupled with the powerful and sophisticated genetic tools available in flies, the NMJ has become an important and popular system for approaching key questions in synapse biology, as well as a highly tractable model for the molecular mechanisms of human neuromuscular and neuronal disorders.

A key hallmark in the history of larval NMJ biology came from the observation that synapses are highly plastic and can respond to a variety of stimuli with changes in neuron morphology and synapse structure, number and efficacy (Budnik *et al.* 1990; Broadie, and Bate. 1993; Jarecki and Keshishian, 1995). To underscore the relevance of the larval NMJ as a model for more complex neurons, mutations that affect memory, learning and decision-making in the adult fly exert profound changes on NMJ structure and function (Zhong and Wu, 1991; Broadie *et al.* 1997; Rohrbough *et al.* 2002; Dubnau *et al.* 2003). The phenotypes of mutations in key genes required at the NMJ have shed considerable light on the mechanisms that underlie memory and learning. Of the memory-impaired mutants revealed by studies in the adult fly, two clear classes have emerged: cAMP-dependent signaling gene products (Davis *et al.* 1995; Davis *et al.*, 1981) and mRNA binding proteins (Dubnau and Tully, 1998; Wan *et al.* 2006; Ashraf *et al.* 2006; Reviewed in Job and Eberwine 2001), and both classes exert significant control over NMJ

morphology and function.

Mutation in genes affecting cAMP and related Ca^{2+}/Calmodulin-dependent signalling, such as *dunce* and *rutabaga*, are detrimental to short-term memory in the adult fly (Tully and Quinn 1985; Tully and Gold, 1993; Davis *et al.* 1995; Davis *et al.* 1996). These same mutations produce defects in neuronal morphological and synaptic excitability at the NMJ through the cAMP cascade (Zhong and Wu, 1991; Zhong et el. 1992). Indeed mutations in the conserved cAMP response element binding protein (CREB) transcription factor is necessary for memory formation in adult flies (Yin *et al.* 1994; 1995), for regulating presynaptic morphology at the NMJ (Davis *et al.* 1996), and for transcription-dependent long-term potentiation in mammals (Bourtchuladze *et al.* 1994). However, alterations in synapse plasticity following signaling are dependent upon changes in synaptic gene expression, and proceed through *de novo* translation. Put simply, assembly of new synapses and changes in synapse efficacy are dependent on *de novo* translation of proteins, often within subcellular regions of the neuron or the cell it innervates (Sigrist *et al.* 2000, 2003 Wang et al; 2009; reviewed in Wang *et al.* 2010). Consistent with this, mRNA binding proteins (RBPs) are required to regulate the changes in synaptic gene expression constituting plasticity. Like genes affecting cAMP signalling, many genes encoding RBPs are highly conserved and are essential for neuronal function in the mammalian neuron, adult fly central nervous sytem, and the larval NMJ. It is through a simple and genetically tractable system such as the larval NMJ that the role mRNA binding proteins play in memory and learning in higher neurons can be elucidated. This is the focus of this thesis.

ii) The larval NMJ offers insights into how mRNA binding proteins, including Syncrip, may regulate neuronal morphology and function

As with both human patients and various experimental models, the larval NMJ has also highlighted the importance of RBPs in neuronal morphology and function. Multiple mutational studies and screens have revealed that translational regulators and mRNA

binding proteins are required for synaptic morphology and function (Table 3.1). Indeed a gain-of-function screen for mutants in synaptogenesis revealed that of 76 genes found to result in aberrant synaptic morphology when over expressed in neuron, 10 (>13%) encoded RBPs (Kraut *et al.* 2001). Interestingly, a number of RBPs that are critical in oogenesis and embryogenesis, are necessary for proper neuronal structure and the NMJ, including Staufen, Pumilio (Dubnau *et al.* 2003), Nanos (Muraro *et al.* 2008), Apontic (Kraut *et al.* 2001), Egalitarian (Kraut *et al.* 2001), and Imp (Boylan *et al.* 2008) (Table 3.1). Oogenesis, embryogenesis, and somatic neuronal plasticity all require local translation to facilitate asymmetric gene expression in large, changing cells, and it is perhaps unsurprising that the same RBPs function in these processes (Reviewed in Martin and Ephrussi. 2009). Indeed, in addition to regulating mRNA localization and translation in oogenesis, Syncrip is implicated in comparable processes in neurons.

In mammalian neurons Syncrip associates with *CamKII*α, *Arc*, and *IP_3R1* mRNAs, likely in RNA transport granules (Bannai *et al.* 2004; Elvira *et al.* 2006; Kanai *et al.* 2004). *CamKII*α, *Arc*, and *IP_3R1* mRNAs are localized within the dendrites of mammalian central nervous system neurons, and their localization is reported to be necessary for proper neuronal function (see Chapter 1). It is tempting to postulate that Syncrip may regulate mRNA localization and translation in neuronal processes. Consistent with this notion, in human dendrites Syncrip interacts with the non-coding translation-regulating RNA BC200 (brain cytoplasmic RNA 200nt) (Duning *et al.* 2008). BC200 is a primate-specific RNA almost exclusively expressed in neurons that is transported to dendritic compartments as part of RNP particles (Tiedge *et al.* 1993; Cheng *et al.* 1997). BC200 inhibits a rate-limiting step in translation through interaction with translation initiation factors eIF4A and Poly Adenylation Binding Protein (PABP) (Wang et al; 2002 and 2005). More recently it has been shown that BC200 RNA expression decreases in human brains with age, particularly between 49 and 86, while in age-matched Alzheimer's patients, brain expression of BC200 increases dramatically in this period (Mus *et al.* 2007). It has been hypothesized

that one mechanism by which Syncrip regulates neuronal expression is through interaction with BC200 (Duning *et al.* 2008). These observations complement separate studies that place Syncrip central in the regulation of neuron-localized mRNAs (See Chapter 1; Hresko and Muechler. 2000 and 2002; Mizutani *et al.* 2000; Bannai et al 2004; Kanai *et al.* 2004; Elvira *et al.* 2006). Despite these data, the role that Syncrip plays, or the mechanism by which it functions, remain elusive in studies focusing on mammalian systems. Research into Syncrip's role in *Drosophila* however, from the oocyte to the larval NMJ, has provided insights into its function in mRNA localization and local translation.

Drosophila Syncrip plays an essential role in establishing axes in oogenesis, through regulating the localization and translation of the key axes-specifying mRNAs, *gurken* and *oskar* (McDermott *et al.* 2012). Syncrip is also expressed in the larval central nervous system and is enriched at the postsynapse of the NMJ (McDermott *et al.* 2012; McDermott *et al.* in preparation), presumably at the postsynaptic membrane specialisation, the SSR. The SSR contains ribosomes, and, during upregulation of translation, either via genetic perturbation or larval crawling, accumulations of the translation initiation factor eIF4E (Menon *et al.* 2004 and 2009; Sigrist et al, 2000 and 2003). As a result the postsynapse of the NMJ is thought to be a site of local translation, though this has never been directly shown. It was hypothesized that Syncrip may bind mRNAs encoding synaptic proteins at the SSR (McDermott et al, in preparation). By immunoprecipitation of Syncrip from larval extracts, followed by reverse transcription and qPCR, McDermott showed that Syncrip specifically binds mRNAs encoding key synaptic proteins, including the conserved tumour suppressor Discs Large (Dlg), the homophilic cell adhesion molecule Fasciclin II (FasII), the voltage-gated potassium channel Shaker (Sh), and others (McDermott *et al.* in preparation). Discs large, Fasciclin II, and Shaker, are all tightly expressed at the pre and postsynapse. Appropriate expression of these proteins is necessary for normal synaptic development and plasticity at the larval NMJ, and also for learning and behaviour in adult flies (Mendoza-Topaz *et al.* 2008; Cheng *et al.* 2001; Cowan and Siegel, 1984) and

mammals (Migaud *et al.* 1998; Meiri *et al.* 1997). These observations place Syncrip as a potential key player in the cellular mechanisms underpinning synaptic plasticity in memory and learning. To understand how Syncrip may regulate synaptic function at the NMJ, it is important to consider the roles of the proteins encoded by its downstream mRNA targets in synapse biology.

fasII mRNA associates with Syncrip. The *FasII* gene encodes three isoforms of a homophilic cell adhesion receptor with similarity to the vertebrate Neural Cell-Adhesion Molecules (NCAMs) and mollusk *Aplysia* Cell Adhesion Molecule (apCAM). FasII has roles in axon bundling (fasciculation), synapse growth, stabilization and structural plasticity at the larval NMJ (Schuster *et al.* 1996a and b; Kraut *et al.* 2001); as well as short-term memory formation in the adult fly (Cheng *et al.* 2001). FasII, and its homologous NMCAMs and apCAM, have been implicated in learning and memory formation and represent key foci of interest in neurobiology (Reviewed in Murase and Schuman 1999; Crissin and Krushel 2000). At the NMJ, FasII is dependent upon the expression of another Syncrip-binding mRNA, *dlg*, for proper synaptic clustering (Thomas *et al.* 1997).

The *discs large* mRNA is a specific downstream target of Syncrip, and encodes a protein central to synapse function. Dlg is the sole and founding member of the conserved family of membrane-associated guanylate kinases (MAGUKs) in *Drosophila*. The MAGUKs contain the highly conserved PSD-95-Discs Large-Zonular Adhesion (PDZ), Src homology (SH3) and guanylate kinase (GUK), domains. Mutation studies originally identified Dlg as a tumour suppressor in the *Drosophila* brain and epithelial septate junctions (Woods and Bryant, 1991) and as a key determinant of epithelial polarity (Reviewed in Humbert *et al.* 2003). In the mammalian central nervous system the Dlg homologue, Post Synaptic Density 95 (PSD-95), scaffolds a range of key proteins that constitute the postsynaptic density, including AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)

receptors, cytoskeletal components, and signaling adapters (Reviewed in Xu, 2011). In PSD-95 mutant mice, two features central to synaptic plasticity, Long Term Potentiation and Long Term Depression, are enhanced and absent respectively (Xu *et al.* 2008). Consistent with placing PSD-95 as central to synaptic plasticity, PSD-95 is required for activity-driven synapse stabilization in mice (Ehrlich *et al.* 2007). In the *Drosophila* central nervous system and retina, Dlg also functions to cluster synaptic proteins (Ruiz-Canada *et al.* 2008). Through its role as a scaffolding protein, PSD-95 is thought to be indispensible for synaptic plasticity and function in mammals (Migaud *et al.* 1998; Carlisle *et al.* 2008; Xu *et al.* 2008; Ehrlich *et al.* 2007; Reviewed in Kim and Sheng, 2004, and Xu, 2011).

The role of Dlg at the larval NMJ is highly similar to that in the mammalian and fly central nervous systems. Dlg clusters Shaker K^+ voltage-gated potassium (Shaker) channels (Zito *et al.* 1997; Tejedor *et al.* 2007), Glutamate receptors (Chen and Featherstone. 2005), and synaptic cell adhesion molecules (Fasciclin II) to the postsynapse (Zito *et al.* 1997; Thomas et al 1997). Furthermore Dlg regulates the ultrastructure of the subsynaptic reticulum, which contain postsynaptic densities and ribosomes (Lahey *et al.* 1994; Budnik *et al.* 1996; Guan *et al.* 1996; Sigrist *et al.* 2000; Mendoza-Topaz *et al.* 2008). Owing to its central role in regulating synapse ultrastructure and postsynaptic composition in both mammals and flies Dlg has been a focus of research, and multiple studies at the larval NMJ have confirmed its key role in synapse growth, structure and plasticity.

In addition to *fasii and dlg, shaker* is a key downstream target of Syncrip*. shaker* encodes the α-subunit of the tetrameric voltage-dependent K+ channel responsible for membrane repolarization after action potentials. In vertebrates, homologues have similar properties (Littleton and Ganetzky. 2000). In *Drosophila*, Shaker (Sh) isoforms are highly expressed in the adult brain, and in particular in the memory-integrating mushroom bodies. Loss-of-function mutations, or pharmacological ablation, affecting *Sh* reveal its role in memory formation (Cowan and Siegel. 1984), sleep (Cirelli *et al.* 2005), and neuromuscular

morphology and transmission (Budnik *et al.* 1996 and 1990).

If Syncrip regulates the translation of transcripts required for synaptic plasticity, morphology, and learning, then it can be hypothesized that mutants deficient in Syncrip would exhibit defects in these neuronal processes. Previously Suzanne McDermott and Carine Meignin had shown that Syncrip mutants exhibited abnormal neuronal morphology at the NMJ (McDermott PhD Thesis, 2009, Edinburgh University). However, overexpression of muscle UAS-Syncrip-GFP in the mutant backgrounds did not rescue the mutant phenotype to wildtype levels. In the absence of a genomic rescue line it was impossible to exclude that it was solely Syncrip deficiency that contributed to the observed morphological phenotypes, and not background genetic alterations. To this end, McDermott and Meignin constructed and generated a rescue line, but time limitations prevented them from analyzing it. A fosmid covering all isoforms of Syncrip except A and H, driven by the endogenous promoter was generated by the *Drosophila* resource community, and transformed into transgenic animals by Carine Meignin. With help from Karolina Varga, the genomic rescue line, $Syp^{Rescue}/Syp^{Rescue}$, was established. Using the genomic rescue line it was possible to understand the function of Syncrip in neuronal morphology. Further, the role of Syncrip in a number of different, but related, neuronal properties can be tested using the genomic rescue line, including local gene expression at the NMJ, and synaptic transmission. From these experiments it is the aim of this thesis to understand the role and mechanism of Syncrip function.

Aims of this chapter

- **To quantify the NMJ morphology of *Syp* loss-of-function mutants relative to the genomic rescue and wildtype controls.**
- **To test if loss of Syncrip affects Discs Large expression in the muscle cytoplasm, the postsynapse, or both.**

- **To measure the efficacy of synaptic transmission in *Syp* loss of function mutants.**
- **To test whether Syncrip is required for the expression of key presynaptic vesicle release proteins in motoneurons.**

Results

i) Syncrip is necessary for proper synaptic morphology

To test whether Syncrip was required for normal neuronal morphology at the NMJ, the branching and bouton number were quantitatively assessed in Syncrip loss of function mutants. An insertion in the *Syp* gene, Syp^{e286}, which is predicted to affect all isoforms, was tested as a homozygote and trans-heterozygote in combination with the deficiency Df(3R)BSC124 (hereon referred to as Syp^{Df124}). All lines developed to pupae, with Syp^{e286}/Syp^{e286}, Syp^{e00286}/Syp^{Df124},and $Syp^{Rescue}/Syp^{Rescue}$ all exhibiting developmental delays relative to wildtype of approximately one, one, and two days respectively. Syp^{e286}/Syp^{e286}, Syp^{e286}/Syp^{Df124} animals however failed to pupate with almost 100% penetrance, while $Syp^{Rescue}/Syp^{Rescue}$ animals developed to viable adults with no noticeable issues. Quantification was restricted to the highly characterized type 1 boutons innervating muscles 6 and 7 of segments 3 and 4. OrR was used as a wildtype strain for comparison. The motoneurons were stained with anti-HRP, which binds to an unknown epitope with high specificity in insect nervous systems, and anti-Dlg to visualize the postsynapse and distinguish type 1b and 1s boutons. To ensure experimental homogeneity, all animals were grown in parallel, size-matched, and fixed in the same day for each repeat of the experiment. In animals deficient in Syncrip a significant neuronal overgrowth phenotype was observed relative to wildtype; a 1.2-fold increase in the number of branches, a 1.5-fold increase in type 1b boutons, and a 1.3-fold increase in total bouton number, per NMJ (Figure 3.1). Bouton size and shape were similar in *Syp* mutants and wildtype, as was the ratio of type 1b boutons to type 1s. The genomic rescue

lines reversed the overgrowth phenotype of the $Syp^{e00286}/Syp^{e00286}$ mutant to that of wildtype. It is therefore concluded that Syncrip is necessary for wildtype NMJ morphology.

Figure 3.1. Syp is required for proper neuronal morphology

Previously, it was shown that RNAi suppression of Syncrip expression in either muscle or neuron resulted in significant and opposing changes to NMJ morphology, suggesting that Syncrip may have distinct roles in regulating morphology neuron and muscle (Chapter 1: McDermott and Meignin; McDermott PhD Thesis, 2009, Edinburgh University). Owing to the 'off-target' effects inherent in many RNAi experiments it is not possible to conclude that Syncrip solely causes this observation. To further test the hypothesis that Syncrip functions in both neuron and muscle to regulate NMJ morphology, Syncrip-GFP was overexpressed in muscle or neurons using the muscle-specific driver MHC-Gal4 or the pan-neuronal driver Elav-Gal4. Larvae were fixed, immunostained, and quantified as above. Initially Syncrip was overexpressed in larval muscle using the C57-Gal4 late embryo/larval muscle driver, however overexpression of Syp-GFP by this driver produced larvae that survived to second instar only. Larvae in which Syp-GFP was driven by MHC-Gal4 were viable, though exhibited delayed development relative to wildtype; two days delay to reach third instar. It is possible that C57-Gal4 is a stronger driver than MHC-Gal4. Overexpression in the larval muscle resulted in under-elaboration of the neuron and a significant decrease in 1b bouton number (0.8-fold) and branching (0.7-fold) relative to wildtype: the opposite phenotype to that observed in *Syp* loss of function mutants. Furthermore the 1b boutons are abnormally large and fused (Figure 3.1 E). Overexpression in the motoneuron generated the same phenotype as the *Syp* loss of function mutants (Figure 3.1 F). While initially surprising, there are a number of possible explanations for this observation (see Discussion). Taken together, these data show that NMJ structure is sensitive to increases in Syncrip expression in both neuron and muscle. As discussed, Syncrip binds *dlg* mRNA expressed in the larvae. As changes in expression of Dlg, and in particular the localization of Dlg protein relative to the postsynapse, are correlated with changes in neuron structure (Koh *et al.* 1999; Zhang *et al.* 2007), it was important to test if Dlg levels or distribution were altered at boutons lacking Syncrip.

ii) Syncrip regulates changes in Discs Large protein levels at the whole NMJ postsynapse, but not at individual boutons

Previously, western blots performed on whole body-wall lysates of wildtype and *Syp* loss-of-function animals revealed that Syncrip deficiency resulted in a ~1.5-fold increase in Discs Large protein. Conversely, in lysate from larvae overexpressing Syncrip-GFP in muscle, the level of Dlg is reduced to ~0.6-fold (Mcdermott *et al.* in preparation). In all cases the two Dlg protein isoforms were detected (Mendoza-Topaz *et al.* 2008) and affected equally, however *dlg* mRNA levels remained unchanged in both Syncrip loss-of-function or gain-of-function lysates relative to controls. While these results indicate that Syncrip regulates post-transcription expression of Dlg, it is not possible to resolve gene expression at the synapse in biochemistry experiments performed on whole body-wall lysate. Moreover, Dlg is expressed in multiple tissues throughout all stages of the fly life cycle (Woods and Bryant 1991; modENCODE temporal expression data) and it is possible that protein levels detected in body-wall lysate may contain signal from non-muscle tissues, including cuticle and epidermis. Increased Dlg expression in muscle may result in extra Dlg protein at the postsynapse, in the cytoplasm, or both. Indeed, the localization of Dlg to the cytoplasm or the postsynapse is an active mechanism for regulating synapse formation and transmission (Koh *et al.* 199; Zhang et el. 2000). As a result it is important to determine how loss of Syncrip regulates Dlg at the NMJ.

To test if Syncrip regulates Dlg expression at the muscle postsynapse, third instar fillet preps from wildtype, *Syp* loss-of-function mutants, and larvae overexpressing Syncrip-GFP in neuron or muscle were fixed, and immunostained for Dlg protein, and tested by fluorescent imaging at muscles 6 and 7 of segments A3 and A4. Care was taken to incubate control and test larvae in the same tube to ensure that antibody exposure was uniform. To limit the effect of helium bulb age on fluorescence imaging, all animals were imaged in the same imaging session, within ~2 hours. Fluorescent imaging of whole muscle and NMJ structures at 40-fold magnification revealed that in larvae deficient in

Syncrip, or overexpressing Syncrip in muscle or neuron, there was no appreciable change in Dlg levels in the muscle cytoplasm (Figure 3.1). Dlg expression was almost entirely restricted to the post synapse in all animals tested. Similarly, imaging of individual boutons at 100-fold magnification revealed that in larvae deficient in Syncrip, or overexpressing Syncrip in muscle or neuron, there was no change in Dlg levels at individual 1s or 1b boutons (Figure 3.2). If the levels of Dlg protein per bouton remain the same in animals deficient in Syncrip, but the number of boutons increases (1.5-fold for 1b), then it follows that Dlg protein levels increase in muscle at the NMJ. This correlates with the biochemical tests, in which *Syp* deficient lysate contained a 1.5-fold increase in Dlg protein relative to wildtype (McDermott et al, in preparation). Conversely, in larvae overexpressing Syncrip-GFP in muscle there are fewer boutons relative to wildtype (0.8-fold for 1b), and as Dlg levels per bouton remain similar to wildtype, therefore there is less Dlg protein expressed in muscle at the NMJ. Again, this is in agreement with biochemical tests, in which overexpression of Syncrip-GFP in larval muscle produced lysate containing reduced (0.6-fold) Dlg protein (McDermott et al, in preparation). It can therefore be concluded that Syncrip regulates post-transcriptional expression of Dlg in muscle at the post synapse, rather than the cytoplasm. However, it is not clear whether the increase in Dlg in muscle in *Syp* loss-of-function larvae is a cause of the NMJ overgrowth phenotype observed, or simply a consequence of it. To interrogate this further, it is necessary to test whether over expression of Dlg is sufficient to produce overgrowth at the NMJ. If the answer is yes, then regulating Dlg translation is one mechanism by which Syncrip may regulate NMJ morphology, and Dlg may represent a key point of control. If however, Dlg overexpression is not sufficient for NMJ overgrowth, then Syncrip may regulate NMJ morphology through a different mechanism, and an increase in Dlg levels could be the consequence, rather than the cause, of overgrowth. While the role of Dlg in regulating the ultrastructure of the synapse, and in particular the SSR, has been a focus of research (Lahey *et al.* 1994; Guan *et al.* 1996, Budnik et al 1996, Mendoza-Topaz *et al.* 2008), and the role of Dlg-regulating factors in NMJ structure have been researched (Koh *et al.* 1999; Zhang *et al.*

2007), no study has examined the direct role of Dlg in NMJ branching and bouton number. This experiment will be conducted as part of postdoctoral research.

Figure 3.2. Levels of Dlg per bouton are similar in *Syp* loss-of-function mutants and controls

iii) Syncrip is necessary for proper synapse transmission

The changes in neuronal morphology observed in *Syp* deficient larvae frequently correlate with altered synaptic transmission. Indeed, Syncrip associates with mRNAs encoding proteins that are necessary for proper synaptic transmission including Dlg (Budnik *et al.* 1996), FasII (Ashley *et al.* 2005) and Shaker (Budnik et al, 1990). Also, mutational studies reveal that other mRNA binding proteins, and in particular translational regulators, necessary for NMJ morphology also regulate synaptic transmission (Table 3.2). To test if the morphological changes exhibited in Syncrip mutants correlated with a change in electrophysiological properties, electrophysiology was performed on *Syp* loss-of-function and genomic rescue lines to quantify the efficacy of transmission. It was not possible to perform the electrophysiology in-house as the laboratory expertise does not lie here. Through collaboration with a world-leading electrophysiology group at the Baylor College of Medicine, Texas, Hugo Bellen and Willie (Yong Qi) Lin measured the electrophysiological properties of the Syncrip mutants.

Figure 3.3. Loss of Syncrip reveals a decrease in vesicle release probability, but does not affect miniature excitatory junction potentials

First, to characterize the release properties of synapses in *Syp* mutant larvae, the spontaneous fusion of synaptic vesicles [miniature excitatory junction potentials (mEJPs)] was measured in the absence of external nerve stimulation. All *Syp* mutant larvae displayed similar mEJP amplitude to wildtype and genomic rescue animals, with no obvious change in frequency (Figure 3.3; Lin and Bellen). This indicates that the muscle in *Syp* mutant larvae responds normally to the release of a neurotransmitter vesicle from the neuron. As a result, it can be concluded that animals lacking Syncrip likely develop synapse with functional architecture e.g. postsynaptic glutamate receptors are appropriately expressed and clustered opposing the presynaptic active zones. A structurally sound synapse is not sufficient for proper synaptic transmission however. In addition, vesicles of glutamate neurotransmitter must be appropriately exocytosed from the presynapse in response to a neuronal action potential.

To test whether Syncrip is involved in exocytosis at the NMJ, motor neurons were severed and stimulated by suction electrode at low frequency stimulation (0.2Hz), and the excitatory junctional potentials (EJPs) were measured in muscle 6 (Figure 3.3 A; Lin and Bellen). *Syp* loss-of-function larvae exhibited clear and significant reductions in EJPs relative to wildtype controls. The genomic rescue line returned the phenotype to wildtype levels, showing that the observed phenotype is caused by loss of Syncrip. While the wildtype mEJPS measured in *Syp* mutants indicate that the postsynapse is fully functional, further experiments may be required to fully exclude the possibility that change in EJPs in *Syp* mutants is caused by changes in the muscle (See Discussion).

To investigate how loss of Syncrip leads to deficient neurotransmitter release, the electrophysiological properties of *Syp* mutants were further examined. The quantal content (QC) of evoked EJPs is the ratio of the average EJP and the average mEJP amplitude. The QC of *Syp* loss-of-function mutants is significantly reduced relative to

wildtype controls (~0.5-fold) and is rescued in genomic rescue animals (Figure 3.3 C; Lin and Bellen). As the QC is proportional to the number of presynaptic release sites, active zones (AZs), and the probability of release, it can be concluded that loss of Syncrip leads to a decrease in the number of AZs and/or a decrease in the probability of vesicle release at the AZs. Immunofluorescence staining for AZs will allow the quantification of AZs in *Syp* loss-of-function mutants relative to control animals, however, the required antibody was only obtained at the end of the DPhil research (See Discussion). While it is not possible to assess if loss of Syncrip leads to loss of AZs without these data, it is possible to examine if loss of Syncrip leads to a change in the probability of vesicle release.

One test that can directly assess the probability of vesicle release is the paired pulses paradigm. For paired pulses stimulation (PPS), two EJPs are evoked in quick succession (ΔT=50ms; Figure 3.3 D) and the amplitudes are measured. The amplitude of the second EJP (P2) is given a ratio to the first EJP (P1); paired pulses ratio = (P2/P1)*100. In wildtype organisms the first (P1) and second (P2) EJPS evoked by the two rapid stimulation events have roughly equal amplitude (P1~P2), and the paired pulses ratio = ~100. If the probability of vesicle release is lower in *Syp* mutants relative to controls, then the second evoked EJP amplitude would be higher than the first (P2>P1) and so the paired pulses ratio would be greater than that for wildtype (>~100). This increase in P2 relative to P1 can be caused by accumulation of vesicles and/or Ca^{2+} at the active zone that did not successfully clear in the first evoked EJP. The resulting increase in the local concentration of vesicles and/or Ca^{2+} at the AZ leads to increased vesicle release at the second evoked EJP. Thus a larger amplitude in the second EJP (P2) indicates a defect in vesicle exocytosis in the first EJP (P1). To test if the probability of vesicle release is lower in *Syp* mutants, PPS was performed in *Syp* mutants and controls and paired pulses ratios calculated. A clear and significant increase in paired pulses ratios was measured in *Syp* loss of function mutants relative to wildtype. The genomic rescue lines restored the paired pulses ratio to wildtype levels (Figure 3.3 D; Lin and Bellen). As PPS is a measure of

vesicle release probability, these data clearly indicate that loss of Syncrip leads to a decrease in the probability of vesicle release.

Electrophysiological measurement of synaptic transmission in *Syp* mutants has revealed a clear deficit in vesicle release relative to wildtype, as evidenced by a decrease in EJPs, and an increase in paired pulses ratio, at synapses that produce wildtype-like miniature excitatory junction potentials. To investigate how Syncrip may regulate vesicle release, it is necessary to examine the relationship between Syncrip and the key proteins that regulate vesicle metabolism.

iv) Loss of Syncrip results in decreased Synaptotagmin 1 protein expression at boutons

The mechanisms that govern vesicle release are active areas of research in multiple systems. The larval NMJ has provided key insights into the essential components of vesicle release, and it has been shown that synaptic transmission is highly sensitive to loss of these proteins (Littleton *et al.* 1994; Broadie *et al.* 1995; Schulze *et al.* 1995; Giagtzoglou *et al.* 2007; and others; reviewed in Schwarz, 2006). The electrophysiological phenotype in Syncrip mutants is consistent with aberrant neurotransmitter release, potentially owing to missexpression of presynaptic vesicle machinery. There are two lines of evidence placing Syncrip as central to vesicle metabolism. First, Syncrip has been shown to bind mRNAs encoding Dlg, FasII, and Shaker, whose presynaptic missexpression perturbs synaptic transmission (See above; McDermott *et al.* in preparation; Ashley *et al.* 2005; Budnik *et al.* 1996). Secondly, Syncrip has been shown to interact with both ubiquitous and neuronal isoforms of the conserved Synaptotagmin proteins, which are required for normal synaptic transmission in multiple organisms (Mizutani *et al.* 2000).

Early mutational studies on Synaptotagmin 1 (Syt1) in *Drosophila*, *Caenorhabditis elegans,*

and mice, revealed defects in neurotransmitter release, and placed Syt1 central to various models of neurotransmission (Yoshihara and Littleton. 2002; DiAntonio et al 1993; Littleton et al 1993; Nonet *et al.* 1993). Though the importance of Syt1 to synaptic transmission is clear, the exact role of Syt1 remains an active point of research. Syt1 has been postulated to dock vesicles to synaptic membrane, to be a fusion clamp during exocytosis, and to act as the Ca^{2+} sensor for the release process. No clear consensus has been formed in the literature however, likely owing to differences in experimental model and interpretation of data, coupled with the challenges that accompany studying processes that occur within 200μs (Reviewed in detail in Koh and Bellen. 2003). Despite the lack of agreement on the role of Syt1, it remains the best candidate in the neuron to sense rapid changes in Ca^{2+}, most likely through the C2B domain, and communicate these to synaptic transmission machinery for synchronous vesicle release (Geppert *et al.* 1994; Yoshihara and Littleton. 2002; Robinson *et al.* 2002; Mackler *et al.* 2002; Fernández-Chacón *et al.* 2001 and 2002; Reviewed in detail in Yoshihara and Littleton 2002; and Koh and Bellen. 2003). As a result, it is possible that the association between Syp and Syt1 proteins could be central to synaptic transmission. From this it was hypothesized that loss of Syncrip affects the expression of the vesicle release machinery, either through direct interaction with Synaptotagmin 1, or indirectly through expression of *dlg*, *fasII* and *shaker* mRNAs.

To test the levels of Syt1, immunofluorescent stainings of Syt1 were performed in, *Syp* loss-of-function mutants, animals overexpressing Syncrip-GFP in neuron or muscle, and control animals. To ensure experimental homogeneity, all animals were grown in parallel, size-matched, and fixed on the same day for each repeat of the experiment. To ensure that each test was exposed to the same concentration of antibody, test and control larvae were incubated in the same tube in each case. To limit the effect of helium bulb age on fluorescent imaging, all slides were imaged in the same session on the same day (within ~ 4 hours). In *Syp* loss-of-function larvae a decrease in Synaptotagmin 1

immunofluorescence was detected at boutons, relative to wildtype and genomic rescue animals (Figure 3.4). The deficit in Syt1 at the bouton however was not identical in each case, even within the same animal, and substantial variability was observed. Indeed, in some cases, a single bouton showed higher Syt1 levels than neighbouring boutons of the same neuron (Figure 3.4, arrow B'). Nevertheless, the reduction of Syt at the boutons of muscle 6 and in segments A2 and A3 was clear and reproducible. In parallel, the expression of other vesicle release proteins were tested by immunofluorescence [e.g. Syntaxin (Syx), Ras Opposite (Rop), and Cysteine String Protein (CSP)] though no change was detected in *Syp* mutants relative to controls. This indicates that *Syncrip* regulates Syt1 specifically, rather than global expression of the vesicle release apparatus. Syncrip binds both Synaptotagmin and a number of cytoplasmic mRNAs. It is possible that Syncrip is regulating Synaptotagmin expression through one, or both, of two possible mechanisms.

Figure 3.4. Loss of Syncrip lease to a decrease in Synaptotagmin1 expression that varies from bouton-to-bouton

As discussed, Syncrip interacts with Synaptotagmin1 (Mizutani *et al.* 2000). It is possible that through this interaction, Syncrip facilitates the localization, or stability of Syt1 at the bouton presynapse. Secondly, Syncrip could be regulating the metabolism of multiple mRNAs in the motoneuron, including *synaptotagmin1* mRNA. Syncrip could perform similar roles in the egg chamber and the motoneuron: in the absence of Syncrip, mRNA could be mislocalized, and potentially ectopically translated, relative to wildtype controls (McDermott *et al.* 2012). In the absence of techniques to assay mRNA distribution in motoneurons (See Chapter 4), the levels and distribution of Syt1 protein was approached. To test if Syt1 protein was mislocalized in *Syp* loss of function motoneurons, Syt1 levels and distribution were assayed using immunofluorescence in the larval ventral nerve cord. In *Syp* mutant animals there was a marked decrease in Syt1 immunofluorescence in the ventral nerve cord relative to wildtype (Figure 3.5). In contrast, Syt1 levels in motoneuron axons were minimal across all lines tested (not shown). These data are consistent with decrease in Syt1 levels at the bouton in *Syp* mutant larvae. The genomic rescue line however, exhibited a dramatic increase in Syt1 fluorescence at the larval brain. This shows that the genomic rescue line does not behave identically to the wildtype. Despite the overexpression of Syt1 in the *Syp* genomic rescue, the simplest interpretation of these data is that Syp is required for proper expression of Synaptotagmin 1. It is not possible however to fully conclude that Syncrip regulates Synaptotagmin expression in absence of a proper rescue however, However, it is still unclear as to how Syncrip regulates Syt1 expression.

Figure 3.5. Loss of Syncrip correlates with decreased Synaptotagmin1 expression in the ventral nerve cord

v) Syncrip binds to multiple mRNAs encoding key synaptic proteins, including dlg and shaker, but not syt1.

As discussed, it is possible that Syncrip regulates Syt1 expression through binding *syt1* mRNA. To test which mRNAs Syncrip binds to, Suzanne McDermott performed immunoprecipitation of Syncrip in native RNP complexes from larval extracts, followed by reverse transcription and qPCR showed Syncrip to precipitate with *shaker, fasii* and *dlg1* mRNA (Suzanne McDermott, PhD Thesis, 2010; McDermott et al, 2012). As discussed in Chapter 1, proper expression of Dlg or Shaker is essential for neuronal morphology and synaptic function, both at the NMJ (Budnik *et al.* 1996; Guan *et al.* 1996; Lahey *et al.* 1994; Tejedor *et al.* 1997; Thomas *et al.* 1997), adult fly brain (Mendoza-Topaz *et al.* 2008; Cirelli *et al.* 2005; Cowan and Siegel. 1984), and in mammalian systems (Carlisle *et al.* 2008; Ehrlich *et al.* 2008; Migaud *et al.* 1998; Meiri *et al.* 1997). Furthermore, in the absence of Syncrip, Synaptotagmin1 expression is decreased in the neuron. While the genomic rescue line leads to an overexpression of Syt1 at the motoneuron soma, the local expression of Syt1 at the boutons are wildtype. One mechanism by which Syp may regulate Syt1 expression, along with Dlg and Sh, is through its role in mRNA localization and local translation. To fully assess the mRNAs that Syncrip binds in muscle and neuron (hereon referred to as Syncrip-target mRNAs), Suzanne McDermott performed RNA-immunoprecipitation of native RNP complexes from larval lysate, followed by deep sequencing and analysis (McDermott et al, in preparation). The sensitivity of deep sequencing is a function of both the molar concentration, and length, of a given transcript. To uniformly quantify the amount of different Syncrip-target mRNAs, transcript levels were quantified as reads per kb of exon model per million mapped reads (RPKM; See Mortazavi *et al.* 2008 for a detailed explanation). Put simply, the RPKM measurement of read density reflects the molar concentration of a given transcript by normalizing for mRNA length. This is achieved through a number of sequential steps. First, reverse

transcription of Syncrip-target mRNA produces multiple 50bp cDNAs. These are then aligned to the different exons of *Drosophila melanogaster* transcriptome (introns are excluded in the exon model). The successful alignment of a 50bp cDNA fragment to an exon is calculated as a single 'read'. The number of reads for a given exon is then normalized to the length of that exon. By considering single mRNA exons, rather than whole genes, it is possible to calculate the prevalence of particular transcript isoforms. Thus the RPKM reflects the abundance of a particular mRNA isoform relative to transcript length. A similar and related parameter, FPKM (Fragments Per Kilobase of exon per Million fragments mapped) similarly quantifies transcript abundance relative to length. The only difference between RPKM and FPKM, is that for RPKM calculation the 50bp cDNA fragments are sequenced from one end only (single end sequenced), whereas for FPKM the 50bp cDNA fragments are sequenced from both ends (paired end sequenced). Single-end sequencing is cheaper than paired end sequencing. Thus while, the total mRNA before Syncrip Immunoprecipitation was quantified via paired end sequencing, the mRNA pulled-down with Syncrip was quantified by single end sequencing. While this difference is not expected to affect the meaning or interpretation of the data, future repeats will employ paired end sequencing only for quantification (experiments underway).

For each transcript with a substantial RPKM, the ratio of the number of reads before and after Syncrip immunoprecipitation was calculated. Deep sequencing data handling and analysis was performed by Russell Hamilton (McDermott *et al.* in preparation; Hamilton *et al.* in preparation). Transcripts enriched by 5-fold or more relative to the total RNA input were focused on (Table 3.4). McDermott and Hamilton found that a number of transcripts that encode key presynaptic proteins were in the resulting list of Syncrip-target mRNAs. Most notably, multiple *shaker* and *dlg* transcripts were enriched, by factors of 15.8 and 10.6 respectively, consistent with previous data. *synaptotagmin1* mRNA was not found to be significantly enriched in the Syncrip-target mRNA list. Although it most likely that Syncrip regulates the expression of key synaptic proteins, such as Syt1, directly through

binding mRNA, it is also possible Syncrip could regulate gene expression through protein-binding, as in certain cases RRM domains have been shown to promote protein-protein interactions instead of protein-RNA binding. Future experiments will have to be performed to assess the mechanism by which Syncrip regulates Syt1 expression.

Table 3.4. Syncrip-target mRNAs.

Discussion

These data show that Syncrip is necessary for proper neuronal structure and transmission at the larval NMJ. In the absence of Syncrip the NMJ grows extra boutons and branches in which to house them, relative to wildtype and genomic rescue animals. Similarly the probability of vesicle release decreases in *Syp* loss-of-function animals relative to controls. These observations are consistent with one another; if synapses have diminished transmitter release, then a greater number of synapses must be grown to maintain excitation-contraction efficacy in the muscle. Though these phenotypes are robust and reproducible, they are the product of complex and developmentally regulated processes in both muscle and neuron. As a result, while these data are consistent with Syncrip being central to gene expression at the NMJ, they are insufficient to deduce exactly how Syncrip functions in this role. Nevertheless, a number of conclusions can be drawn that will be discussed here:

1. Syncrip is necessary for NMJ structure.
2. Syncrip is required for proper neurotransmission.
3. Syncrip is likely to have roles in the neuron and muscle that contribute to NMJ structure and function. These roles may be distinct.

It is interesting that overexpression of Syp-GFP in the motoneuron generated the same phenotype as the *Syp* loss of function mutants. While initially surprising, there are a number of possible explanations for this observation. One likely explanation is that Syncrip has distinct roles in muscle and neuron with distinct downstream mRNA targets. Indeed, there is precedent for this in other key regulators of synaptic morphology, including Pumilio, Nanos, and Leucine-Rich Repeat Kinase 2, which have different binding partners in the pre and post synapse (Menon *et al.* 2004; Menon *et al.* 2009; Lee *et al.* 2010). If this is the case for Syncrip, then the aberrant NMJ structure and function

observed in *Syp* mutants will be a function of both muscle and neuronal Syncrip deficiency. This can complicate analysis of the phenotype of the deficiency, particularly as muscle and neuron are sensitive to the expression patterns of one another. For example, expression of Dlg in the neuron can rescue Dlg-deficiency in muscle (Budnik *et al.* 1996). Taken together, the data from animals overexpressing or lacking Syncrip, show that Syncrip is necessary for correct NMJ morphology, potentially through distinct regulation of pre- and postsynaptic mRNAs. There are a number of experimental approaches that can dissect the pre and postsynaptic roles of Syncrip. First, using the Gal4 system it is possible to overexpress Syncrip-GFP in neuron or muscle in animals that are deficient in genomic Syncrip, in attempt to generate a 'neuronal rescue' and a 'muscle rescue'. There are, however, caveats to this approach in this case. Firstly, Syncrip-GFP overexpression generates a non-wildtype NMJ morphology phenotype when expressed in the *Syp* loss-of-function backgrounds, that resembles overexpression of Syncrip in widtype muscle (Suzanne McDermott and Carine Meignin; McDermott PhD Thesis, 2009, Edinburgh University). It is possible that even in animals deficient in wildtype Syncrip, overexpression of Syncrip-GFP via Gal4 still produces excess Syncrip activity. Second, the Syncrip-GFP construct is unlikely to entirely replicate endogenous Syncrip function. CG17838 is a large gene (>45 kb) and encodes 8 annotated transcripts and protein isoforms of Syncrip (A-H), while the Syncrip-GFP construct was cloned from a single cDNA with greatest sequence identity to isoform (CG17838-F) (Figure 1.5).

To distinguish between the neuronal and muscle roles of Syncrip it is necessary to circumvent these limitations; all endogenous Syncrip isoforms have to be downregulated in muscle or neuron with high specificity. To approach this problem, powerful new genetic tools employing the *Minos* Mediated Integration Cassette (MiMIC) have been employed (Venken *et al.* 2011). Put simply, a collection of over 4,000 insertions of the MiMIC transposable element into random *Drosophila* genes has been generated, and each MiMIC insertion line facilitates recombination-mediated cassette exchange (RCME) of any

sequence of DNA into a gene. 2,293 of these insertions map to 1,541 annotated genes and the library is growing. MiMIC technology allows any exogenous DNA sequence to be added to any one of these 1,541 genes. The exogenous DNA will then be transcribed, spliced, and translated in frame with the gene in which the MiMIC element resides. For example, a fluorophore, such as Enhanced Green Fluorescent Protein (EGFP), can be inserted into a MiMIC insertion site in a given gene to "trap" the endogenous protein products of that gene with a fluorescent marker. In parallel with MiMIC, Hugo Bellen (Baylor College of Medicine, Texas) developed highly specific RNAi constructs directed against GFP. The anti-GFP RNAi lines are predicted to have no off-target effects in *Drosophila*. If the endogenous Syncrip protein isoforms were trapped with GFP, then anti-GFP RNAi could be driven in either muscle or neuron to downregulate Syncrip expression in either tissue with great specificity. This would allow experimental dissection of the roles of Syncrip in the muscle and neuron of the NMJ. There are four MiMIC insertions in the CG17838 gene (Table 3.3). Two insertions, MI03981 and MI06413, will allow all isoforms of Syncrip to be trapped with EGFP, while the remaining two MiMIC insertions, MI02101 and MI05398, will trap isoforms A and H, and A,B,C,D,E,F,H respectively. Using MiMIC technology, in collaboration Hugo Bellen, the Syncrip proteins are being trapped with EGFP at all insertion sites. Insertions MI03981 and MI06413 will be most useful in trapping all Syncrip protein isoforms with EGFP, while MI02101 and MI05398 will trap specific isoforms, allowing for isoform-specific experiments in the future. Though these lines are in production, it has not been possible to complete them during the course of the DPhil as MiMIC resources were published 31/06/2012. Anti-GFP RNAi experiments in muscle and neuron will be completed as part of post-doctoral research.

As with neuronal structure, synaptic transmission is a product of communication between two cell types. As a result, further experiments are required to fully dissect the role of Syncrip in synapse transmission in neuron and muscle. While the wildtype mEJPS measured in *Syp* mutants indicate that the postsynapse is fully functional, to fully exclude

the possibility that change in EJPs in *Syp* mutants is caused by changes in the muscle, two experiments should be performed. First, it is necessary to fully test if the glutamate receptors are appropriately clustered apposing active zones in *Syp* mutants. This can be approached by testing the immunofluorescence of the Glutamate Receptor subunit IIC (GluRIIC or GluRIII), a subunit shared between both types of Glutamate receptor complexes in muscle, and Bruchpilot (Brp), a marker for presynaptic active zones (AZs), in *Syp* mutants relative to controls. In wildtype animals virtually all AZs and Glutamate receptors are apposed to one another across the synapse, and deviation from one another leads to aberrant synaptic transmission (Marrus and DiAntonio. 2004). The antibodies for this experiment have been obtained only recently (anti-GluRIIC, a gift from Aaron DiAntonio; Brp, DSHB), and time constraints prevent the data being presented here. In the absence of these reagents, the levels and distribution of the glutamate receptor subunit IIA, which represents one of two heterotetrameric glutamate receptor complexes in the postsynapse, was examined in *Syp* mutants and controls. There was no appreciable change in GluRIIA levels or distribution in the *Syp* loss-of-function (Figure 3.6).

While it appears that GluRIIA-containing glutamate receptors are appropriately expressed in *Syp* mutants, it cannot be excluded that the levels or distribution of GluRIIB-receptors and/or Brp has changed in the *Syp* mutants relative to wildtype and contributes to the defect in synaptic transmission. Secondly, to confirm that postsynaptic Glutamate receptors are functional, EJPs from muscle 6 can be measured in response to pressure injection of the neurotransmitter L-glutamate directly onto the muscle in *Syp* mutants and control larvae. Both of these experiments are crucial to dissecting the role of muscle and neuronal Syncrip in regulating synaptic transmission, and will be performed as part of postdoctoral research. Until these data are available, it is not possible to fully exclude that defects in muscle also contribute to the change in EJP measured in *Syp* mutants. Consistent with this notion, it is noted that it is technically challenging to obtain a level resting potential in *Syp* mutant larvae bodywall muscle for obtaining electrophysiological

recordings (Lin, personal communication); this could indicate a defect in muscle electrophysiology.

Figure 3.6. Loss of Syncrip, or overexpression in muscle, does not affect GluRIIA expression

At the bouton, loss of Syncrip leads to a clear, albeit variable, reduction in Syt1 in motoneurons, while the expression or other key presynaptic vesicle release components remain unchanged. While the genomic rescue does not fully rescue the reduction in Syt1 at the bouton, the levels are elevated relative to loss-of-function mutants and less variable. It is intriguing that unlike the deficits in neuronal structure and synapse electrophysiology observed in *Syp* mutants, the downregulation of Syt1 at the bouton was not perfectly rescued by the genomic line. Indeed, investigation of Syt1 expression in the motorneuron cell bodies in the ventral ganglion revealed a dramatic overexpression of Syt1 in the genomic rescue line. This observation may offer insights into Syncrip function. It is possible that the genomic rescue line inappropriately expresses Syncrip owing to changes in the local chromatin context of the fosmid insertion. Regulation of CG17838 may be dependent of sequences outside of the gene and promoter, e.g. regulatory *trans*-acting DNA sequences. As a result, in the genomic rescue, Syncrip may be over expressed or under expressed in the larval CNS, or expressed at inappropriate developmental stages.

While it is possible that missexpression of Syncrip in the ventral nerve cord leads to the increase in Syt1 expression, it is unlikely, as neither loss-of-function or gain-of-function *Syp* animals exhibit elevated Syt1 levels in the CNS relative to controls. Another possibility is that the Syncrip isoforms absent from the genomic rescue line are required for proper rescue of wildtype Syt1 expresssion. The genomic rescue line encodes 6 of 8 Syncrip isoforms, A and H are excluded. While expression of B,C,D,E,F, and G, is sufficient to fully rescue deficiencies in neuronal structure and transmission, isforms A and/or H may be required to rescue Syt1 expression in a wildtype manner. In the absence of isoforms A and H, Syt1 levels are partially rescued at the bouton, but concomitant with this is an overexpression of Syt1 at the cell body in the ventral nerve cord. For example, while isoforms B,C,D,E,F, and G may rescue Syt1 expression at the bouton, isoforms A and H may be required to suppress excess Syt1 expression in the ventral nerve cord.

Alternatively, though Syt1 is fully expressed in the genomic rescue line, isoforms A and H may be required for trafficking of Syt1 protein or mRNA to the bouton, leading to an excess of ectopically expressed Syt1 protein in their absence. This explanation is however unlikely. RTPCR was previously employed to detect the expression of different isoforms throughout development, and isoforms A and H were not detected earlier than the pupal stage. As a result, isoforms A and H are not predicted to have a role in the larval stage. It is possible that isoforms A and H may be expressed at low levels, below the threshold of detection, or may be expressed transiently, at a crucial stage of development. To fully understand the function of Syncrip it is necessary to dissect the different roles of distinct isoforms. While this is not possible from the data here, and beyond the scope of this thesis, employing reverse genetics to mutate specific isoforms of Syncrip would shed light on the different roles of Syncrip isoforms.

In summary, these data here clearly place Syncrip as key to neuronal morphology and synaptic transmission. Owing to the conserved role of Syncrip in mRNA binding, the key nature of Syncrip-target mRNAs in synapse function, and the requirement of Syncrip for mRNA localization and local translation in oogenesis (McDermott et al, 2012; McDermott et al in preparation; Hamilton et al, in preparation), it was hypothesized that Syncrip may regulate neuronal structure function, at least in part, through the localization and local translation of neuronal mRNAs. To this end, techniques were developed to try and test the distribution of Syncrip-target mRNAs, such as *sh* and *dlg*, in the motoneuron.

Chapter 4: Characterization of the presynaptic distribution of Syncrip-target mRNAs

Introduction

i) Syncrip may regulate NMJ structure and function through the localization of neuronal mRNAs

Loss of Syncrip leads to changes in neuronal morphology, bouton number, synaptic transmission, and most likely, a reduction of Synaptotagmin1 expression in the motoneuron. Although it is unknown whether these different phenotypes are inter-related, it is possible to envisage some mechanisms *a priori* that might relate Synaptotagmin function with mRNA transport or translation. For example, it is possible that mRNA arrives at the synapses in association with Synaptotagmin-bound vesicles. To understand further how Syncrip regulates these key neuronal functions, the mechanism of Syncrip function must be characterized in more detail.

Owing to the conserved role of Syncrip in mRNA binding, the key nature of Syncrip-target mRNAs in synapse function, and the requirement of Syncrip for mRNA localization and local translation in oogenesis (McDermott et al, 2012; McDermott et al in preparation; Hamilton et al, in preparation), it was hypothesized that Syncrip regulates neuronal structure function, at least in part, through the localization and local translation neuronal mRNAs. To this end, techniques were developed that allow the distribution of Syncrip-target mRNAs, such as *sh* and *dlg*, to be assayed at the motoneuron bouton. Once established, these techniques would allow the role of Syncrip in regulating mRNA localization in the neuron to be tested.

ii) mRNA fluorescent in situ hybridization is challenging in neurons

mRNA Fluorescent *in situ* hybridization (*FISH*) is a powerful tool for examining the endogenous levels and localization of transcripts, with modern methods increasingly achieving single molecule sensitivity (Femino et al 1998; Raj *et al.* 2008; Batish *et al.* 2011). Moreover, mRNA *FISH* is often compatible with protein immunocytochemistry, allowing for mRNAs to be detected relative to important proteins. Despite the ubiquitous success of mRNA *FISH* across a range of cell types and tissues, including the developing nervous system, mRNA *FISH* of specific mRNAs in mature neurons, has proved technically challenging.

Though there are examples of neuronal mRNA *FISH* in the literature, most focus on *ex planta* (Swanger *et al.* 2011; Sotelo-Silveira *et al.* 2011; Lyles *et al.* 2006) or cultured neurons (Bassell *et al.* 1994; Batish *et al.* 2011; Poon *et al.* 2006; Lyles *et al.* 2006). Further, those examples that show mRNA localization in whole nervous systems, e.g. mature brains and developing embryos, are limited to gross imaging encompassing multiple neurons and are unable to resolve single cells (Wanaka *et al.* 1990; Esclapez *et al.* 1993; Swanger *et al.* 2011; Daul *et al.* 2010). Neurons present a number of technical challenges that can confound *in situ* protocols, and it is only recently that ISH techniques have increased in sensitivity and spatial resolution to approach the complex structures of neurons. First, probe penetration can be difficult; neurons are frequently coated by myelinated sheaths and glial cells *in vivo*, both of which can impede probe penetration. It is challenging to balance structural preservation in intact tissue with the need for probe penetration, which can incorporate large modified nucleotides. To circumvent this, neurons can be cultured without the glial microenvironment. Though some cultural preparations do form functional synapses (e.g. the *Aplysia* sensory and motor neurons; Wang *et al.* 2009), axons do not always fully develop and neurons often do not exhibit the full electrical connections central to their function *in vivo*. Moreover, cultured neurons inevitably develop in the absence of surrounding tissues, which may affect function. Some preparations do exist that can circumvent these issues, for example in the common

goldfish *Carassius auratus*, the giant axon can be dissected and fixed without myelin or glial, but these *ex planta* preparations likely perturb neuronal function to some extent (Sotelo-Silveira *et al.* 2011). Second, RNA copy number is thought to be low in neuronal processes. In all cells RNA numbers are typically lower than that of the proteins they encode, and in a long thin projection of cytoplasm the number of RNA molecules present can be lower still. Indeed, recent evidence suggests that in cultured neurons mRNA is transported to dendritic processes as single molecules (Batish *et al.* 2011). A major problem with such low signal levels is that even small degrees of unwanted background, such as non-specific probe binding, can significantly mask the *bona fide* mRNA signal. As a result eliminating background signal is often the key to obtaining useful mRNA *FISH* data in neurons, and often has to be optimized from system-to-system (Kim *et al.* 2010). In many cases the low amount of RNA in processes relative to the cell body soma can lead to image saturation of the cell body before the signal in the processes can be detected. To circumvent this, and increase the sensitivity of *FISH*, tyramide-signal amplification can be used (Wilkie *et al.* 1999; Lyles *et al.* 2006), however this can lead to two problems. Firstly, tyramide-signal amplification is non-linear, and subsequently, non-quantitative. Secondly, as mentioned, the small difference between specific and non-specific probe binding is a major problem for mRNA *FISH* in neurons, and this can be exacerbated by non-linear signal amplification.

Another issue is that neurons contact other cells, sometimes forming many connections with different cell types, and it can be difficult to isolate a specific neuron for imaging. The neuron must be made accessible to imaging, either by removal from the nervous system, or by exposing the neuron *in situ*, by dissection of intact tissue, e.g. by fillet prep or equivalent. When imaged in intact tissue, non-neuronal tissues can provide contaminating autofluorescence, particularly if damaged. For example, in a live fillet prep it was found here that muscle autofluorescence increases dramatically after 15-20 minutes. While mRNA *FISH* has been performed at the NMJ, the experiments have been targeted

towards mRNAs expressed only in muscle (Currie *et al.* 1995; Sigrist *et al.* 2000; Karr *et al.* 2008; Ganesan et al 2011). To test if Syp-target mRNAs were enriched in the presynapse, these published protocols were directed against mRNAs expressed in both muscle and neuron.

Aims of this chapter:

1. **To optimize and test if different mRNA *FISH* technologies can detect mRNA in motoneurons**
    A. **To test if existing mRNA *FISH* techniques detect neuronal mRNA at the NMJ.**
    B. **To test if multiple small, singly labeled, DNA probes can be used to detect neuronal mRNA at the NMJ.**
    C. **To test if branched DNA probes can be used to detect neuronal mRNA at the NMJ.**
2. **To test if mRNA can be imaged in live motoneurons.**
    A. **To develop a cloning strategy to produce Syncrip-target mRNAs tagged with MS2 loops for live imaging.**
    B. **To develop quantitative methods of analyzing the presynaptic localization of MS2-RNAs relative to controls.**

Results

i) Existing fluorescence in situ hybridization techniques do not show mRNAs at the NMJ presynapse

To test if existing mRNA *FISH* protocols would reveal mRNAs at the bouton presynapse, riboprobes incorporating digoxigenin (DIG) modifications were made with complementarity to the Syncrip-target mRNA *dlg*. Dlg protein is highly localized throughout *Drosophila* development (Woods and Bryant, 1991), expressed at the presynapse (Lahey *et al.* 1994), and the *dlgA* transcript is known to exhibit an asymmetric distribution in the blastoderm embryo (Lécuyer *et al.* 2007). Further, it is possible to overexpress Dlg in specific tissues using Gal4 drivers (Brand and Perrimon. 1993) and UAS-Dlg-GFP; this reagent is a useful tool for testing probe specificity. For these reasons *dlg* mRNA was chosen as a good

candidate for examining presynaptic mRNA localization. It should be noted however, that no NMJ-localizing mRNA is published in the motoneuron. While the absence of a positive control makes developing new mRNA *FISH* protocols challenging, it is the aim of this chapter to test a number of methods to find NMJ-localizing mRNAs. Initially, a protocol that has been reported to specifically detect mRNA in larval muscle was employed. Larvae were fixed, washed and treated as published in Karr *et al.* 2008 (Hereon referred to Karr *et al.* 2008 protocol; See Materials and Methods for detailed protocol). Briefly, larvae were fixed for 15 minutes in 4% paraformaldehyde at room temperature, then 45 minutes on ice. Following multiple washing steps in phosphate buffer saline (PBS) with 0.1% Triton-X, washing was continued in PBS and 0.1% Tween-20. Larvae were then incubated in Hybe- buffer containing yeast tRNA, sheared salmon sperm DNA, and Heparin at 56^{o}C for 4 hours to block non-specific binding. Riboprobes were transcribed *in vitro* using T3, T7, or SP6 polymerases from cDNA sequences from the Berkeley Drosophila Genome Project (BDGP) Gold collection (See Materials and Methods), and hydrolysed from full length to approximately 150bp to improve probe penetration. Larvae were subsequently incubated with riboprobes (1ng/μl) over night at 56^{o}C in Hybe+ (with 5% dextran sulphate), then washed for over 24 hours in wash buffer, before being incubated with anti-DIG primary antibodies over night at 4^{o}C. Secondary antibodies were incubated for 2 hours at room temperature and larvae were mounted in ProLong Gold medium and left to cure overnight before imaging.

Owing to the demands of fixing each larva for 15 minutes at room temperature, then 45 minutes on ice, and then washing at 4^{o}C, fixing larvae from five different genotypes in parallel can be technically demanding and require great organization. For example, the washing steps for one line must be performed while another line is fixing on ice, while another line is being dissected. To try and circumvent this, larvae were fixed and dehydrated in -20^{o}C methanol and stored at -20^{o}C. While this allowed larvae to be fixed over a great period of time, and rehydrated and tested in parallel, it was found that

methanol treatment increased the background fluorescent signal in the larval muscle for an unknown reason (data not shown). Instead, to fix a suitable number of larvae for each genotype in an experiment while maintaining experimental homogeneity, dissection had to be performed in a single session, frequently lasting ~7 hours. This limited the number of larvae per genotype per experiment to 4. While each mRNA *FISH* experiment presented here has been repeated, and assayed in more than 12 NMJs per experiment, a greater n number would be preferred.

Comparison of *dlg* probe with *dlg sense* strand, a control for non-specific binding, showed that no *FISH* could be detected in the neuron at the NMJ by the Karr *et al.* 2008 protocol (Figure 4.1). While there was significant difference between *dlg* signal in the muscle relative to non-specific control, and no change in the wildtype animal, (See Chapter 5), indicating that the probes were specifically binding *dlg*, no signal could be detected over background within the neuron, as marked by the presynaptic epitope recognized by an anti-HRP antibody. It is possible that the presynaptic mRNA levels are far lower than in the muscle, and the mRNA *FISH* probes do not detect the target mRNA above background. To test if an increase in levels of presynaptic *dlg* mRNA would lead to an increase in *FISH* signal in the neuron, mRNA *FISH* was performed in animals over expressing Dlg-GFP in motoneurons, driven by the pan-neuronal driver Elav-Gal4. Larvae were selected by GFP fluorescence in salivary glands to confirm expression of Dlg-GFP, fixed and dissected as above in parallel with wildtype controls. No increase in presynaptic *dlg* levels was detected presynaptically in animals overexpressing Dlg-GFP in neurons relative to controls (Figure 4.1, C-D).

Figure 4.1. Karr *et al*. *FISH* protocol reveals *dlg* mRNA signal in muscle, but not in neuron

ii) Attempting improved probe penetration did not reveal presynaptic mRNA

As discussed probe penetration is a major hurdle in performing mRNA *FISH* in neurons, particularly in the context of glia, muscle, and other surrounding tissues that may sequester or block probe penetration into the neuron. Consistent with this, probe penetration into muscle was not problematic, and was shown to be specific (Chapter 5). To improve probe penetrance into neurons, the fixed larvae were treated with Proteinase K for 1, 3, or 5 minutes. However no change in fluorescent signal was detected presynaptically, and defects in neuronal morphology were detected (Figure 4.1, E) even at 1 minute exposures. To attempt to improve probe penetration, while preserving tissue structure, an unpublished larval mRNA *FISH* protocol developed by Carine Meignin was employed (Summarized in Figure 4.8). This protocol involved a number of significant changes to that published in Karr et al 2008. The fixing, washing, and hybridization steps employed different reagents, and larvae were treated with Proteinase K for 3 minutes, then dehydrated and stored in methanol at -20^{o}C (Materials and Methods). Furthermore no blocking steps were used and washing steps were reduced in number and duration. This protocol was a work-in-progress, and had only been performed a handful of times. Nevertheless the preliminary data produced by Carine Meignin appeared to show *dlg* mRNA signal colocalizing with the presynaptic marker anti-HRP (not shown). To test this result further, the mRNA *FISH* protocol developed by Carine Meignin was repeated five times in total, across more than 50 animals in total. Despite the issues previously reported with Proteinase K treatment and larval mRNA *FISH*, with this protocol the Proteinase K treatment did not lead to loss of tissues structure. It is possible that by reducing the number and duration of washes significantly, the fillet preparations were agitated less, and minor tissue damage cause by Proteinase K did not lead to gross changes in NMJ morphology. Furthermore, imaging at the NMJ revealed fluorescent signal within the neuron, something not detected with the protocol from Karr *et al.* 2008. The presynaptic

dlg signal was highly variable from experiment-to-experiment and was broadly similar to the non-specific probe however (*dlg sense*) (Figure 4.2). Negative controls in which no probe was used showed no presynaptic signal, indicating that the *dlg* and *gfp* probe were hybridizing to the sample. To investigate the effect of probe concentration on non-specific binding, mRNA *FISH* was performed with probes ranging from 1ng/μl to $5x10^{-5}$ ng/μl (1/100 probe dilution to 1/20,000), however, non-specific binding seemed broadly similar to test *dlg* probe (probe dilution 1/20,000 shown in Figure 4.2).

Owing to the great variation from NMJ-to-NMJ in both *dlg* test and non-specific probe control, coupled with comparably variable signal in the muscle, it was challenging to quantify the presynaptic signal. Instead, a different method was employed to test if there was a difference between the *dlg* test and non-specific probe control. In collaboration with Graeme Ball, the Image Analysis expert employed in the Advance Microscopy Unit (Micron) housed in the Department of Biochemistry, a program was written that would mask the name of each imaging file during analysis. This allowed each file to be assigned as either a 'positive', in which substantial presynaptic fluorescent signal was detected, or a 'negative' in which no presynaptic fluorescent signal was observed (Figure 4.2, D and E). As a result, images of NMJs treated with *dlg* or non-specific probe could be analyzed blind in parallel. It was found from over 100 unique images (imaged, processed and contrasted identically), that there was no difference between the percentage of 'positives' found with *dlg* or non-specific probe (Figure 4.2, F). It was therefore concluded that the presynaptic signal detected with this protocol represented, at least in the most part, non-specific binding events. To attempt to circumvent this issue, a different and relatively new mRNA *FISH* protocol was employed.

Figure 4.2. Carine Meignin protocol detects variable and non-specific presynaptic signal

iii) mRNA FISH with Stellaris oligo probes in the oocyte and larva

To detect mRNAs within the neuron it was decided that a methodology that maintains tissue structure, while allowing probe penetration, and produces a strong signal to background ratio, was necessary. One way that tissue penetration can be enhanced is by using smaller probes. The probe size used thus far is approximately 150nt, as measured by gel electrophoresis. To improve probe penetration 20nt oligonucleotide (oligo) probes were purchased from Stellaris. Each probe mixture contained 48 different 20nt oligo probes, each targeted against a unique sequence in the *dlg* transcript (summarized in Figure 4.8). The probes were designed against the regions of the *dlg* transcripts that are free from secondary structure and available for binding using Stellaris in-house algorithms. Each probe contains one fluorophore, allowing the target transcript to be decorated with multiple fluorophores, in principal providing high sensitivity and quantitative assessment of target transcript levels. As no published study has employed oligo probes for mRNA *FISH* in the larval fillet prep, a new protocol had to be developed. Owing to the challenges of performing mRNA *FISH* on the larval NMJ, a proof-of-principle protocol was first engineered for oocyte, in which robust positive controls would allow the technique to be tested.

Figure 4.3. Stellaris DNA probes reveal mRNA localization in the oocyte, but not at the larval NMJ

In the oocyte, the TGFα-like factor Gurken, is restricted to the dorso-anterior corner of the stage 9 oocyte through localization of *gurken* mRNA, where it establishes dorso-ventral fate in the surrounding maternal follicle cells (Neuman-Silberberg and Schupbach. 1993 and 1994) (Figure 4.3, A and A'). The localization of *gurken (grk)* mRNA to the dorso-anterior corner of the oocyte is highly asymmetric, and localized *grk* is present at high levels. As a result *grk* oocyte localization is a useful positive control to test Stellaris probe technology before progressing to the larval NMJ. *gurken* mRNA forms dynamic interactions with the electron-dense sponge bodies in the dorsoanterior corner of the oocyte thought to be central to anchoring and translation regulation (Delanoue *et al.* 2007; Weil *et al.* in preparation). Sponge bodies are marked by the DEAD-Box RNA helicase Me31B, and at 100-fold magnification, proper *grk* localization should interdigitate with sponge bodies (Weil *et al.* in preparation). To test if Stellaris probes can detect fine mRNA distribution, *grk* mRNA was labeled relative to Me31B-GFP. No published protocol could be found for using multiple singly labeled oligo probes in the oocyte, however, studies have been published using this technology in the *Drosophila* embryo (Little *et al.* 2011) and larval imaginal wing disc (Raj et al 2008). Combining elements from these published protocols, while testing the effect of varying hybridization temperature, allowed the development of a functional protocol for mRNA *FISH* in the oocyte using Stellaris oligo probes.

Following mRNA *FISH grk* mRNA was clearly and specifically labeled at the dorsoanterior corner of the stage 9 oocyte (Figure 4.3, B to B''). The distribution of Me31B-GFP was unaltered in this experiment, indicating that oocyte structure was maintained through the mRNA *FISH* procedure. To test if the fine localization of *grk* mRNA was maintained, *grk* distribution was tested relative to Me31B-GFP at 100x magnification. Consistent with previous observations in live cells (Weil *et al.* in preparation), *grk* mRNA and Me31B-GFP interdigitate at the dorsoanterior corner (Figure 4.3, C-C''). This indicates that in the

oocyte, Stellaris probes can specifically detect *grk* mRNA while maintaining cell structure. Compared to conventional mRNA *FISH* techniques in the oocyte, such as TSA amplification, there is relatively high background-to-signal when using Stellaris oligo probes. To test if the background signal could be mitigated at lower concentrations of probe, the *grk* probe was diluted to 1/1000. While the lower background fluorescence signal was detected at 1/1000 probe, lower *grk* signal was detected also (Figure 4.3, D-D'). Despite this, detection of *grk* mRNA using Stellaris oligo probes has provided a useful method for researching mRNA biology in the oocyte, and has provided a proof-of-principle of the technique.

To test if Stellaris oligo probes could detect presynaptic mRNA at the NMJ, a protocol was developed for mRNA *FISH* on the larval fillet prep. Developing from the oocyte-optimized protocol, hybridization temperatures, times, and probe concentrations were kept the same, while fixation and washing methods were borrowed from the Karr *et al.* 2008 protocol (See Materials and Methods for full protocol). It was found that significant fluorescence in the muscle obscured any fluorescent signal that may have been present in the neuron (Figure 4.3, E to E''). The fluorescent signal detected in the muscle with the *dlg* Stellaris probe did not resemble the punctate structures found with the Karr et al 2008 protocol (Figure 4.1) and appeared identical to a non-specific Stellaris probe used (not shown). It was concluded that the fluorescent muscle signal was non-specific background. To try and circumvent this, extra washing steps and shorter hybridization times were employed. In addition, the *dlg* probe was diluted by 10-fold to 1/10,000, substantially less than that recommended by the manufacturers and other published protocols. Even with these steps, no decrease in background muscle fluorescence was detected (Figure 4.3, F-F''). In the oocyte, *grk* mRNA is present at a high copy number, and could be detected above the background fluorescence found with Stellaris oligo probe mRNA *FISH*. In the motoneuron however, mRNA levels may simply not be high enough over muscle background to be

detected. To improve on signal-to-background levels, while continuing to use a small oligo probe to aid tissue penetration, a different protocol was tried.

iv) mRNA FISH in the oocyte and larvae with Affymetrix branched DNA technology

Affymetrix Quantigene ViewRNA Cell Assay (hereon referred to as the Affymetrix protocol) employs novel branched-DNA technology to allow single molecules of mRNA to be detected in single cells. As with Stellaris probes, the Affymetrix probes are small oligo nucleotides (20–100nt), however, Affymetrix technology allows each mRNA to be labeled with a greater number of fluorophores than with Stellaris probes by employing branched DNA technology (Figure 4.8, D). As with the Stellaris protocol, the Affymetrix *FISH* system employs multiple small oligo probes to bind to the target mRNA. These probes (named target-probes) however, do not contain any fluorophore. Instead, after incubation of the target-probes, the sample is incubated with a longer (~100nt) oligonucleotide probe, the pre-amplification probe, that recognizes a degenerate sequence in each of the target probes. Next, multiple amplification probes decorate the pre-amplification probe, and each amplification probe can in turn bind multiple small fluorescent oligos. The result is that each small target probe can recruit a large number of fluorophores to the target mRNA (summarized in Figure 4.8). In theory this technology provides sufficient signal per mRNA to allow for detection of single mRNAs. However, employing multiple types of probes requires a greater number of permeabilization, hybridization, and washing steps, often with different buffers at each step. Care would have to be taken to ensure that tissue integrity was maintained during these steps.

To test the principles of the Affymetrix technique, the oocyte-localized mRNA *gurken* was again labeled for in egg chambers expressing Me31B-GFP. Following the manufacturer's protocol however, no *grk* mRNA could be detected at the oocyte dorsoanterior corner and Me31B-GFP signal was clearly disrupted (Figure 4.4, A'-A''). Moreover, gross damage to

the stage 9 egg chambers was observed (arrowheads, Figure 4.4, A-A'', B-B''). Indeed, from a starting population of >100 oocytes of stage 9 or younger, fewer than 5 were identifiable in each slide, and all showed signs or significant damage; most younger stages had been broken down completely by the process. Even in the older stage 14 oocytes, which are typically more resistant than stage 9 egg chambers, breakages could be seen in the cuticle (arrowheads, Figure 4.4, C-C''). The Affymetrix protocol is optimized for cell culture, and has not been published outside of this system. Moreover, the Affymetrix buffers and reagents come pre-mixed in a commercial kit with unknown compositions. While the Stellaris protocol was optimized in the oocyte from a combination of protocols in other systems, and altering conditions in line with first principles, it was challenging to optimize the Affymetrix protocol when the reagents are unknown. To try and mitigate the tissue damage to the oocytes, hybridization temperatures and the duration of washing steps were altered, but to no effect.

Despite the difficulties with oocyte mRNA *FISH*, it was decided to test the Affymetrix technology in the larva as the larva may be more resistant that the oocyte. As with the oocyte, significant tissue damage was the main obstacle to data collection. Almost all muscles exhibited tearing (yellow line, Figure 4.4, D), holes, and stretching, and nuclei with stretched and deformed shapes (arrowheads, Figure 4.4, D). Though each larva contains 14 pairs of muscles 6 and 7, frequently none were preserved well enough for imaging. Those muscles that could be imaged showed punctate fluorescent structures throughout the muscle. Both *dlg* probes and the non-specific binding control probe, *lacZ,* showed highly variable but comparable signal intensities and distributions (Figure 4.4, E-H). Upon request, Affymetrix sent Dr. Paul Turner, the European Field Application Scientist, to assist with trouble-shooting in the laboratory. Despite further modifications being made to the protocol, including a longer fixation period for larvae to increase their resistance to the procedure, no improvement could be made to the disruption to tissue, and no difference between *dlg* probe and non-specific probes could be detected. Owing to

the dramatic problems with the protocol, Affymetrix have offered to replace the kit free of charge to test if the issues are caused by a faulty component. This is in progress.

Figure 4.4. Affymetrix mRNA *FISH* protocol generates non-specific signal, and significant tissue damage, in both the oocyte and NMJ

v) Testing the localization of other mRNAs at the NMJ

While three different methods have been employed to detect presynaptic mRNA at the NMJ, only one, from Karr *et al.* 2008, has produced specific signal that it is appreciably different to non-specific controls, *albeit* in muscle (summarized in Figure 4.8, below). Owing to these reasons the Karr *et al.* 2008 protocol was optimized (Chapter 5) and employed as the principal mRNA *FISH* protocol for all further experiments. It is possible that while *dlg* mRNA may not be present at the NMJ presynapse, other mRNAs, including Syncrip-target mRNAs, may be. To test this, mRNA *FISH* was performed using the Karr *et al.* 2008 protocol with *shaker, fasii*, and *eiF4e* probes. As with the *dlg* mRNA*, shaker*, *fasii* and *eIF4E* signal was detected throughout the muscle in foci, but not presynaptically (Chapter 5). While it is possible that any one transcript type is present at copy number below the threshold for imaging in the bouton, the total mRNA content of the presynapse may be high enough to detect. To this end, probes were designed that would detect all cytoplasmic mRNAs in the neuron. Almost all mature transcripts have multiple adenine repeats at the 3'UTR, often several hundred, and the poly adenylated [poly(A)] tail has long been used to biochemically distinguish between bulk mRNA transcripts and other, non-coding RNAs, including ribosomal RNA (first in Aviv and Leder, 1972). To test if bulk mRNA signal could be detected at the presynapse two different *FISH* probes were used to target the polyadenylation sequence. mRNA *FISH* was performed with fluorescent DNA oligo poly Thymine (T) probes and RNA poly Uracil (U) probes, both directly conjugated to Alex-568 fluorophores. Both tests revealed mRNA distributed throughout the muscle, with foci of post synaptic mRNA proximal to the neuron, but no signal in the neuron could be detected above the signal in the muscle (Figure 4.5).

Figure 4.5. mRNA *FISH* of poly adenylated mRNA detects signal throughout the muscle, foci at postsynapse, but no signal at the presynapse

vi) Testing mRNA FISH in motoneuron axon bundles

It is possible that probe penetration is hindered by muscle. Boutons penetrate into muscle during development, and are almost entirely surrounded by the postsynaptic SSR structures, which may hinder probe diffusion into the bouton structures. To mitigate both of these issues, mRNA *FISH* was performed with *dlg, shaker*, and *eiF4e* on whole fillet preparations and axons, rather than boutons, were imaged. As a negative control a *gluRiiA* antisense probe was used; *gluRIIA* mRNA is not expressed in neuronal tissue (Schuster *et al.* 1991 and 1993). The mRNA *FISH* protocol published by Karr *et al.* is labour intensive and long; hybridization, washing, and antibody staining takes place over four days. Owing to extensive washing and hybridization steps the larvae are subject to frequent mild mechanical disruption and changes in conditions as buffers are exchanged. As a result it is challenging to maintain structural integrity of the larval motoneuron axon bundles, which are incredibly long (up to 1mm), thin (<15μm), and delicate. In contrast, perfect motoneuron axon structure can be readily maintained when performing a simple overnight antibody staining without mRNA *FISH.* To try and improve the integrity of motoneuron axon bundles, buffers were applied gently with great care onto the larvae during washing steps. This increased the time of each step in the protocol. Following these changes, a few motoneuron axon bundles were maintained in most experiments, though numbers were low in each case (≤5). mRNA signal in the motoneuron axon bundles initially appeared absent. Upon increased contrast enhancement however, *dlg, shaker,* and *eiF4e* probes revealed mRNA signal that was not present in the *gluRIIA* negative control (not shown). The mRNA signals were very low and required high exposure time (0.4s) to detect, and harsh contrasting to visualize. Many axon bundles had become detached from the ventral nerve cord, muscle, or both. As a result it was challenging to consistently image each axon bundle at the same region, though care was taken to try and image axons distal to the ventral nerve cord, and proximal to the

innervating muscle. Coupled with the low number of samples in each repeat of the experiment, it was decided that further experiments were required to show that *dlg, shaker,* and *eiF4e* mRNAs were being specifically detected in motoneuron axons. A useful experiment would be to test the *dlg, shaker,* and *eIF4E* mRNA signals in motoneuron axons overexpressing these genes, relative to wildtype controls. Before further experiments however, this protocol needs to be optimized to allow maintenance of motoneuron axon bundles. Damage to tissue integrity could affect *FISH* probe hybridization. Further, it would be useful to be able to follow an intact neuronal bundle from the ventral nerve cord to the bouton, say on muscles 6 and 7, to follow mRNA levels from cell body to the synapse. Until then it is concluded here that while it is likely that possible mRNA signal is detected in the motoneuron axon, further experiments are required to reinforce this observation owing to technical challenges in the assay, low signal, and low numbers.

vii) Abrogating axonal trafficking does not increase presynaptic mRNA signal

To test the mRNA FISH protocol in motoneuron axons and boutons, it may be necessary to increase the concentrations of the target mRNAs. Once mRNA can be detected in the neuron, *albeit* at artificially elevated levels, then it may be possible to optimize the protocol to detect transcripts at endogenous levels. In larval motoneurons inhibition of the dynein molecular motor, by overexpression of the p150 subunit, blocks axonal transport and leads to large aggregates of proteins that are otherwise present in far smaller particles (Martin *et al.* 1999; Xiong *et al.* 2010). It is possible that axonal mRNA may be trafficked in the motoneuron, and may accumulate in these large protein aggregates. Neuronal mRNAs may accumulate in these aggregates, particularly, but not exclusively, if trafficked by dynein. Large aggregates in the axon may sterically block axonal mRNA transport by other motors, or indeed completely different modes of localization. At increased concentrations in the presynaptic aggregates mRNAs may be robustly detectable by

fluorescence microscopy. To test this, UAS-Glued^{DN} was expressed in motoneurons using the pan-neuronal driver Elav-Gal4, and mRNA *FISH* was performed to detect *dlg, shaker,* and *eIF4E* mRNAs. In motoneuron bundles overexpressing Glued^{DN} the neuronal epitope recognized by anti-HRP antibody was present in large aggregates (1-3μm) consistent with previous studies (Martin *et al.* 1999; Xiong *et al.* 2010). These aggregates represent the accumulation of macromolecules caused by abrogated dynein-mediated transport. mRNA *FISH* revealed that the neuronal aggregates did not contain enriched levels of *dlg, shaker,* and *eIF4E* mRNAs (Figure 4.6). While motoneuron axon maintenance is challenging during mRNA *FISH,* bouton structures are perfectly preserved. To test if neuronal overexpression of Glued^{DN} lead to an increase in presynaptic mRNA at the bouton, larvae were incubated with *dlg, shaker,* and *eIF4E* probes and imaged at the muscle 6 and 7 NMJ. mRNA *FISH* revealed that there was no increase in mRNA signal in the bouton upon overexpression of Glued^{DN} in neurons (Figure 4.7). Low levels of fluorescent signal are detectable in axons, but not in boutons. It is possible that probe penetration is occurring at least in motoneuron axons. As overexpression of Glued^{DN} does not lead to an increase of signal in transport aggregates, it is tempting to conclude that *dlg, shaker,* and *eIF4E* mRNAs are not trafficked by Dynein. The data here, however, is too preliminary to draw such conclusions from. Ultimately it has not been possible to clearly show that any signal in the motoneuron axon correlates to mRNA, owing to low sample numbers and variations in signal, both originating from poor tissue integrity.

Figure 4.6. Abrogation of axonal transport does not lead to accumulation of mRNA in axons

Figure 4.7. Abrogation of axonal transport does not lead to accumulation of mRNA in boutons

viii) Imaging endogenously expressed RNA in live motoneurons

Thus far a number of different approaches have been employed to visualize neuronal mRNAs at endogenous levels *in situ* (Figure 4.8). There are a number of reasons as to why mRNA *FISH* signal may be absent or unclear: low copy number (below the threshold for detection), the housing of mRNA in inaccessible granules and structures, poor probe penetration etc. Also, as discussed, it is well observed that mRNA *FISH* in neurons is challenging even in systems where examples of neuron-localized mRNAs are documented. Where mRNA *FISH* has not been possible in other neuronal systems, the MS2 system has been employed to test the localization of endogenously transcribed mRNAs live *in vivo* (Brechbiel and Gavis. 2008). The MS2 system is a genetically encoded, two-component system, that allows endogenously expressed mRNAs to be decorated with fluorophores (Bertrand *et al.* 1998). Put simply, a gene of interest is tagged with repetitive MS2 sequences from the MS2 bacteriophage that, when transcribed into RNA, form multiple repeats of a RNA stem-loop structure, termed the 'MS2 loop'. In parallel, the MS2 bacteriophage coat protein (MCP) is expressed fused to a fluorescent protein, such as GFP. When expressed in the same cell, the RNA MS2 loop is specifically bound by the MCP-GFP fusion, thereby labeling the RNA with GFP (Figure 4.9). Any MCP protein that is not bound to cytoplasmic MS2-RNA is shuttled to the nucleus by a conjugated nuclear localization signal (NLS). While initially employed in yeast, the MS2 system has since been used to study mRNA localization and dynamics in neuronal process in mammalian and insect systems (Rook and Kosik. 2000; Tiruchinapalli *et al.* 2003; Ashraf *et al.* 2006; Brechbiel and Gavis. 2008). Many of the technical challenges met with mRNA *FISH* in motoneurons, as well as artifacts caused by fixation in general (Schnell *et al.* 2012), can be avoided by live imaging. That said, live imaging of MS2 RNA is not without challenges (See Discussion). In order to achieve neuron-specific signal, any MS2 RNAs will be expressed using the neuron-specific Gal4 driver Elav. While this will

remove much background from non-neuronal tissues (leaving only autofluorescence), the overexpression of RNAs could affect their subcellular distribution, as could the addition of RNA MS2 loops. As a result it is imperative that all experiments employing the MS2 system are carefully controlled. Consistent with this, it was decided that all MS2 constructs would be inserted into the same region of the *Drosophila* genome, to maintain equal expression levels across MS2-tagged RNAs; chromatin context is known to affect the expression levels of transgenes in a tissue-specific manner (Markstein *et al.* 2008). To this end a bacterial site-specific integrase, named PhiC31 (ΦC31), would be used to integrate MS2-constructs into specific genomic regions. PhiC31 integrase catalyses the irreversible integration between two specific sequences, named attB or donor sites, and attP or docking sites. attP docking sites can be introduced into the *Drosophila* genome by conventional p-element transgenesis, and then repeatedly used to reintroduce different genetic elements into the same genomic location. Owing to the popularity of PhiC31 as a tool for site-specific integration, large collections of *Drosophila* stocks, each containing an attP docking site in a different region, have been amassed and screened. All MS2 constructs were integrated into *attp2*, a docking site on chromosome three that is known to express highly and specifically in neurons when driven by Gal4 (Markstein *et al.* 2008).

Figure 4.8. Four different mRNA *FISH* techniques used to detect neuronal mRNA at the NMJ

Figure 4.9. the MS2 system: a two-component system for tagging endogenously expressed RNA with fluorophores *in vivo*

To generate MS2-constructs that could be integrated into the attp2 site and driven in specific tissues by Gal4, the pUASTattB plasmid was obtained from community resources (Figure 4.10. Frontiers in Genetics, FlyC31 resource, University of Geneva, http://flyc31.frontiers-in-genetics.org/index.php). A number of changes were made to the pUASTattB plasmid to facilitate expression of MS2-tagged cDNAs through an ordered subcloning strategy (Figure 4.10). First, in order to subclone entire cDNA sequences into pUASTattb, which encode the transcripts of interest and can range from 1-4kb in size, unique restriction enzyme sites would be needed that are absent from the cDNA. Owing to the size of the cDNA inserts, it is much more probable that a restriction enzyme that recognizes a 8nt site, rather than a 6nt, would be useful. To increase the number of 8nt restrictions sites, in the pUASTattb multi-cloning site (MCS), a 33nt sequence containing Pme1, Pac1, and Asc1 sites was subcloned into the pUASTattb MCS, creating the new plasmid pUASTattbMCS' (Figure 4.10 B). Subsequently the DNA sequence encoding 6 MS2 loops was subcloned into the centre MCS', flanked by 8nt restriction sites. This allowed specific cDNAs to be inserted upstream or downstream of the MS2 loops, allowing the cognate RNA to be tagged with 3' or 5' MS2 loops respectively. The new plasmid, named pUASTattB6xMS2 would allow any sequence to be tagged with MS2 loops and inserted into specific regions of the genome. To this end the pUASTattB6xMS2 plasmid is a useful tool for the RNA localization community and will be made available to the community following publication through The Frontiers in Genetics, FlyC31 resource, University of Geneva, http://flyc31.frontiers-in-genetics.org/index.php (Figure 4.10 C). Following construction of the pUASTattB6xMS2, cDNA sequences of Syncrip-target mRNAs had to be chosen to make MS2 constructs.

Figure 4.10. Subcloning and transformation strategy for generating presynaptic RNAs tagged with MS2 loops

Extensive cDNA libraries cover most genes in *Drosophila.* The Berkeley *Drosophila* Genome Project (BDGP) Gold Collection contains over 8,000 clones and is the most highly validated cDNA collection. Information on the extraction method, tissue of origin, developmental stage of origin, and sequence of each cDNA is available. From the BDGP Gold Collection cDNAs encoding *dlg, shaker, eIF4E and FasII* transcripts were amplified by PCR and subcloned into pUASTattb6xMS2 (Figure 4.10 D - E). The cDNAs chosen to generate MS2-tagged *dlg, shaker, eIF4E and FasII* RNAs were chosen, and confirmed by squencing before subcloning into the pUASTattB6xMS2 vector. While it was not possible to use a cDNA that encompassed the sequences of all transcripts produced from a given gene, care was taken to select cDNAs that represent most annotated transcripts (Figure 4.11). The resulting constructs were then integrated into *attP2* docking sites via PhiC31 site-specific integration by Genetic Services to generate transgenic animals (Cambridge, Massachusetts).

Figure 4.11. cDNAs used to generate MS2 constructs

To image RNA in the living NMJ, it would be necessary to have the boutons fluorescently labeled by a marker to aid identification. To this end a number of potential markers were tested that label boutons with red fluorescence (GluRIIA-RFP, a gift from Stephan Sigrist; UAS-Chc-mCherry, a gift from Simon Bullock; UAS-mCD8-mCherry; UAS-myr-RFP). It was found that expression of UAS-mCD8-mcherry in motoneurons labeled both boutons and motoneuron axons clearly and specifically. UAS-mCD8-mcherry was recombined with Elav-Gal4 (C155 variant) to produce a permanent stock that could be crossed with MS2 constructs for live imaging by Ana Maria Valles. As *dlg* mRNA is known to localize in the blastoderm embryo (Lecuyer et al 2008) and exhibits a tightly-localized presynaptic protein distribution (Lahey *et al.* 1994), *dlg6xMS2* decorated by MCP-GFP (hereon annotated as *dlg**GFP) was imaged first. A custom-built upright Olympus Deltavision optimized for live imaging of fillet preps, was used to test if *dlg6xMS2* localized to the NMJs of muscles 6 and 7 (segments A2 and A3). GFP fluorescence, likely representing *dlg**GFP, was clearly detectable within boutons (Figure 4.12 A-B). While *dlg**GFP signal was present in some NMJs, it was absent from others, even within the same animal (Figure 4.12 A-C). No variation was detected from bouton-to-bouton with a given NMJ i.e. boutons innervating muscles 6 and 7 at a given hemisegment either all contained GFP signal, or all were devoid of GFP signal. No pattern could be detected across different hemisegments from animal-to-animal.

Figure 4.12. Presynaptic *dlgGFP signal varies from NMJ-to-NMJ and requires quantitative measurement**

The *dlg**GFP signal appeared to be uniform and evenly distributed across the bouton and branches, and signal was absent from motoneuron axon bundles. This was a surprising observation, as actively localized mRNA in a variety of cell types is generally present in punctate particles corresponding to different mRNP complexes. This is true in both mammalian and insect neurons. Distinct mRNA particles were detected in mammalian dendrites using *CaMKII*α RNA fused to 8 MS2 loops in Rook and Kosik. 2000. In the *Drosophila* larval peripheral nervous system, *nanos* RNA tagged with 18 MS2 loops was shown to form distinct particles in class IV dendritic arborization (Da) neurons (Brechbiel and Gavis. 2008). Further, in the *Drosophila* adult brain, particles of *CaMKII* mRNA were visualized in post synaptic specializations using the MS2 system, however, the number of MS2 loops used was not published, and the authors were unable to remember (Ashraf *et al.* 2006; Prof. Sam Kunes, personal communciation). To test if *shaker, eIF4E* and *fasII* formed presynaptic particles, *eIF4E**GFP, *shaker**GFP, and *fasII**GFP were imaged. As with *dlg**GFP, *eIF4E**GFP, *shaker**GFP, and *fasII**GFP exhibited even distribution at some NMJs, but were absent from others in no detectable pattern, and were absent from motoneuron axon bundles. One possible reason that RNA particles may not be present in these experiments is that there may a problem with expression of *dlg*6xMS2, *eIF4E*6xMS2, *shaker*6xMS2, *shaker*24xMS2, and *fasII6xMS2*. While every step of subcloning was confirmed by DNA sequencing, it is possible that an error in plasmid construction, or in choice of cDNA sequence, may produce transcripts that do not localize properly. To test if MS2 constructs that have been shown to form particles in the *Drosophila* adult central neuron can form particles at the larval NMJ, *CaMKII*MS2 (Ashraf et al 2008) was driven in motoneurons. As CaMKII protein localizes to boutons, it could be expected that *CaMKII*MS2 would also (Tao-Cheng et al 2006; Shakiryanova *et al.* 2011). *CaMKII*MS2 exhibited a non-particulate distribution at the NMJ similar to that of *dlg*6xMS2, *eIF4E*6xMS2, *shaker*6xMS2, *shaker*24xMS2, and *fasII*6xMS2 (Figure 4.15). To test if a greater number of MS2 loops would reveal mRNA particles, while mitigating

recombination during sub-cloning, a novel 24xMS2 sequence designed to be non-repetitive (A gift from Jeffrey Chao, then Albert Einstein College of Medicine, New York) was employed to produce *shaker*24xMS2. While *shaker*24xMS2 gave a greater fluorescent signal than *shaker*6xMS2, the uniform RNA distribution, variability from NMJ-to-NMJ, and absence of signal from motoneuron axon bundles was the same (Figure 4.16). There are a number of possible explanations for non-particulate RNA distribution of *dlg**GFP, *eIF4E**GFP, *shaker**GFP, and *fasII**GFP relative to other published studies with neuronal MS2 constructs (See Discussion).

Figure 4.13. Quantitative assessment of presynaptic MS2-RNA*GFP signal

Figure 4.14. Overexpression of GFP in motoneurons labels every NMJ in the third instar larvae

ix) Unbiased quantification of presynaptic mRNA localization

The *dlg*6xMS2, *eIF4E*6xMS2, *shaker*6xMS2, and *fasII*6xMS2 constructs all show comparable levels of signal in some NMJs but not others. To test if the levels or frequency of MS2-RNA observed at boutons was related to MS2-RNA sequence, the 6xMS2 loops alone (PUASTattb6xMS2 plasmid) were transformed as above to generate control animals expressing only the 6xMs2 loops without a fused cDNA. These animals are referred to as empty6xMS2 and were designed to serve as a negative control for MS2-RNA localization to boutons.

Surprisingly, empty6xMS2 exhibited a similar distribution as *dlg*6xMS2, *eIF4E*6xMS2, *shaker*6xMS2, and *fasII*6xMS2, at NMJs. As the empty6xMS2 control contains no cDNA, it lacks any mRNA signals necessary for localization, and would likely represent distribution by passive diffusion. If the distribution and levels of empty6xMS2 reflect the free diffusion of the MS2 loops alone, then it is important to quantitatively test if other MS2 constructs localize to a greater or lesser extent. mRNA particles can be counted in individual dendrites and can provide a convenient mechanism of quantification of mRNA localization when normalized to neuron size (Brechbiel and Gavis. 2008). In the absence of particles however, a different method for quantification of neuronal mRNA signal had to be developed. A number of different challenges had to be overcome when designing tools to analyze these data:

1. As the empty6xMS2, *dlg*6xMS2, *eIF4E*6xMS2, *shaker*6xMS2, and *fasII6xMS2* constructs all exhibit localization to some NMJs and not others, with no apparent pattern, a non-biased method must be applied.
2. NMJs that do exhibit GFP signal can vary in intensity, so intensity must be quantified with sensitivity.

3. The size and number of boutons in a given image can vary widely from image-to-image. As a result presynaptic fluorescence must be normalized to neuron volume in a 3D image.
4. Finally, as high exposure times (0.4s) are required to detect presynaptic GFP signal in these experiments, autofluorescence in muscle is a highly variable contaminant that must be considered in analysis.

To this end, in collaboration with Graeme Ball, the image analysis expert in the Advance Microscopy Unit (Micron) housed in the Biochemistry Department, a systematic set of imaging commands were, tested, refined, and programmed into a macro for ImageJ that could be applied to multiple images in a high-throughput manner. Briefly, by standardizing the imaging of NMJs, it was possible to automatically define the presynaptic volume in a 3D image stack using mCD8-mcherry signal to create a Mask. Following refinement of the Mask through subsequent iterations, the mean GFP intensity of voxels are calculated inside and outside of the neuron (See Materials methods for code and detailed explanation). The mean presynaptic voxel intensity can then be considered as a ratio, or differential, to the postsynaptic mean voxel intensity (Figure 4.12). As a proof of principle, the macro was then applied to a four different images with varying neuronal shape and volume, muscle autofluorescence, and presynaptic signal, one of which exhibited presynaptic GFP signal (Figure 4.13). Both the ratio [Presynaptic signal/Postsynaptic signal (Figure 4.13 A-D)] and differential [Presynaptic signal – Postsynaptic (Figure 4.13 A'-D')] of presynaptic and postsynaptic signal correlates with the images. It is unclear whether presenting presynaptic signal as a ratio, or as a differential, to background fluorescence is more useful. Both data types correlate in test cases (Figure 4.12) and both data types will be presented here. It is presumed that a genuine phenotype would be equally reflected in both data types.

To quantitatively assess whether there is a difference between presynaptic levels of *dlg*6xMS2, *eIF4E*6xMS2, *shaker*6xMS2, and *fasII*6xMS2 RNA relative to empty6xMS2 controls, >30 NMJs across 5 animals from each genotype were imaged live. To maintain experimental homogeneity, all animals across all genotypes were dissected and imaged in the same day. Images were discarded from analysis only if contaminating material from dissection obscured the neuron e.g. fat body, motoneuron axon, etc. Statistical analysis of these data revealed that the presynaptic levels of *dlg*6xMS2, *eIF4E*6xMS2, *shaker*6xMS2, and *fasII6*xMS2 RNA and empty6xMS2 were largely similar. When considered as differentials, all MS2 constructs were enriched at the NMJ to the same extent (Figure 4.13 F). When considered as ratios, there was a small but significant ($p<0.05$) difference between *fasII6*xMS2 enrichment relative to the empty6xMS2 control (Figure 4.13 F'). Owing to the uncertain and small difference between *fasII6*xMS2 and empty6xMS2, this result is challenging to interpret (See Discussion).

Figure 4.15. *CaMKII*MS2*GFP MS2-RNA does not form particles at the NMJ presynapse

It is possible that empty6xMS2 is not a perfect negative control. The MS2 loop sequence is ~200nt, while *dlg*6xMS2 is >2700nt, and may diffuse to a far greater extent than the full-length cDNA. The hydrodynamic radius of a macromolecule is known to dictate diffusion rates in axons (Provok and Poo 1992). As a result, a MS2-construct that is similar in size to full length MS2-RNAs may serve as a better negative control. If particular sequence elements in *dlg* mRNA were necessary for presynaptic localization, then the removal of these elements by deletion would render *dlg* incompetent for localization to boutons.

To test if specific sequences were necessary for *dlg* localization to the presynapse, the 5'UTR and 3'UTR were separately amplified as PCR fragments containing the 1st 800nt and last 3rd 800nt of *dlg* respectively. Both were subcloned into pUASTattb6xMS2 and transformed as before. Quantitative analysis was performed on the presynaptic levels of full length *dlg*6xMS2, *dlg*1st800nt6xMS2, and *dlg*final800nt6xMS2 across >30 NMJs for each construct. When the data are considered as a ratio, full length *dlg*6xMS2 localizes to a greater extent than both *dlg*1st800nt6xMS2, and *dlg*final800nt6xMS2 (Figure 4.13). Surprisingly, full length *dlg*6xMS2 also exhibits significantly greater presynaptic localization than empty6xMS2 in these experiments, whereas in previous experiments full length *dlg*6xMS2 has localized to the same extent as the empty 6xMS2 control (Figure 4.13 E - F). When the data are considered as a differential however, there is no significant difference between full length *dlg*6xMS2, *dlg*1st800nt6xMS2, *dlg*final800nt6xMS2, and empty6xMS2 (Figure 4.13 E'). This is consistent with previous data where *dlg*6xMS2 localizes to the same extent as the empty6xMS2 control (Figure 4.13 F). Owing to the similar localization levels of different MS2-RNAs to boutons, it is challenging to confirm that these experiments are examining a biologically meaningful process. Multiple mRNA *FISH* techniques failed to detect mRNA localized to the NMJ presynapse, and live experiments using the MS2 system has detected little difference between test MS2-RNAs and negative controls. While it is clear that mRNA localization to synapses and axons

occurs in other systems, it has not been possible to directly observe this in the larval motoneuron so far. While a lack of evidence is not evidence of absence, it is necessary to consider that mRNA localization does not occur to the same extent in the mature larval motoneuron as in other systems. In summary it has not been possible to develop techniques to assay neuronal mRNA localization, and to subsequently test if Syncrip regulates neuronal mRNA biology.

Discussion

A number of different techniques, both in fixed and live cells, have not detected neuronal mRNA at the NMJ over negative controls. Here novel approaches have been employed to assay motoneuron mRNA localization. While some techniques appear to reveal presynaptic mRNA signal, blind and quantitative image analysis has confirmed that these data are similar to a variety of negative controls. To this end it is impossible to draw conclusions of how, or if, mRNA if is distributed in larval motoneuron axons. These data are insufficient to distinguish between an inability to detect presynaptic mRNA, or the absence of it. The mRNA *FISH* and MS2 methods, and their pitfalls and limitations, will be discussed sequentially.

i) mRNA FISH is challenging in neurons

As discussed, it is well established that mRNA *FISH* is challenging in mature neurons. Consistent with this, development of *FISH* protocols for the detection of presynaptic mRNA at the NMJ has been confounded here by a number of key issues:

1. Neuron and muscle both express many of the same synaptic proteins. As the muscle cell is many times greater in volume than the boutons that innervate it, *bona fide* signal in muscle can mask that in the presynapse. Thus it was not possible to simply increase probe levels to try and detect low levels of presynaptic mRNA, as signal in the muscle would overwhelm presynaptic signal.

2. Muscle cells are highly prone to non-specific probe binding when fixed. While the reason for this is unknown. Therefore, even probes targeted against mRNA expressed only in neuron would generate substantial background in muscle.
3. Probes did not appear to penetrate into the NMJ presynapse. While in muscle, *dlg* riboprobes gave a significantly higher signal than non-specific controls, in neuron neither a variety of test probes, or non-specific control probes, gave any presynaptic signal. Indeed, even probes targeted against poly(A) mRNA tails did not yield any signal above background.
4. The *in situ* process was long and labour intensive, taking more than four days from dissection to imaging. This is largely owing to the need for multiple washing steps to remove non-specific signal. As a result, the optimization and trouble-shooting of mRNA *FISH* protocols proceeds at a relatively slow pace.

ii) All MS2-RNAs tested localize to the bouton to a similar extent in an identical distribution

It was initially thought that the MS2 system could be employed to circumvent the considerable challenges facing mRNA *FISH* at the NMJ presynapse. Using the MS2 system at the NMJ however, presented a host of technical challenges, many of which had not been anticipated:

1. Presynaptic signal was very low and not present in particles. As a result high exposure times were required during imaging and novel tools had to be designed for signal quantification.
2. The muscle was highly autofluorescent. Further, it was found that within 15-30 minutes of dissection, autofluorescence in body wall muscle would increase dramatically. In studies where fillet preps have been cultured for over 150 minutes, muscle background may not have been an issue as fluorescent reporters were present in sufficiently high copy number (Ataman *et al.* 2008).

3. Signal variation was high. During the course of multiple experiments it was anecdotally observed that variation in signal was greatest from animal-to-animal, rather than from NMJ-to-NMJ within the same animal. During image acquisition it would be common to obtain 4 NMJs from one larva that clearly showed presynaptic signal, and then 4 NMJs from the next larva that showed no presynaptic signal. Though care was taken to ensure that each larva was treated identically, it is possible that sub populations of larvae existed that localized mRNA to the presynapse to different degrees. This could, for example, correlate with crawling activity. Contraction of crawling muscles is known to lead to changes in the organization of presynaptic proteins at the NMJ and the same could be true of mRNA (Shakiryanova *et al.* 2011).
4. The localization of only one mRNA isoform can be studied at one time with the subcloning strategy outlined here. As nothing is known about the mRNA localization of *dlg, fasii, shaker* and *eIF4E* in neurons, it is possible that the isoforms chosen are not competent for localization in neurons, i.e. they lack neuron-specific localization elements present in other isoforms.
5. The subcloning of plasmids contain repeats of MS2 viral DNA was challenging. Despite helpful advice from those with direct experience in handling MS2 loops (Elizabeth Gavis, Princeton University), recombination of the repetitive loop sequences in *E.coli* was frequent. As a result, a number of variables had to be interrogated, along with a number of different strains of recombination-deficient bacteria. The time spent cloning was substantial, and prevented further investigation into MS2-RNA localization in the motoneuron.

iii) mRNA localization in motoneuron axons may operate by a number of mechanisms

While the MS2 system has been successfully employed to study mRNA localization in neurons, it has not given insight into mRNA localization at the NMJ presynapse using the

approaches reported here. Nevertheless there are some important observations to consider from these data. First, it is possible that *dlg**GFP, *eIF4E**GFP, *shaker**GFP, and *fasII**GFP are present in particles, but that low fluorescent signal prevents their resolution. This could be caused by low mRNA copy number in mRNP particles, or by low occupancy of 6xMS2 loops by MCP-GFP, or both. Six MS2 loops was sufficient to resolve RNA particles in the larval trachea, and the same DNA sequence was used here (Jayan Nair, PhD Thesis, 2008). While a greater number of MS2 loops will recruit more fluorophores to the RNA, a larger fusion to the RNA of interest is more likely to interfere with the native RNA structure that may be necessary for localization. Moreover, the MS2 loops are encoded by repetitive viral DNA sequences that are highly recombinogenic, and a subcloning protocol had to be developed to suppress recombination (See Materials and Methods). Subcloning is aided by using a lower number of MS2 sequences.

Second, while the even distribution of MS2-RNA in the boutons is at odds with the particulate distribution observed in other neuronal MS2-RNAs (Rook and Kosik. 2000; Tiruchinapalli *et al.* 2003; Ashraf *et al.* 2006; Brechbiel and Gavis. 2008), it is important to note that these studies focus on dendrites rather than axons. While particulate MS2-RNA distribution has also been observed in axons (Yan *et al.* 2009), a distinctive and evenly distributed, non-particulate, MS2-RNA distribution has also been reported in axons (Bi *et al.* 2006). It is possible that different trafficking mechanisms exist for different RNA in different neuron types, or even different appendages within the same neuron. It is interesting that *CaMKII*MS2, and *CaMKII3'UTR*MS2, both of which are housed in particles in the adult fly brain, are present at the NMJ presynapse in an even distribution. It is possible that the localization elements present in the *CaMKII* 3'UTR that target it to large mRNP granules in the brain neurons do not signal the same fate in the motoneuron. It is even possible that an even distribution of macromolecules at the NMJ may be biologically meaningful. Indeed proteins crucial to presynaptic function, Shaker and CaMKII (Tejedor

et al. 1997; Shakiryanova *et al.* 2011), are both evenly distributed throughout the bouton presynapse, and CaMKII becomes clustered into foci only upon muscle contraction.

Third, the fact that all MS2-RNAs tested here, including the negative controls, localize to the presynapse to the same extent may reflect the biological process that governs mRNA localization. It is possible that all poly(A) mRNA is targeted for transport to the presynapse to the same extent (empty6xMS2 is poly adenylated), or simply that all mRNA diffuses to the presynapse during development, and that translational control governs changes in local gene expression. Consistent with this, a paper published at the end of the PhD, claims that mRNA localization is the rule, rather than the exception, in cultured mammalian neuron dendrites (Cajigas *et al.* 2012). The authors achieve single-molecule mRNA *FISH* with affymetrix branched DNA probes, just as attempted in this study. They show that over 30% of mRNAs, including *dlg*, are present in rat hippocampal dendrites, with some transcripts present in small mRNPs containing single molecules. If similar rules of distribution operate in the larval motoneuron, then perhaps it is unsurprising that all MS2-RNAs localized to a similar extent when expressed by the same driver. New findings such as (Cajigas *et al.* 2012) demand new thought on the prevalence of mRNA localization in neuronal appendages, and the mechanism of trafficking. It is widely discussed in the neuronal trafficking field that active transport is necessary to deliver macromolecules to extremities in a physiologically relevant time scale (recently; Wong *et al.* 2012; Moughamian *et al.* 2012; Lloyd *et al.* 2012). As a result it is generally thought that passive diffusion of macromolecules along axons is unlikely to contribute to neuronal function.

Despite this seeming consensus, there are relatively few studies that examine the rate of macromolecule diffusion in axons, while empirical evidence shows that large macromolecules do diffuse in axons, *albeit* at $1/5^{th}$ of the rate in free water (Propov and Poo 1992). Indeed, expression of GFP protein in motoneurons will label every bouton

completely, presumably demonstrating that diffusion is sufficient to bring macromolecules to the presynapse (Figure 4.14). While it is clear that active localization of mRNA in dynamic particles is central to many neuronal systems, it is possible that alternative mechanisms operate in parallel, or even dominate in certain cases. While the simplest answer maybe that the MS2 system does not recapitulate the behaviour of endogenous, non-tagged, transcripts, it is worth bearing in mind that mRNA localization in neurons is a rapidly growing field with much still to be understood.

Figure 4.16. *shaker*24xMS2 gives greater presynaptic signal than *shaker*6xMS2 and similar distribution

It is possible that *dlg, fasII* and *shaker* mRNAs do not localize to the presynapse, but that Syncrip orchestrates control of expression through entirely different mechanisms. mRNAs are thought to be subjected to post-transcriptional regulation by binding hundreds of RBPs and other regulatory mRNP complexes (including micro-RNA-containing RNPs). To understand how Syncrip contributes to this regulation it is necessary to know where on target mRNAs Syncrip binds, the RBP recognition element (RRE). This is possible using new, transcriptome-wide techniques such as PAR-CliP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation). This information, coupled with the RNA-Seq data showing the most significant Syncrip-target mRNAs, would allow any consensus Syncrip-binding sequence or structure to be found. This information would provide powerful insights into the *in vivo* interactions between Syncrip and its target mRNAs and may shed light on how Syncrip contributes to the life cycle of these transcripts. These experiments, however, are beyond the scope of this thesis. Instead, while the role of Syncrip in regulating mRNA localization in neurons remains elusive, the localization of Syncrip-target mRNAs was pursued in the muscle. Neuronal structure and synaptic transmission are a function of both neuron and muscle biology at the NMJ, and it is necessary to appreciate how Syncrip functions in both cell types to elucidate its role. To this end, protocols were developed to test the localization of Syncrip-target mRNAs in muscle.

Chapter 5: The levels and distribution of muscle mRNAs are responsive to genetic alterations of synaptic plasticity

Introduction

The formation of a functional NMJ requires communication between muscle and neuron, both in development and synaptic plasticity. To this end, the regulation of gene expression in muscle, as well as in neuron, is critical to NMJ structure and function. Indeed, mutational studies have shown that neuron and muscle can regulate the expression pattern of one another. During development of the NMJ in the stage 17 embryo, presynaptic innervation is necessary to cluster post-synaptic scaffolding proteins, which in turn organize post-synaptic ion channels (Chen and Featherstone, 2005). In the larval NMJ, expression levels of FasII patterns on one side of the synapse can affect the expression levels in the other, and a balance between neuron and muscle is necessary for proper synaptic growth (Schuster *et al.* 1996). Moreover, presynaptic protein complexes can even traverse the synapse, and travel from neuron to muscle, to regulate the shared synapse (Ataman *et al.* 2008). As Syncrip is expressed in both neuron and muscle, it is essential to appreciate Syncrip's role both pre and postsynaptically to understand how Syncrip functions. While it has not been possible to assay mRNA localization in motoneurons, and subsequently Syncrip's role in neuronal mRNA distribution, muscle is more amenable to mRNA *FISH* than neurons. If mRNA localization and local translation are central to plastic changes at synapses, then it is crucial to understand the processes that regulate mRNA localization in muscle.

i) mRNA localization and local translation at the NMJ is a small but contentious field

Overexpression of Syncrip in muscle leads to undergrowth of branches, and expression of RNAi against Syncrip in muscle leads to an overgrowth of the NMJ (Chapter 3; McDermott, PhD thesis, 2009). Taken together, these data indicate that Syncrip in the muscle can regulate NMJ structure. As Syncrip is a conserved mRNA-binding protein, it is likely that Syncrip regulates changes in NMJ structure through expression of mRNAs encoding key synaptic proteins. Indeed, the changes in neuronal morphology observed in *syp* loss-of-function mutants resemble mutants that fail to regulate local translation at the NMJ postsynapse (Menon et al 2004). As discussed in Chapter 1, larval NMJs have to grow extensively to maintain contractile efficacy at muscles that increase in size dramatically from first to third instar. The number of boutons increases up to 10-fold and active zones, the sites of vesicle release, are added to each bouton (Lnenicka and Keshishian, 2000). New boutons are budded from existing boutons, either at the ends of branches, or from between two existing boutons on a branch (Zito *et al.* 1999). As synapse assembly requires the addition of new proteins to the bouton, it is tempting to speculate that translation of postsynaptic proteins occurs at the site of new bouton assembly, at the NMJ. Indeed, a number of key observations have supported this notion, and linked postsynaptic local translation to NMJ activity. First, the NMJ can respond to increases in activity with local changes in postsynaptic protein levels that correlate with increases in synaptic strength. Crawling increases the motor activity of larvae, and after a few hours, large (frequently >500nm) cytoplasmic bodies containing the eukaryotic translation initiation factors eIF4E and poly(A) binding protein (PABP) appear at the NMJs. Subsequently glutamate receptors appear at these eIF4E-enriched sites (Sigrist *et al.* 2000, 2003). Second, genetic mutation that results in increase synapse activity, such as mutants in the *shaker* K^+ channel, or aberrant translation regulation, such as *Pumilio* mutants, correlate with increased frequency of eIF4E and PABP bodies at the NMJ (Sigrist et a. 2000 and 2003; Menon *et al.* 2004). Third, electron microscopy has revealed polysomes to be

present at the NMJ postsynaptic subsynaptic reticulum (Sigrist et al 2000). Finally, immunofluorescence has revealed a number of translational regulators, including Syncrip, to be enriched at the bouton (McDermott *et al.* in preparation; Menon *et al.* 2004 and 2009). As a result, the SSR is thought to be the site of local translation, and the critical point of *de novo* bouton formation at the NMJ. Despite the attractiveness of this model, it is important to recognize that mRNA has never been shown to colocalize with eIF4E and PABP bodies at the NMJ, and that while ribosomes are present at the NMJ postsynapse, there is no evidence to suggest that there is an enrichment of ribosomes relative to the rest of the muscle cytoplasm.

If local translation at the NMJ is critical to the plastic growth of new boutons, then it follows that mRNA must be present at the SSR, even transiently. Though the localization of proteins to the post synapse is an active area of research, there are relatively few studies that examine mRNA localization in the larval muscle. While a few studies have examined the localization of mRNA directly, these studies have focused on only *gluRIIA* mRNA and have reported widely different results. Initially, it was reported that *gluRIIA* mRNA is enriched at the NMJ postsynapse around each bouton in an even distribution, while being absent from the branch or trunk regions of the NMJ and the muscle cytoplasm (Sigrist *et al.* 2000). While it was proposed that this localized mRNA was the substrate for eIF4E-bodies that represent site of translation, it is important to note that eIF4E bodies do not have a similar distribution to the *gluRIIA* mRNA: *gluRIIA* mRNA localizes to boutons, while eIF4E-bodies appear in between boutons and at the NMJ trunk (Sigrist *et al.* 2000, 2003; Menon *et al.* 2004). While the localization of *gluRIIA* mRNA to boutons was consistent with the hypothesis that local postsynaptic translation affected synapse efficacy, a previous study had shown *gluRIIA* mRNA to be distributed throughout the embryonic and larval muscle in distinct foci, and not enriched at the NMJ (Currie *et al.* 1995). Further, subsequent studies that have focused on *gluRIIA* mRNA localization at the NMJ have measured widely different results to that of Sigrist *et al.* 2000, in agreement with Currie *et*

al. 1995. Indeed, while quantitative analysis of *gluRIIA* mRNA localization revealed that a low number of mRNA foci are adjacent to boutons, most mRNA is actually localized *away* from the NMJ, in the centre of the muscle (Karr *et al.* 2008; Ganesan *et al.* 2012). Even overexpression of *gluRIIA* mRNA did not lead to enrichment at the boutons, and no statistically significant colocalization between *gluRIIA* mRNA and eIF4E protein was observed, either at the boutons or throughout the muscle cytoplasm (Ganesan *et al.* 2011). Owing to the widely different *gluRIIA* mRNA distributions reported by Sigrist *et al.* 2000, and Currie *et al.* 1995, Karr *et al.* 2008, and Ganesan *et al.* 2011, it is challenging to reconcile these studies with one another. More recently, Speese *et al.* have shown that *par-6* mRNA, which encodes the asymmetry regulating protein Par-6 at the NMJ postsynapse, is present throughout the muscle, but enriched at the NMJ (Speese *et al.* 2012). It is entirely possible that all studies make sound observations, but that muscle mRNA localization is a dynamic process that can change rapidly. For example, the differences in mRNA localization may be attributable to changes in larval crawling activity. Nevertheless it is important to recognize that research into mRNA localization in larval muscle is largely restricted to a handful of publications focusing on *gluRIIA*, and these studies differ substantially in their findings. Previous work by McDermott and Meignin (McDermott *et al.*, in preparation) has shown that Syncrip is present in the muscle nuclei and at the subsynaptic reticulum. From these two subcellular locations Syncrip is poised to participate in post-transcriptional mRNA regulation.

Aims of this chapter

- **To optimize mRNA *FISH* protocols in muscle.**
- **To test the localization of key mRNAs in muscle.**
- **To test if key processes in synaptic plasticity regulate mRNA levels and distribution:**

a) Synaptic transmission

b) cAMP and Ca^{2+} (secondary messengers and signaling)

c) Trafficking and Microtubule organization

d) Postsynaptic Translation

- **To test different methods for imaging live mRNA in muscle *in vivo*.**

Results

i) Systematic optimization of mRNA FISH protocols provides highly sensitive detection of muscle mRNA

mRNA *FISH* in larval motoneurons proved challenging (Chapter 4) as no published protocols existed, and no neuron-localized mRNA was known to serve as a positive control. In the muscle however, three different laboratories had published mRNA *FISH* data in the larval muscle at the time of experimental design (Currie *et al.* 1995; Sigrist *et al.* 2000; Karr *et al.* 2008), and a fourth has been published more recently (Speese *et al.* 2012). As discussed, Sigrist *et al.* 2000 found *gluRIIA* mRNA to be enriched at the NMJ, while Karr *et al.* 2008, and Currie *et al.* 1995, did not. Unfortunately, no mRNA *FISH* protocol is published in Sigrist *et al.* 2000, and a request for a detailed protocol from the corresponding author gained no response[1]. The protocol in Karr *et al.* 2008 (and again in Ganesan *et al.* 2012) was published in the greatest detail, and the last author, Dave Featherstone (University of Illinois, Chicago), offered helpful advice on reproducing the technique. As a result, the protocol published in Karr *et al.* 2008 was employed to detect mRNA in the muscle.

To test if mRNA *FISH* would reveal mRNAs enriched at the bouton postsynapse, riboprobes incorporating digoxigenin (DIG) modifications were made complementary to the Syncrip-target mRNA *dlg*. As discussed, Dlg protein is highly localized at the NMJ

[1] The first author of Sigrist *et al.* 2000, Stephan Sigrist, now Freie Universität Berlin, was able to provide a detailed mRNA *FISH* protocol, however the protocol had to be located and translated to English, and it was only during the second year of this three year DPhil that the protocol was obtained.

postsynapse (Budnik *et al.* 1990), presynapse (Lahey *et al.* 1994), in asymmetric cell junction (Woods and Bryant, 1991) and the *dlgA* transcript is known to exhibit an asymmetric distribution in the blastoderm embryo (Lécuyer *et al.* 2007). As a result *dlg* mRNA is a good candidate for asymmetric distribution. After initial testing, *dlg* mRNA signal was observed to be distributed throughout muscle in distinct foci, likely representing mRNP structures (Figure 5.1). While *dlg* mRNA was present at boutons, there was no enrichment at the postsynapse relative to the rest of the muscle cytoplasm. Indeed, *dlg* mRNA was largely distributed away from the NMJ, resembling *gluRIIA* mRNA published in Karr *et al.* 2008 and Ganesan *et al.* 2012. To test if the mRNA *FISH* protocol was competent to detect other muscle transcripts, probes complementary to *fasII* mRNA were used in wildtype animals. As with *dlg* mRNA, *fasII* mRNA was present throughout the cytoplasm in discrete foci of varying size. While *dlg* and *fasII* mRNA signal were greater than that of the sense probe controls, the level of non-specific signal was still substantial. High levels of background signal can mask *bona fide* signal and complicate analysis. To improve the signal-to-background ratio it was necessary to determine if the DIG-conjugated riboprobes, the antibodies, or both, were responsible for non-specific signal. To test if the *dlg* riboprobe was specifically detecting *dlg* mRNA, mRNA *FISH* was performed in animals overexpressing UAS-Dlg-GFP in muscle, driven by the late mesodermal driver C57-Gal4. Overexpression of UAS-Dlg-GFP in muscle lead to a dramatic increase in the levels of *dlg* mRNA, while no change was detected in the *dlg* sense probe control (Figure 5.1). Similarly, overexpression of UAS-FasII in muscle lead to a marked increase in *fasII* mRNA signal relative to controls. Intriguingly, *dlg* and *fasII* mRNA exhibited similar distributions in both wildtype muscle, and muscle overexpressing either transcript. While the size of mRNA foci the cytoplasm increased upon overexpression, the approximate number, shape, and distribution, of foci did not. Moreover, while some mRNA foci increased in size and intensity dramatically, others remained the same size as in wildtype. It is possible that the different mRNA foci serve different and distinct roles in the muscle cytoplasm, and that excess mRNA is funneled

into some mRNP structures, and not others. Close inspection of the distribution of increased levels of *dlg* mRNA reveals that, in addition to spherical foci of varying sizes in the cytoplasm, complex nuclear structures can also be resolved (Figure 5.2 e, 1-5). It is possible that these foci represent multiple sites of transcription.

Figure 5.1. mRNA *FISH* in larval muscle reveals *dlg* and *fasII* mRNA to be distributed throughout the muscle, and not at boutons. Non-specific control probes produce substantial background fluorescence

Figure 5.2. Overexpression of UAS-Dlg-GFP and UAS-FasII in muscle confirms mRNA *FISH* probe specificity and reveals mRNA foci of varying sizes in cytoplasm and nuclei

Drosophila larval bodywall muscles are single, syncytial myofibers containing multiple nuclei that are thought to be polyploid. The DNA content of the muscle nuclei increases dramatically throughout larval development, and tightly correlates with muscle growth (Demontis and Perrimon, 2009). The multiple, closely-packed, mRNA foci that appear in the nuclei could represent multiple copies of a gene during transcription. When driven by C57-Gal4, *dlg* and *fasii* mRNA could be observed in complex structures in every nucleus of both muscles 6 and 7. Inspection of wildtype muscles revealed that *dlg* and *fasII* mRNA structures were present in muscle nuclei, though rarely (typically one nucleus per muscle; Figure 5.3, *dlg* shown, yellow box and inset). While there is a precedent for syncytial muscle localizing mRNA through selective transcription in particular nuclei in mammals (Burszjtajn *et al.* 1989; Rotundo 1990), no pattern could be found here. *dlg* and *fasii* mRNA structures were observed in both nuclei proximal and distal to the NMJ, and while the structures were always observed on the nucleus periphery, there was no correlation between the position of mRNA foci in the nucleus and the NMJ.

Figure 5.3. mRNA *FISH* in wildype larval muscle reveals *dlg* mRNA to be present in complex nuclear structures in some nuclei

While it was clear that the *dlg* and *fasII* DIG-conjugated riboprobes specifically hybridized to their mRNA targets, non-specific background was still high in muscle. To test if the antibodies used to detect DIG-conjugated riboprobes were binding non-specifically in muscle, *FISH* was performed with, and without, riboprobe (Figure 5.4, X). mRNA *FISH* in the absence of riboprobe, the 'no probe' control, produced fluorescent signal in the muscle that was similar to that recorded in the sense probe control. This suggests that background signal observed in muscle is caused by non-specific binding of antibodies, rather than riboprobes. Different antibodies can behave very differently from system-to-system. To test if different antibodies may reduce the background signal, three different combinations of primary and secondary antibodies were chosen based on their published use in larval muscle. The three antibody combinations were named X, Y, and Z (Table 5.1), and each were tested in parallel with *dlg* antisense probe, sense probe, and no probe (Figure 5.4). Examination of the muscle clearly showed that combination Z produced the highest level of non-specific antibody binding, as shown by the 'no probe' control. In contrast, the antibody combinations X and Y both produced low levels of non-specific background. As combination Y produced a higher fluorescent signal intensity than X (possibly owing to a higher affinity for DIG, or differences in fluorophore properties), combination Y was employed for all mRNA *FISH* hereafter. In addition to the change in antibodies, systematic testing lead to further optimization of the mRNA *FISH* protocol. First, greater mRNA signal was achieved by raising the hybridization temperature from 56^{o}C to 57^{o}C. Second, the pre-absorption of anti-DIG antibodies with fixed *Drosophila* oocytes removed contaminants from the antibody stock that contributed to non-specific binding (data not shown). As a result, the original protocol published by Karr *et al.* was slightly modified to improve mRNA detection and lower non-specific signal in these experiments (Figure 5.4, A-B). Owing to the marked difference between mRNA signal in tests and controls, and the demonstration that probes respond to changes in endogenous

mRNA levels, it was not necessary to evaluate mRNA *FISH* data by blind or automated analysis.

Table 5.1. Three different antibody combinations used during mRNA *FISH.* Combination Y produced the highest signal:noise.

Antibody combination	Primary		Secondary	
	Antibody	Concentration	Antibody	Concentration
X	Sheep anti-Digoxigenin AP FAB fragments. Roche: 11093274910	1/500	Donkey anti-Sheep Alexa 594. Invitrogen: A-11016	1/250
Y	Anti-Digoxigenin from mouse IgG1κ (clone 1.71.256). Roche: 11333062910	1/500	Donkey anti-mouse Alexa 594 IgG (H+L) *2 mg⁄mL Invitrogen: A-21203	1/500
Z	IgG Fraction Monoclonal Mouse Anti-Digoxigenin Jackson Immuno (Stratech): 200-002-156	1/1500	DyLight 594-AffiniPure Goat Anti-Mouse IgG (H+L): Jakson Immuno (Stratech);115-515-003	1/500

Figure 5.4. Comparison of the three different combinations of antibodies, named X, Y and Z, reveals that combination Y produces the highest signal intensity and at least non-specific bunding in muscle mRNA *FISH*

ii) An optimized mRNA FISH protocol reveals that mRNA encoding key synaptic proteins is distributed throughout the muscle in distinct cytoplasmic and nuclear foci

It has been shown here that *dlg* and *fasII* mRNA are distributed throughout the third instar muscle, and neither is enriched at the NMJ. Using the optimized mRNA *FISH* protocol the distributions of other mRNAs that encode key synaptic proteins were examined. Shaker, the voltage-gated K^+ channel, is localized to both the pre and post synapse of the NMJ (Gho and Ganetzky, 1992; Martínez-Padrón and Ferrus 1997; Sheng et al 1993; Tejedor *et al.* 1997), and *shaker* mRNA, like *dlg* mRNA, associates with Syncrip (Table 4; McDermott *et al.* in preparation; Hamilton *et al.* in preparation). To test if *shaker* mRNA is enriched at the postsynapse, mRNA *FISH* was performed on wildtype animals with *shaker* antisense riboprobes. *shaker* mRNA was distributed throughout the muscle in a similar distribution to *dlg*, and *fasII* mRNA, with very few foci proximal to the NMJ. While nuclear structures housing *dlg*, and *fasII* mRNA were rare, every muscle nucleus examined across multiple animals (n>12) contained high intensity *shaker* mRNA structures (Figure 5.5 A-B, arrows). The role of the nuclear mRNA foci is unknown, though it appears that different mRNAs occupy these structures to different extents, particularly as *dlg, fasII* and *shaker* mRNAs appear to be expressed at similar levels. If these mRNA foci do represent sites of transcription, then the number of nuclear foci could reflect the levels of transcription of a given gene in a muscle, or even the mechanism of nuclear export for a particular transcript (See Discussion). To test if other transcripts encoding synaptic proteins were housed in nuclear foci, or enriched at the NMJ, an antisense *gluRIIA* riboprobe was employed. As discussed, *gluRIIA* mRNA was reported to localize to the boutons in wildtype larva (Sigrist *et al.* 2000). To test if *gluRIIA* mRNA is enriched at the NMJ postsynapse, a *gluRIIA* antisense riboprobe was employed for mRNA *FISH. gluRIIA* mRNA was distributed throughout the muscle cytoplasm, and not enriched at the NMJ (Figure 5.5 D). *gluRIIA* mRNA was not found in nuclear foci. The distribution of *gluRIIA*

observed here is similar to that reported by research from two other research groups (Currie *et al.* 1995; Karr *et al.* 2008; Ganesan *et al.* 2012) but at odds with the data from another (Sigrist *et al.* 2000). No explanation is given for this discrepancy here, though the *gluRIIA* mRNA *FISH* data presented in this thesis are robust, reproducible, and substantially different to non-specific control probes (not shown). Moreover, the distribution of *gluRIIA* mRNA is similar to that of other transcripts encoding postsynaptic proteins, such as *dlg, fasII,* and *shaker*. In later studies it was proposed that *gluRIIA* mRNA was kept at the NMJ postsynapse under resting conditions, and the local translation of eIF4E protein at the NMJ in response to activity induced GluRIIA translation at the synapses (Sigrist *et al.* 2000 and 2003; Menon *et al.* 2004, 2009;). If eIF4E is translated locally at the NMJ, then *eIF4E* mRNA must be present at the NMJ. Examination of muscle from wildtype larvae however revealed that *eIF4E* mRNA is distributed throughout the muscle in foci of varying size (Figure 5.5 C). While there are a few mRNA foci that are proximal to the NMJ, the distribution of eIF4E transcripts appears even throughout both muscles 6 and 7, and similar to other mRNAs. Unlike *shaker* mRNA, *eIF4E* mRNA was not observed in large nuclear structures.

Figure 5.5. An optimized mRNA *FISH* protocol reveals that mRNA encoding key synaptic proteins is distributed throughout the muscle in discrete foci

Though the distribution of mRNAs presented here does not show any enrichment at the NMJ postsynapse, these data are not wholly inconsistent with local translation occurring in the SSR for a number of reasons (See Discussion). First and foremost, every mRNA tested was present at NMJ boutons. While these foci are few in number, and are proximal to a couple of boutons per NMJ at most (often none), they could represent a small proportion of the total muscle mRNA that is actively translated at the NMJ. The NMJ is a highly polarized system, with the postsynapse representing less than 0.1% of the total surface area of the muscle, yet regulating contraction entirely. If the postsynapse is the site of local translation at the NMJ, then it is critical to understand how mRNA localization to the NMJ is regulated. Many mRNAs in neurons are localized in response to activity, and it is hypothesized here that neuromuscular activity, and its downstream signaling events and translation, are critical to regulating mRNA distribution and levels in the muscle.

iii) Genetic alteration of neuromuscular activity reveals changes in the levels and distribution of mRNA

The macromolecular organization of the NMJ postsynapse is sensitive to neurotransmission. In the embryo, electrical transmission stimulates the muscle, and causes the expression, and then clustering, of postsynaptic scaffolding molecules, which in turn recruit glutamate receptors to assemble into functional synapses (Broadie and Bate, 1993; Chen and Featherstone 2005). Subsequently in the larva, increased neuromuscular transmission can affect the dynamic distribution of proteins. Upregulated transmission leads to increased levels of the ubiquitous secondary messenger molecules, Ca^{2+}, and cAMP, both of which can lead to transient changes in the phosphorylation, and concomitant distribution, of proteins between the muscle cytoplasm and SSR (Koh *et al.* 1999; Zhang *et al.* 2007). Secondary messenger signaling is not limited however to redistributing macromolecules, but can also elicit long-term changes in gene expression at

synapses that constitute plasticity. For example, elevated levels of cAMP leads to a down-regulation of the cell adhesion molecule FasII at the NMJ synapse, and activates the CREB transcription factor to induce long-term changes in gene expression necessary for changes in synapse structure and function (Schuster *et al.* 1996a and 1996b; Davis *et al.* 1996). Despite the research into how neurotransmission correlates with changes in synapse structure and function, little is known about if, or how, mRNA levels and localization respond to these processes and contribute to plasticity. Moreover, while mRNA localization in larval muscle has been measured in a few previous studies, none have shed light on the mechanisms that regulate mRNA localization. To examine how mRNA levels and localization in muscle respond to changes in plasticity, mRNA *FISH* was performed in a number of mutant animals. In each mutant a key cellular process that contributes to synaptic plasticity was perturbed (See Figure 5.6 for a detailed explanation):

1. Synaptic transmission
2. cAMP and Ca^{2+} secondary messengers and signaling
3. Trafficking and Microtubule organization
4. Postsynaptic Translation

Genetic mutants, each deficient in one category of processes, were systematically tested for changes in mRNA levels and distribution via mRNA *FISH*. Comparison of these data would provide insight into how, and if, muscle mRNA responds to neurotransmission.

Figure 5.6. 1) Synaptic transmission; 2) secondary messenger and signal transduction; 3) organization of macromolecules; and 4) postsynaptic translation, all contribute to synaptic plasticity in muscle

iv) The levels and distribution of dlg mRNA are sensitive to changes in synaptic transmission

To test if *in vivo* physiological stimulation of NMJs regulated changes in mRNA distribution and levels, it was decided that a previously reported assay, in which extensive crawling is induced in larvae, would be employed. In this crawling assay, wildtype third instar larvae are raised under standard conditions at 18^{o}C before being transferred onto moist, non-nutritious, agar plates at 29^{o}C. It has been shown that moving larvae from the soft slurry of food, to a hard warm agar plate, will induce increased crawling rates as the larvae search for food (Sigrist et al 2003; Steinert *et al.* 2006). This crawling is reported to induce a number of experience-dependent changes in both neuron and muscle, including elevated postsynaptic translation.

Transferring larvae from food slurry at 18^{o}C, to an agar plate at 29^{o}C, changes a number of variables at once, and so this experiment must be carefully controlled. In previous studies, the temperature-sensitive mutation of the presynaptic K^{+} channel Paralytic (Para) has been used as a control. In these studies, *para*^{ts1} larvae were raised in parallel with wildtype larvae at the permissive temperature (18^{o}C) in standard food. Upon transfer to 29^{o}C agar plates the *para*^{ts1} larvae were reported to be unable to crawl, owing to perturbed vesicle release at the restrictive temperature, while wildtype larvae began to crawl. While eIF4E was reported to accumulate at the NMJ of wildtype animals, no such change was observed in *para*^{ts1} larvae, thereby controlling for changes in gene expression that may have been caused by increased temperature or contact with the agar plate. As a result, it was concluded that increases in postsynaptic eIF4E was the product of increased crawling. To reproduce this assay the *para*^{ts} stock was obtained from the National Institute of Genetics, Japan. The *para*^{ts1} stock had to be sent three times as the adult flies, larvae, and pupae were dead upon arrival of the first packages. The second stock sent contained

very few living adult flies and no living larvae. The stock was cultured both at 18°C and 21°C and required great care to maintain; even at permissive temperatures the adult flies would lay very few eggs, and the progeny were very sickly. To improve and maintain the stock, *para*^{ts} was balanced on X chromosome to produce heterozygous females. While the health of the balanced stock was improved, the *para*^{ts1} mutant must be homozygous to ablate muscle contraction at elevated temperatures. Even at permissives temperatures however, larvae homozygous for the *para*^{ts1} rarely moved, crawled, or ate food, and very few third instar progressed to pupae, while wildtype larvae exhibit normal movement and survival rates. While the recipe of food that stocks are raised on can sometimes help to maintain sick lines, the food used here is sufficient to maintain multiple weak stocks. Owing to the sickness of the para^{ts1} stock, it was concluded that *para*^{ts1} did not constitute a good control in this assay.

Without the *para*^{ts1} stock, it was challenging to control a crawling assay experiment. While different options were tested, none were found to be satisfactory controls. Initially, it was hypothesized that while larvae transferred to non-nutritious warm agar plate would crawl in search of food, larvae transferred to a warm agar plate with food would lie relatively still and eat. While not a perfect control, as one set of larvae is fed while another is not, this was tested. Upon transfer to elevated temperature however, larvae with food left the food and crawled extensively. Lowering the crawling temperature from 29°C to 25°C did not stop control larvae from increasing their crawling considerably. As both test and control larvae crawled to the same extent, it was decided that larvae kept in food was not an appropriate control for this experiment. As result, it was not possible to control this experiment properly. Instead, a different approach was employed to increase neuromuscular activity.

Loss-of-function mutants in the voltage-gated K^+ channel Shaker, such as *sh*^{14}, exhibit increased synaptic excitability and an increase in NMJ branches and bouton number

(Budnik *et al.* 1990). Shaker repolarizes the neuronal and muscle membrane potential after an action potential. As a result, *Sh* loss-of-function mutants exhibit constitutive hyperexcitability, leading to bouton overgrowth. To test if hyperexcitability regulates mRNA levels or distribution, mRNA *FISH* was performed on wildtype and sh^{14} loss-of-function mutants.

All mRNA *FISH* experiments presented in this chapter were performed in parallel, using animals reared, fixed, stained, imaged, and image-processed, under identical conditions. All experiments were repeated a number of times, though all data presented within a given figure is from the same repeat. As a result all mRNA *FISH* data presented in this chapter are comparable across different figures (please note different contrast adjustments where noted, CA0, CA1, CA2 etc.).

Examination of *dlg* mRNA in Sh^{14} mutants reveals a dramatic increase in *dlg* mRNA levels throughout the muscle relative to wildtype (Figure 5.7). Adjustment of the image contrast revealed that Sh^{14} muscles contained distinct foci of *dlg* mRNA similar to that in wildtype controls, however the foci were much brighter in Sh^{14} mutants. In addition, mRNA signal was present outside of cytoplasmic foci in Sh^{14} mutants, in a uniform, haze-like distribution, while all mRNA signal in wildtype muscle was present in foci (Figure 5.7). It is interesting to note that while overexpression of UAS-Dlg-GFP in muscle lead to an increase of foci size, but no signal outside of the foci, Sh^{14} loss-of-function mutants exhibited elevated *dlg* levels that were distributed much more evenly throughout the muscle. Thus loss of Shaker, and the concomitant change in membrane excitability, likely lead to a change in both the distribution and levels of *dlg* mRNA (See Discussion). To fully test if synaptic transmission leads to changes in *dlg* mRNA levels and localization, it is necessary to conditionally increase synaptic transmission at the NMJ, and then perform mRNA *FISH*. To this end, heat-activated channels that would conditionally activate either neuron or muscle were tested however, technical challenges limited interpretation of these data (see discussion).

If increased synaptic transmission in *shaker* mutants, rather than other effects, leads to the changes in *dlg* mRNA levels and localization, then the processes downstream of transmission may also regulate *dlg* mRNA in a similar manner. To further approach if, and how increased neurotransmission regulates muscle mRNA, the processes downstream of synaptic transmission was be interrogated.

Figure 5.7. Loss-of-function *shaker* mutants exhibit elevated *dlg* mRNA levels and *dlg* mRNA free from punctate foci

v) dlg mRNA levels and distribution are independently regulated by cAMP and CaMKII signaling pathways

The increased membrane excitability of *Sh* loss-of-function mutants will upregulate multiple downstream signaling pathways (Figure 5.6). Most notably, synaptic transmission will lead to increases of the Ca^{2+} and cAMP secondary messengers in muscle. cAMP and Ca^{2} signaling are both required for NMJ synaptic plasticity, as well as learning and memory in adult flies (See Chapter 1; Tully and Quinn 1985; Tully and Gold, 1993; Davis *et al.* 1995; Davis *et al.* 1996; Ashraf *et al.* 2008). Moreover, cAMP and Ca^{2+} pathways are intimately linked; elevated levels of Ca^{2+} can lead to increases in cAMP concentration through activation Ca^{2+}/Calmodulin Dependent Adenylyl cyclases, such a Rutabaga (Figure 5.6). As a result, to distinguish between the roles of Ca^{2+} and cAMP signaling on mRNA regulation, mutants that affect either pathway, but not both, were examined.

Under resting conditions, CaMKII is inactive owing to an interaction between its autoinhibitory and catalytic domains. Elevation of Ca^{2+} and calmodulin signaling weaken this inhibitory interaction, and CaMKII is freed to autophosphorylate a conserved regulatory threonine residue (T286 in mammals and T287 in *Drosophila*) (Hanson and Schulman, 1992). Using this knowledge, researchers have developed a mutant allele of CaMKII, called CaMKII-T287D, which mimics T287-phosphorylation in flies and is constitutively active (Koh *et al.* 1999). To test if upregulated Ca^{2+} dependent-signaling lead to changes in *dlg* mRNA levels or distribution, UAS-CaMKII-T287D was expressed in muscle, and *dlg* mRNA was stained for via mRNA *FISH.* Overexpression of CaMKII-T287D in muscle lead to varied change in *dlg* mRNA distribution. Most muscles exhibited a wildtype-like distribution, with *dlg* distributed throughout muscle in distinct foci. A number of muscles however, showed a haze-like *dlg* mRNA signal free from foci, similar to that seen in *shaker* mutants, enriched at the postsynapse (Figure 5.8 B and C). Neither case

showed a noticeable change in *dlg* mRNA levels. While it is exciting to consider that mRNA is localized to the postsynapse in response to elevated CaMKII activity, examples of this are few (2 of 24 NMJs observed across 6 animals). Even when observed, other NMJs in the same animal would not show NMJ-enrichment of *dlg* mRNA. As a result, it is possible that localization of *dlg* mRNA in animals expressing CaMKII-T287D is sensitive to other factors, such as the activity of a specific NMJ (See Discussion). Surprisingly, overexpression of CaMKII-T287D in muscle resulted in an increase in cytoplasmic anti-HRP signal. Anti-HRP antibodies specifically bind an unknown epitope in the insect nervous system. Sugar moieties conjugated to sodium channels have been suggested as a possible epitopes, though not shown. It is possible that upregulation of Ca^{2+}-dependent signaling leads to changes in the distribution of the anti-HRP epitope in the muscle. Alternatively, it is possible that non-specific antibody binding is increased in animals expressing CaMKII-T287D; increased contractility is likely to change the muscle protein content.

It is important to note that while central to Ca^{2+}-dependent signaling, CaMKII is not the only change in signaling induced by Ca^{2+}. Increased Ca^{2+} leads to increased levels of the secondary messenger cAMP (Figure 5.6). To understand how mRNA levels and localization are regulated in muscle, it was necessary to interrogate if Ca^{2+} influx regulates mRNA through CaMKII, cAMP, or both. As mentioned above, increased NMJ activity can lead to increases in cAMP concentration through activation of Ca^{2+}/Calmodulin Dependent Adenylyl cyclases, such a Rutabaga. Dunce is a cAMP phosphodiesterase that catalyses the removal of cAMP from muscle cytoplasm (Figure 5.6). Consequently, loss-of-function *Dunce* mutants exhibit elevated cAMP levels, and an increase in both bouton numbers and transmission quantal content (Davis *et al.* 1996). To test if increased levels of the secondary messenger cAMP regulates the levels and distribution of muscle mRNA, mRNA *FISH* was performed in *dunce*^{1} loss-of-function mutants. Loss of Dunce lead to a dramatic increase in *dlg* mRNA levels relative to wildtype controls (Figure 5.9). Adjustment

of contrast revealed that *dlg* mRNA was present both in larger foci, and in an even haze, distributed throughout the muscle. No enrichment of *dlg* mRNA was observed at the postsynapse.

It is interesting to speculate that the two distinct signaling pathways, consisting of CaMKII or cAMP, may regulate *dlg* mRNA in distinct manners. While an increase in cAMP will increase the levels of *dlg* mRNA, but not alter the enrichment at the synapse, activation of CaMKII can recruit *dlg* mRNA to the boutons, but not alter the mRNA levels (See Discussion). To understand how CaMKII and cAMP may regulate mRNA levels and distribution, it was necessary to interrogate the processes downstream of these signaling pathways. One such process is the reorganization of macromolecules at the synapse.

Figure 5.8. Constitutive activation of CaMKII in muscle leads to a varied change in *dlg* mRNA distribution

Figure 5.9. Loss of Dunce increases the levels of *dlg* mRNA in muscle and increases non-foci mRNA signal

vi) The levels and distribution of dlg mRNA change with perturbations to microtubule organization, but not dynein-dependent trafficking

Neuromuscular activity can change local gene expression at the NMJ through two distinct mechanisms. First, *de novo* translation produces new proteins to assemble into either pre-existing or novel synapses. Translation-dependent changes in gene expression at the synapse represent long-term changes associated with memory formation in central neurons and are limited by the time frame of protein expression. Second, the distribution of existing proteins can be altered to change the copy number present at synapses. Relocating proteins between synaptic and extra-synaptic regions of the cytoplasm is thought to facilitate more rapid, short-term, changes in synaptic gene expression. For example, phosphorylation by PAR-1 and CaMKII kinases regulates the translocation of Dlg between the synapse and extra-synapse cytoplasm, a process thought to be dependent upon trafficking along the cytoskeleton (Koh *et al.* 1999; Zhang *et al.* 2007). It has been shown here that in mutant muscles in which synaptic transmission is altered, the distribution of *dlg* mRNA is changed from punctate foci housed away from the synapse, to an even 'haze' distribution throughout the muscle. To test if the *dlg* mRNA is localized into foci by microtubule-dependent trafficking, both the microtubule organization, and the molecular motor proteins that move along it, were perturbed.

Initially, mRNA *FISH* was performed on larvae lacking fully functional Futsch. Futsch is a microtubule organizing protein with homology to mammalian Microtubule Associated Protein 1B (MAP1B), a microtubule-organizing protein (Hummell *et al.* 2000; Roos *et al.* 2000). MAP1B expression is correlated with regions of the vertebrate brain that show activity-dependent plasticity (Caceres *et al.* 1986; Reviewed in Muller *et al.* 1994). While many animals with mutations in *Futsch* are sick, homozygous lethal and challenging work with, the point-mutant *futsch*^{K68V} is homozygous-viable and exhibits a disorganized

microtubule network and aberrant NMJ development (Hummell *et al.* 2000; Roos *et al.* 2000). Inspection of *dlg* mRNA in *futsch*^{K68V} mutant larvae revealed that *dlg* mRNA levels were elevated relative to wildtype, and distributed throughout the muscle free from distinct cytoplasmic foci (Figure 5.10). While it may seem surprising that *dlg* mRNA levels rise in response to a disorganized cytoskeleton, there are a number of possible explanations (See Discussion). In addition to the increased levels of mRNA, the change in *dlg* mRNA distribution in *futsch*^{K68V} mutant muscle is striking; the large cytoplasmic foci in wildtype muscle are replaced by an evenly distributed 'haze'. The even distribution of mRNA resembles that observed in mutants with elevated synaptic transmission and elevated secondary messenger signaling (*Shaker* mutant Figure 5.7, and *Dunce* mutant Figure 5.9). It is interesting to consider that the localization of mRNA between the free cytoplasm, and punctate foci, is a regulated process. Wildtype muscles show mRNA housed in foci, while mutant muscles with elevated neuromuscular signaling exhibit a haze-like distribution of mRNA. To approach how mRNA localization in muscle is regulated, it is necessary to better understand the mechanisms by which *dlg* mRNA is recruited to, or freed from, cytoplasmic foci.

Figure 5.10. Loss of function *futschK68V* mutant exhibits elevated *dlg* mRNA levels free from punctate foci

Owing to the requirement of fully functional Futsch to house *dlg* mRNA in foci, it is possible that microtubule-dependent transport is necessary to regulate the formation of *dlg* foci. The molecular motor Dynein is required to localize mRNA in different systems by active transport along microtubules, and is present throughout larval muscle (Duncan and Warrior 2002; Clark and Davis 2007; Lloyd *et al.* 2012). It was hypothesized that Dynein is necessary for microtubule-dependent formation of *dlg* mRNA foci. To test this hypothesis, microtubule transport by the dynein molecular motor was abrogated in muscle by overexpression of the p150 subunit Glued^{DN} (See Chapter 4 for an explanation of Glued^{DN} function). Examination of *dlg* mRNA distribution in animals overexpressing Glued^{DN} however, revealed no change in mRNA distribution (Figure 5.11). This was an initially surprising result. If proper microtubule organization was necessary to organize *dlg* mRNA into foci, then it would seem likely that microtubule-dependent trafficking was central to mRNA localization in muscle. It is important to remember however that in *futsch^{K68V}* mutants, the microtubules of both muscle and neuron are disrupted, and that changes in *dlg* distribution may be indirect. For example, it is known that mutations in *Futsch* alter NMJ morphology, which likely leads to changes in NMJ transmission (Hummell *et al.* 2000; Roos *et al.* 2000). As mutants with altered NMJ transmission and signaling have been shown here to affect *dlg* mRNA localization (*Shaker* mutant Figure 5.7, and *Dunce* mutant Figure 5.9), it is possible that *futsch^{K68V}* mutants lead to altered *dlg* mRNA distribution through elevated NMJ transmission rather than deficient trafficking along microtubules in muscle. It is possible that elevated NMJ transmission, and downstream signaling, regulates *dlg* mRNA localization to facilitate the requirements of the muscle for Dlg protein. To fully understand how the punctate organization of *dlg* mRNA is regulated in response to Dlg expression, it was necessary to test *dlg* mRNA distribution in mutants in which Dlg translation is perturbed.

Figure 5.11. Overexpression of UAS-Glued^{DN} in muscle decreases the levels, but not the distribution, of *dlg* mRNA

vii) Loss of Pumilio reveals the relocalization of cytoplasmic mRNAs to the NMJ

To test if increased translation at the NMJ correlated with increased mRNA levels at the NMJ, mRNA *FISH* was performed in animals with loss-of-function mutations in the translational regulator Pumilio. Pumilio (Pum) is a conserved RBP that is well known for regulating abdominal segmentation in the early *Drosophila* embryo through the local translation of *hunchback* mRNA. Maternal *hunchback (hb)* mRNA is distributed throughout the early embryo, but binding of Pumilio to specific sequences in the *hb* 3'UTR blocks translation from the posterior region, restricting Hb translation to the anterior regions of the embryo (Murata and Wharton. 1995; Chagnovich and Lehmann. 2001).

Subsequent research has shown that Pumilio is enriched at the NMJ postsynapse, regulates translation in larval muscle, and is required for proper NMJ morphology (Menon *et al.* 2004 and 2009, Table 1, Chapter 3). In the absence of Pumilio, a dramatic decrease in NMJ growth is observed concomitant with an increase in eIF4E and GluRIIA protein levels at the NMJ. Biochemical experiments in the same studies revealed that Pumilio specifically binds to *eIF4E* and *gluRIIA* transcripts. As a result it was concluded that Pumilio regulates NMJ structure through local translation of eIF4E and GluRIIA. In parallel, informatics approaches, supported by biochemical and imaging data, revealed that Pumilio also binds to the 3'UTR of *dlg* mRNA and represses Dlg expression in the central nervous system of the adult fly (Chen *et al.* 2008). To this end, Pumilio is thought to regulate the expression of Dlg, eIF4E, and GluRIIA expression through direct interaction with the cognate transcripts at the NMJ postsynapse.

Thus far it has been shown that mRNA localization in muscle is sensitive to mutations that affect synaptic transmission and secondary messenger signaling. Translation of key synaptic proteins is a fundamental step in synaptic plasticity and is thought to occur

downstream of synaptic transmission and secondary messenger signaling. To test if mRNA localization is regulated in response to elevated local translation, mRNA *FISH* was performed in animals with loss-of-function mutations in *Pumilio*. Mutations in *Pumilio* gene were chosen that give strong phenotypes in both embryogenesis and NMJ synaptic plasticity (Forbes and Lehmann. 1998; Menon *et al.* 2004). pum^{MSC} is an inversion mutant with the breakpoint in the 8th intron of the *Pum* gene, while pum^{et7} and pum^{et9} are ethyl methane sulfonate (EMS)-induced nonsense mutations. None of the *Pum* mutants used here are protein null in larvae, and all exhibit residual protein expression in both neuron and muscle (Menon *et al.* 2004).

In the *Pumilio* loss-of-function mutant $pum^{et7/et9}$, large structures (>μ2m) consisting of multiple foci of *dlg* mRNA appeared proximal to the NMJ (Figure 5.12, arrowheads). These structures were never observed more than 5μm away from a bouton in *pum* mutants, and were absent from wildtype controls. In the *Pumilio* mutant $pum^{et7/MSC}$ however, no such structures were observed. A possible explanation for this is that pum^{MSC} mutations have been reported to produce weaker morphological and electrophysiological NMJ phenotypes owing to the preservation of PUF RNA binding domain in pum^{MSC}, but not pum^{et7} and pum^{et9} mutants (Sonoda and Wharton. 1999). It is possible that expression of the Pumilio RNA-binding domain in the $pum^{et7/MSC}$ mutant, but not the $pum^{et7/et9}$ mutant, rescued the mRNA localization phenotype.

Thus far two different classes of *dlg* mRNA distribution have been observed 1) cytoplasmic foci housed distal to boutons and 2) a haze-like distribution throughout the muscle that can occasionally be enriched at boutons. In the absence of Pumilio, another distinct distribution of *dlg* mRNA is observed, 3) the presence of large structures, made of multiple cytoplasmic foci, proximal to boutons. While Pumilio regulated Dlg expression in the central nervous system, this has never been demonstrated in larval muscle. It is reported however that *Pum* mutants exhibit elevated levels of eIF4E and GluRIIA protein

at the postsynapse, reportedly through local translation at the NMJ (Menon *et al.* 2004 and 2009). If translation of eIF4E and GluRIIA is upregulated at the NMJ in the absence of Pumilio, then so might localization of *eIF4E* and *gluRIIA* mRNA to the postsynapse. To test if increases in eIF4E and GluRIIA protein correlated with changes in mRNA distribution at the NMJ, mRNA *FISH* was performed on *Pum* mutants using *eIF4E* and *gluRIIA* antisense probes. The loss-of-function $pum^{et7/et9}$ mutant exhibited clear enrichment of *eIF4E* mRNA at the NMJ (Figure 5.13 A – B). Interestingly, the enrichment formed a haze-like distribution (similar to earlier observations; Figure 5.7 and 5.8) only the haze of small mRNA foci was localized to the NMJ and not distributed throughout the muscle (see Discussion). In some cases, the increase in *eIF4E* mRNA at the NMJ was so dramatic in the $pum^{et7/et9}$ mutants that contrast adjustment was required to resolve the mRNA distribution. The $pum^{et7/MSC}$ mutant however showed an increase in *eIF4E* mRNA proximal to boutons, but housed in large foci (Figure 5.13, D) arrows). It is possible that expression of the PUF-domain in the $pum^{et7/MSC}$ mutant regulates the distribution of *eIF4E* mRNA to large foci, rather than a haze. $pum^{et7/et9}$ mutants also showed an increased enrichment of *gluRIIA* mRNA at the NMJ relative to wildtype controls (Figure 5.14). The enrichment of *gluRIIA* mRNA at the NMJ was less dramatic than for *eIF4E,* but was still robust and absent from non-specific controls (not shown).

To test if loss of Pumilio lead to a global increase in mRNA localization to the NMJ, mRNA *FISH* was performed on *Pumilio* mutants with *shaker* antisense probes. As Pumilio is not predicted to bind to *shaker* mRNA or to regulate Shaker translation (Chen *et al.* 2008), it is not expected that loss of Pumilio would affect *shaker* mRNA localization. No difference in *shaker* mRNA levels or distribution was observed in *pum* mutants relative to wildtype controls (not shown). It is clear from these data that mRNA localization is regulated in response to the translation status of the postsynapse. In the absence of Pumilio, eIF4E and GluRIIA protein are specifically overexpressed, reportedly by translation at the SSR. Here it has been shown that upregulation of translation of eIF4E and GluRIIA correlates

with an increase in recruitment of the cognate mRNAs to the postsynapse. To further approach the mechanism of mRNA localization however, progress must be made in two key areas. First, experiments must move beyond the examination of mutants. While useful, mutational studies are fraught with complications and potential artifacts (See Discussion). Second, the variable changes in mRNA localization observed in different mutants, sometimes differing from muscle-to-muscle in the same animal, suggest that mRNA localization in muscle is a dynamic process. To characterize a process capable of rapid changes, it is necessary to employ live-cell imaging.

Figure 5.12. Loss of Pumilio leads to large *dlg* mRNA foci proximal to synapses

Figure 5.13. Loss of Pumilio leads to enrichment of *eIF4E* mRNA at the NMJ

Figure 5.14. Loss of Pumilio leads to a slight and varied enrichment of *gluRIIA* mRNA foci at the NMJ

viii) Testing methods for imaging mRNA in live muscle

While these data presented here have shown that mRNA distribution is sensitive to the neurotransmission, signaling, and translation processes that constitute synaptic plasticity, the mechanisms by which this occurs remain elusive. To interrogate these mechanisms further it is necessary to develop a live-cell system in which mRNA localization can be assayed. If mRNA localization within muscle is a dynamic process that responds to neuromuscular activity, then it is important to measure these processes within the time frame that they occur. For example, by applying stimulating buffers to a living fillet prep, it may be possible to test whether increased NMJ activity does lead to mRNA leaving cytoplasmic foci and forming an even distribution throughout the muscle. Moreover, the proteins necessary for mRNA localization could be investigated readily if an assay for live mRNA imaging existed. Injection of inhibitory antibodies into muscle, or application of pharmacological agents, could acutely inactivate specific proteins, and shed light on their role in mRNA distribution. Moreover, these experiments would be performed in wildtype animals, free from developmental deficiencies arising from mutation (see Discussion). To this end, existing methods for imaging RNA in live cells were applied to larval muscle.

Microinjection of fluorescently-labeled mRNA into cells has proved to be a powerful technique for studying the mechanisms of mRNA localization. As discussed, *gurken* (*grk)* mRNA is localized to the dorsoanterior corner of the oocyte to specify primary embryonic axes. While the distribution of *grk* mRNA was known, microinjection of *grk* mRNA revealed the two-step pathway by which the mRNA was localized (MacDougall *et al.* 2003). Further, co-injection of *grk* mRNA with inhibitory chemicals and antibodies revealed that proper localization was dependent upon dynein trafficking along microtubules, followed by anchoring by the RBP Squid (MacDougall *et al.* 2003; Delanoue *et al.* 2007). A key advantage of the *in vivo* microinjection method is that the experiment is rapid,

requiring expression of no exogenous genetic components to function and no generation of transgenic animals. Thus, while the *cis*-acting mRNA sequence necessary for *grk* mRNA localization had been mapped to a 400nt region in the coding sequence by testing the localization of gene-fusions in transgenic animals (Saunders and Cohen. 1999; Thio *et al.* 2000), microinjection of fluorescently-labeled *grk* mRNA sequences quickly revealed a 64nt sequence to be necessary and sufficient for *grk* mRNA localization (Van de Bor *et al.* 2005).

Beyond *gurken*, the microinjection of fluorescently-labeled mRNA into the oocyte and embryo has provided powerful insight into the proteins and RNA sequences that govern mRNA localization in development (Wilkie and Davis 2001; Hughes *et al.* 2004; dos Santos *et al.* 2008). If the same technique could be applied to the larval bodywall muscle, similar progress could be made in understanding how mRNAs encoding key synaptic transcripts are localized to cytoplasmic foci, or to the NMJ. To test if the larval bodywall muscles could be microinjected, Alexa 488 dye was suspended into the hemeolymph-like buffer HL3 and loaded into a microneedle. With practice, injection of dye into muscle 6 of abdominal segments 2 and 3 was performed rapidly and with relative ease (Figure 5.15 A). It was found that muscles had to be pierced for the shortest period of time possible, and as superficially as possible. The muscles instantly contracted upon piercing and would frequently snap the needle if pierced too deeply, or held in place for too long. The dye rapidly spread throughout the entire muscle within 30 seconds.

To test if microinjection of fluorescently labeled *dlg* mRNA would recapitulate the distribution observed via mRNA *FISH*, *dlg* mRNA was transcribed *in vitro* incorporating modified Uracil residues conjugated to Alexa 568 and injected into muscle. As a negative control, RNA encoding GFP was injected in parallel into muscles of the same animal. Despite testing a range of mRNA concentrations and suspension buffers, microinjection of *dlg* mRNA formed a similar distribution to *gfp* mRNA (Figure 5.15 B and C). Microinjected

mRNA would frequently accumulate at the site of injection (arrows) and adhere in static structures to the muscle striations proximal to the site of injection. Microinjected mRNA would not spread throughout the muscle or form foci even when left for 60 minutes. To test if other mRNAs would localize to punctate foci in muscle, *shaker* mRNA was also microinjected, but with the same results (not shown). It is not clear why mRNA microinjected into muscle does not behave as endogenous mRNA, though it is important to note that even in the oocyte, where microinjection is a prominent technique, there are examples of mRNAs that will not localize when microinjected, e.g. *oskar*. A microinjected mRNA will only mimic the endogenous transcripts if the proteins necessary for its localization are present in the cytoplasm. For example, proteins necessary for mRNA localization may be recruited in the nucleus. While it was not possible to develop an assay for microinjection of mRNA, focus was applied to using an alternative method for visualizing mRNA in living cells, the MS2 system.

Figure 5.15. Injection of fluorescently-labeled mRNA into living muscle does not recapitulate the distribution observed via mRNA *FISH*

As described in Chapter 4, the MS2 system employs two genetically encoded components to label endogenously expressed mRNAs decorated with fluorophores *in vivo* (Chapter 4; Figure 4.9). One disadvantage of the MS2 system is that multiple transgenes must be expressed in the same cell. This can be both complicated to construct, and cause sickness in the transgenic animal. For example, to decorate MS2-RNA with MCP-GFP in muscle, and label boutons for reference, it was necessary to construct a line that contained three transgenes 1) a muscle-specific Gal4 driver 2) UAS-MCP-GFP and 3) a red bouton marker. This line could then be crossed to animals expressing MS2-RNA. To this end, transgenic animals expressing these constructs were created by recombining and 'crossing in' different transgenes. UAS-mCD8-mCherry was found to label all membranous structures, including the SSR, within muscle. Owing to the dense membrane structure of the SSR, boutons were readily discernable over background fluorescence. It was found that viable flies that expressed 1) C57-Gal4 muscle driver 2) UAS-MCP-GFP and 3) UAS-mCD8-mCherry were very rare (<1/200 eclosed) and required extensive cosseting at 21^{o}C to establish a stock. This line, named Muscle line 1, had the genotype:

UAS-MCP-GFP,UAS-mCD8-mCherry ; C57-Gal4
II III

By crossing Muscle Line 1 with a fly expressing a MS2-RNA, the localization of that RNA could be visualized in live muscle relative to boutons. Using the MS2 constructs described in Chapter 4, *dlg*6XMS2, *eIF4E*6XMS2, *shaker*6XMS2, *fasII*6XMS2, and *gluRIIA*6XMS2 were crossed with Muscle Line 1 to examine their subcellular distribution in living muscle. As controls, Muscle Line 1 was examined alone (i.e. without any MS2-tagged RNA) or crossed with *empty*6XMS2 RNA (expressing the MS2 loops alone). In no case however, could MS2-RNA signal be detected in muscle cytoplasm. As a result, no difference could be found between any MS2-RNA and the *empty*6XMS2 control, or Muscle Line 1 alone (Figure 5.16; *dlg*6XMS2 shown). There are a number of reasons as to why tagging mRNA

with MS2-loops may prevent the mRNA localizing in the same manner that non-tagged mRNA does (See discussion). One possibility was that the low levels of mRNA in the cytoplasm were being obscured by the high fluorescence in the nuclei throughout the muscle. MCP-GFP that is not bound to MS2-RNA is shuttled to the muscle nuclei by a nuclear localization signal (NLS) and in all cases fluorescence in muscle nuclei was very high. Even at 100x magnification, images of boutons contained contaminating fluorescence from the nuclei, even when the nuclei were not in the field of view. Confocal imaging, rather than wide-field, may have improved this. To try and circumvent this issue, a different line expressing MCP-RFP, which is less bright than MCP-GFP, was constructed. The stock, named Muscle Line 2, also labeled boutons (via UAS-Dlg-GFP) while driving UAS-MCP-RFP specifically in muscle, and had the genotype:

UAS-MCP-RFP ; C57-Gal4, UAS-Dlg-GFP
II III

Muscle Line 2 also required great care and cosseting to establish a stock (>1/100 originally eclosed). By crossing the MS2-RNAs to Muscle Line 2, their subcellular distribution could be assayed. Unlike Muscle Line 1, Muscle Line 2 did produce fluorescent signal in the muscle cytoplasm when crossed to MS2-RNA. The cytoplasmic MS2-RNA*RFP signal however was remarkably varied from muscle-to-muscle and all MS2-RNAs exhibited the same range of distributions, none of which recapitulated the endogenous mRNA distributions. Within the same animal, muscles could show multiple small non-spherical structures with uniform distribution, large structures (>5μm) adjacent to nuclei, or no cytoplasmic signal. No difference or pattern could be detected across the different MS2-RNA constructs. As a result it was concluded that MS2-constructs tested here did not recapitulate the distribution of endogenous mRNA (Figure 5.16). There are many possible explanations as to why the MS2 system does not work in larval muscle (See Discussion). Unfortunately, in the absence of a method for imaging mRNA in live muscle, it is not possible to probe the mechanisms that govern mRNA localization

dynamics in muscle. Nevertheless, while the mechanisms behind mRNA localization in muscle remain elusive, these data presented here show that mRNA localization in somatic muscle changes in response to perturbations in synaptic transmission, secondary messengers and signaling, and translation.

Figure 5.16. The MS2 system in larval muscle produces excessive nuclear fluorescence and variable cytoplasmic RNA distributions

Discussion

i) mRNAs encoding synaptic proteins are distributed throughout muscle, and rarely at synapses

It is well accepted that local translation occurs at the NMJ postsynapse. The postsynapse is enriched with translational regulators, such as Nanos, Pumilio, and Syncrip, and polysomes have been detected in the SSR (McDermott *et al.* in preparation; Sigrist *et al.* 2000; Menon *et al.* 2004, 2009). Moreover, activation of the NMJ, either through extended crawling or genetic perturbation, leads to enrichment of the translation initiation factor eIF4E at the postsynapse, followed by the appearance of new Glutamate Receptor foci. Taken together, these observations strongly suggest that translation occurs at the NMJ in muscle. It is surprising then, to find that mRNAs encoding key synaptic proteins are distributed throughout muscle, and rarely present at boutons, rather than localized to boutons as previously reported (Sigrist *et al.* 2000). Indeed, a similar distribution was observed in wildtype muscle for *dlg, fasII, shaker, eIF4E* and *gluRIIA* mRNA, indicating that these mRNAs, whose products are central to synaptic plasticity, could be distributed by similar mechanisms. Owing to complete reproducibility, extensive controls, and optimization of existing mRNA *FISH* methods to high levels of sensitivity, these results are presented here with great confidence. Thus if local translation does occur at the postsynapse, then it is a prerequisite that mRNA must be housed at boutons at some point. It is of note that while translational regulators such as Pumilio, Nanos, and Syncrip, are evenly enriched around the whole circumference of every bouton, mRNA lies close to single boutons only in infrequent foci. Thus it would be predicted that while all *dlg* mRNA at a bouton will colocalize with Pumilio and Syncrip, only a small fraction of Pumilio and Syncrip localized to a bouton will overlap with *dlg* mRNA. If translation is restricted to the postsynapse, it is tempting to postulate that mRNA availability at the NMJ is a key point of regulation. mRNA could be trafficked from extrasynaptic foci to the postsynapse only

when translation was required. Consistent with this concept, all transcripts examined here are overwhelmingly away from the synapse under resting conditions. Indeed, even in recent studies that show *par-6* mRNA to be localized to the NMJ, most *par-6* is present in the extrasynaptic muscle cytoplasm (Speese *et al.* 2012). To test this concept, conditional perturbation of NMJ signaling, coupled with live imaging may be necessary.

An overwhelmingly extra-synaptic distribution of mRNAs encoding key synaptic proteins is described here. Even in mutants, such as $Pum^{et7/et9}$, where *eIF4E* mRNA is enriched at the postsynapse, only a small fraction of muscle mRNA is actually proximal to the NMJ. As a result, it is important to consider that while local translation at postsynapses likely occurs in muscle, translation may not be restricted solely to the NMJ. While translation factors such as Nanos, Pumilio and eIF4E are clearly enriched at the NMJ, they are present throughout the muscle cytoplasm also (Ganesan *et al.* 2012; Menon *et al.* 2004, 2009). While polysomes have been found at the postsynapse, no study has examined ribosome distribution throughout the muscle. Moreover, it has been shown by 3D reconstruction of entire muscles, that removal of microRNA translational repression of *gluRIIA* and *gluRIIB* mRNA leads to ectopic expression of GluRIIA and GluRIIB throughout the muscle (Karr *et al.* 2008). Thus while local translation at the NMJ is a compelling concept, with substantial correlative evidence, it is unlikely that a cell as large as a muscle (A3 muscle 6 is over 30μmx10μmx60μm) would solely restrict translation to a region representing ~0.01% of its volume. One way in which the location of Dlg translation could be tested is by using photoconvertible protein fusions. Photoconvertible fluorophores can be permanently switched from one colour to another upon exposure to specific wavelengths of light. Dlg fused to the photoconvertible fluorophore mEOS, for example, would fluoresce in the green spectrum. Upon photoconversion Dlg-mEOS would permanently fluoresce in the red spectrum. Thus, by photoconverting Dlg-mEOS to the red spectrum, and then testing for emergence of green fluorescence, it is possible to test for *de novo* translation of Dlg. This experiment was designed early in the thesis, and Dlg-

mEOS was kindly provided by Dr. Ulrich Thomas and Oliver Kobler (unpublished reagent, Leibniz Institute for Neurobiology). However a key problem with this experiment was that newly translated fluorophores can take hours to mature to fluorescence. Fillet preps, however, exhibit poor electrophysiology after one hour in culture, indicating stress and death. As a result it would not be possible to photoconvert Dlg-mEOS at the boutons of a fillet prep, and image the appearance of new Dlg-mEOS protein. A possibility for future experiments would be to photoconvert Dlg-mEOS in intact larvae at NMJs present at the bodywall periphery, such as those innervating muscles 26 and 27, and follow appearance of new Dlg protein at the synapse or muscle cytoplasm (Owald *et al.* 2010). A key focus for future work will be to understand where translation occurs in muscle. This knowledge will allow a more profound interpretation of mRNA localization within muscle.

ii) mRNAs encoding synaptic proteins are present in multiple nuclear foci

It is interesting that *shaker* mRNA is housed in bright foci in every muscle nucleus, while *dlg* and *fasII* are rarely present in the nucleus, and *eIF4E* and *gluRIIA* never observed in the nuclear foci. This difference could reflect different transcriptional profiles, or even different export mechanisms. Only recently it was shown that large mRNP structures containing the A-type Lamin LamC, and mRNA encoding synaptic proteins, were exported from muscle nuclei through a novel pathway involving budding of the nuclear membrane (Speese et al 2012). It is possible that nuclear mRNA foci observed represent large mRNP complexes en route to nuclear exit through membrane budding. While the nuclear mRNA foci reported here are always near the periphery of the nucleus, and it is tempting to imagine them as large export complexes, it is important to note that not all mRNAs exit the nucleus through membrane budding. Indeed Speese *et al.* reported that *dlg* mRNA does not colocalize with the large LamC complexes near the nucleus periphery, and so does not exit the nucleus via Lamin-dependent nuclear budding. *Shaker* mRNA was not tested (Speese *et al.* 2012, supplementary table S2). It would be interesting to test if

shaker mRNA colocalizes with LamC. As LamC mRNP complexes are known to export the nucleus following prolonged neuromuscular activity, this could potentially tie *shaker* mRNA with activity-dependent changes in mRNA distribution. How mRNA localization ties in with the assembly of new synapses is a question central to neuroscience. As proper expression of Shaker is necessary for proper synapse structure and function, it would be interesting to assay *shaker* mRNA localization following *in vitro* activation, as employed in Speese *et al.* 2012 (see also Ataman et al 2008).

iii) mRNA levels and localization respond to changes in synaptic transmission, signaling, and translation

To assess how neuronal transmission may regulate mRNA levels and localization, mutants with perturbations in key neuromuscular processes have been studied here. In a number of such mutants a change in both mRNA levels and distribution have been observed. Intriguingly, while overexpression of UAS-Dlg-GFP in muscle lead to an increase of foci size but no signal outside of the foci, a number of mutants with elevated neuromuscular activity exhibited elevated *dlg* levels that were distributed evenly throughout the muscle in a 'haze'.

It appears that the localization of mRNA, both to large foci and to the postsynapse, and the levels of mRNA, can be independently regulated. For example, while increased membrane excitability in a *shaker* mutant lead to increased *dlg* mRNA, the mRNA was evenly distributed throughout the muscle in a haze. Conversely, in animals in which CaMKII activity was upregulated, a downstream product of increased membrane excitability, *dlg* levels appeared similar to wildtype but exhibited enrichment at the NMJ. These observations can be explained by a number of different possibilities. First, it is possible that both the enrichment of mRNA to the NMJ, and overexpression of mRNA throughout the muscle, achieve the same goal: to increase mRNA levels at the NMJ. If translation does occur at the NMJ, and *dlg, shaker, eIF4E* and *fasII* transcripts are housed

in foci largely away from the NMJ, then it is a requirement that mRNA is relocated to the NMJ. While mutants in *CaMKII* may be competent for this relocation, *Shaker* mutants may not, potentially owing to developmental defects. In the absence of mRNA relocalization mechanisms, *dlg* mRNA levels may simply continuously elevate, in response to the growing NMJ's requirement for Dlg, until *dlg* mRNA fills the whole muscle cytoplasm, and thus the NMJ postsynapse. Alternatively, it is worth considering that while *dlg* mRNA is housed in large foci under resting conditions, smaller *dlg* particles that are released in response to increased membrane excitability, signaling, or translation, may preferentially localize to the NMJ. For example, constitutive activation of CaMKII may increase the probability that *dlg* mRNA is relocated from large cytoplasmic foci to the postsynapse (Figure 5.8). The same process may occur in mutants with elevated membrane excitability; elevated *dlg* levels may saturate the NMJ anchoring machinery and then fill the entire muscle cell.

Central to these explanations is the notion that the *dlg* mRNA foci observed throughout the muscle cytoplasm represent translationally silent complexes, such as transport, processing or storage particles etc, and it is only mRNA that is released from these foci in a 'haze' that are competent for translation. Consistent with this notion is the observation that as with mRNA, the translation initiation factor eIF4E is distributed throughout the muscle in small foci, and *gluRIIA* mRNA foci do not colocalize eIF4E, indicating that muscle mRNA foci are translationally silent (Ganesan *et al.* 2012). The "haze-like" distribution of mRNA likely represents multiple small mRNP complexes, possibly containing a few or even individual transcripts. It is increasingly understood that transcripts are continuously associated with a changing troupe of proteins that regulate transcription, editing, localization, translation, and degradation. The transition from a larger foci to a smaller foci could reflect the remodeling of a mRNP complex to accommodate new RBPs, and a new phase in the transcripts life cycle. For example, it is tempting to hypothesis that release of mRNA from large static structures to smaller foci

may facilitate rapid mRNA localization. It is also interesting to note that changes in mRNA localization in different mutants could not only vary from larva-to-larva, but also from NMJ-to-NMJ within the same animal. In larvae expressing CaMKII-T287D, *dlg* mRNA was mostly observed throughout the cytoplasm, though in 2/24 NMJs observed *dlg* mRNA appeared in a "haze-like" distribution, enriched at the postsynapse. It is possible that at any one point the translational needs of a muscle may differ to other muscles in the same abdominal segment.

It is known that local translation can be regulated in a synapse-specific, and stimulus-specific, manner (Wang *et al.* 2009). Also, mRNA can be localized in neuronal postsynapse in response to activity (Lyford *et al.* 1995; Steward *et al.* 1998). It is interesting to consider that muscle mRNA may be localized in an activity-dependent manner. Also, it is possible to test this hypothesis by exploiting mutants in which muscles within the same animal are innervated to different extents. Mutants in the conserved homeodomain transcription factor Prospero, which is involved in neuron growth from embryogenesis to plasticity in adult flies, results in larvae that show weak, strong, delayed, or absent, muscle innervation (Broadie and Bate, 1993a and 1993b; Griffiths and Hidalgo. 2004;). As a result it is possible to directly compare the same muscle types, in the same segment of the same larva, that are innervated to different extents. It may also be informative to assess how the levels and distribution of ribosomal proteins may change in muscles that are innervated to different extents. To this end, antibodies specific for the 6S ribosomal subunit have been ordered.

iv) The caveats of studying NMJ biology in mutants

It is important when analyzing these data presented here, to consider two important caveats. First is a caveat that it is central to all mutational studies in multicellular

organisms; it is challenging to distinguish between developmental and somatic phenotypes in mutants. It is not possible to conclude whether a mutant phenotype reflects the role of the missing protein at a given stage in development; is the result of developmental adaptions that occur previous to compensate for the protein deficiency; or a function of both. This is particularly true in the nervous system, as multiple cell-types, such as central neurons, motoneurons, and muscle, must develop in homeostasis with one another to maintain circuit efficacy. For example, mutants in the Ca^{2+} sensor Synaptotagmin 1 at the larval NMJ led to an increase in spontaneous vesicle release. As a result, it was proposed that Syt1 functions as a fusion clamp, as well as a Ca^{2+} sensor, that regulates vesicle release from the presynapse (DiAntonio and Schwarz, 1994; Littleton and Bellen, 1994). However, using photochemical inactivation, it was possible to acutely ablate Syt1 expression from third instar larvae with spatial and temporal specificity. Larvae that lacked Syt1 in the third instar only, but had expressed Syt1 during embryogenesis and larval development, exhibited no increase in spontaneous neurotransmitter release. As a result it was concluded that the increase in spontaneous vesicle release observed in loss-of-function *syt* mutants was a development adaption, and did not reflect the role of Syt1 at the third instar synapse (Marek and Davis 2002).

In addition, the cell biology of neuron and muscle are intimately and reciprocally linked across the synapse. Changes in neuron or muscle gene expression can be communicated across the synapse to regulate the partner cell. One example of this is that both muscle and neurons are sensitive to the balance of proteins across the synapse. For example, while elevation of FasII levels on either side of the neuron leads to aberrant neuron morphology, equal elevation of FasII in both neuron and muscle produces wildtype morphologies (Schuster *et al.* 1996). Similarly, increased expression of Dlg in neuron can rescue a muscle deficiency in Dlg (Budnik *et al.* 1996). Moreover, synaptic transmission between motoneuron and muscle is subject to retrograde signaling. As a result, the perturbation of a process in muscle can affect presynaptic signaling, and *vice versa*. For

example, in addition to regulating the postsynaptic localization of Dlg protein and postsynaptic bouton structure, muscle CaMKII also negatively regulates retrograde signaling that controls the assembly of presynaptic active zones and the homeostasis of neurotransmitter release (Koh *et al.* 1999; Haghighi *et al.* 2003). As a result, perturbing CaMKII function in muscle, will affect presynaptic neurotransmitter release. It is for these reasons that it is impossible to use genetic mutants to finely dissect the mechanisms that regulate mRNA in muscle. Instead, these data presented here provide a first step towards showing that mRNA levels and distributions are sensitive to changes in neurotransmission, and the downstream events that contribute to plasticity. To further probe the mechanisms that govern mRNA localization in synaptic plasticity, conditional perturbation of the NMJ is required.

v) Conditional perturbation of neuromuscular processes is necessary to elucidate mRNA localization mechanisms

In this study the fundamental processes that contribute to synaptic plasticity, and their regulation of muscle mRNA, have been examined using mutants. While these data provide insight into the processes that regulate mRNA levels and distribution, they do not probe how rapid physiological changes in neuromuscular transmission regulate mRNA. To approach the mechanisms that govern these processes a number of different approaches must be employed. First, it is necessary to conditionally alter NMJ activity and assay mRNA levels and localization. Unfortunately, it was not possible to control an experiment in which larval crawling is induced, in wildtype and *para*^{ts} animals, owing to the sickness of *para*^{ts} mutant larvae. An alternative methodology to crawling is to stimulate neuromuscular activity, and bouton growth, through the repetitive *in vitro* application of buffers containing high levels of K^+ and Ca^{2+} onto a live fillet prep (Ataman *et al.* 2008). mRNA *FISH* could then be employed on wildtype fillet preps treated in parallel with either activating buffers or saline controls. While *in vitro* stimulation of fillet preps over a two hour time period is no doubt complicated by some artifacts, this is a useful strategy for future research. Further

to these approaches, a number of recent advances allow conditional control of neurotransmission using selectively-permeable ion channels. One such tool depends on the tissue-specific expression of UAS-Transient Receptor Potential A1 (TRPA1). TRPA1 is a warmth-activated cation channel that is expressed in heat-sensing sensory neurons. When expressed in specific neurons, including larval motoneurons, TRPA1 can be used to induce action potentials upon increasing temperature from 21^{o}C to 27^{o}C (Puver *et al.* 2009; Ataman *et al.* 2008).

Similarly to TRPA1, the light-sensitive channel Channelrhodopsin-2 (ChR2), isolated from green algae, can be used to activate specific neurons, only using blue light instead of heat. Animals that have been fed retinal, and express UAS-ChR2 in larval motoneurons, will induce neuromuscular activity upon exposure to blue light (Ataman *et al.* 2008; Schroll et al 2006). While both have been demonstrated to acutely activate larval motoneurons, neuronal activity is measured in each case through electrophysiological recordings, a technique in which the Davis lab does not have expertise or equipment. As a result, it is challenging to verify that either TRPA1 or ChR2 is actually increasing neuromuscular activity upon exposure to warmth or blue light, an important proof-of-principle. Nevertheless, UAS-TRPA1 was expressed in larval motoneurons and muscle, and activated at 27^{o}C for 4 hours, and *dlg* mRNA levels and distribution were compared to wildtype animals under the conditions. No appreciable difference could be detected between wildtype and animals expressing TRPA1 (not shown). As it is not possible to test if expression of TRPA1 in larval motoneurons is inducing neuromuscular activity within this time frame, it cannot be concluded that *dlg* mRNA levels and distribution do not change in response to increased transmission.

One method by which NMJ activity may be directly assayed in the absence of electrophysiological recordings, may be through live-cell imaging of Ca^{2+}-sensitive fluorophores in the muscle and neuron. Ca^{2+} levels rise in both the neuron and muscle

adjacent to the synapse upon synaptic transmission, and fluorophores that change fluorescence with fluctuations in Ca^{2+} levels, can give an indirect indication of activity. Ca^{2+} indicators can be loaded into cells, directly injected, or genetically encoded (reviewed in Macleod *et al.* 2012). To this end, genetically encoded calcium indicators have been obtained from Troy Littleton (Massachusetts Institute of Technology) and are being tested. Another test for conditional NMJ activation could be to measure uptake of endocytotic dyes such as FM-143 (Verstreken *et al.* 2008). These indicators will confirm in TRPA1, or other methods of conditionally activating the NMJ, are functioning. Conditional activation of the NMJ represents a key target for future research. The experiments described here can shed light on how mRNA responds to rapid changes in NMJ activity free from developmental defects arising from mutation.

vi) Live cell imaging of muscle mRNA

The changeable levels and distribution of mRNA described in this chapter, with variation between muscles in the same animal, hint at a dynamic system for localizing mRNA in muscle. Live imaging of mRNA in muscle would provide insights into the dynamics of this system, and allow the key proteins involved to be studied. The mechanisms that govern muscle mRNA localization could be interrogated by acute inactivation of proteins in muscle, either through injection of inhibiting antibodies and chemicals, or through more recently introduced protein inactivation methods, such as TEV-cleavage and FlAsH-FALI (Marek and Davis. 2002; Pauli *et al.* 2008). To this end, it was attempted to employ the MS2 system to image mRNAs in living muscle. Despite the use of different MS2-RNAs, fluorophores, and Gal4 drivers, it was not possible to robustly reproduce the mRNA distribution observed by mRNA *FISH*. As mRNA *FISH* is considered a 'gold standard' for localization studies, it was concluded that MS2-RNAs did not recapitulate the *in vivo* localization. It is possible that Gal4-driven overexpression, or addition of the RNA sequence containing the MS2 loops, interfered with mRNA localization. Moreover, nuclear

fluorescence from the MCP-GFP or MCP-RFP proteins made imaging the muscle cytoplasm challenging. One key issue with studying mRNA localization in muscle using the MS2 system was the sickness of the stocks created to drive MS2-RNA, and bouton markers, under Gal4-sensitive promoters. Both Muscle line 1 and Muscle line 2 contained three transgenes, and were crossed to fourth to produce larvae in which MS2-RNA was decorated by fluorophores. The burden of expressing four transgenes can be substantial, particularly in muscle, which is a highly genetically sensitive tissue. It is possible that the constitutive expression of transgenes affected the health of the larvae. This may in turn, affect crawling, NMJ activity, and mRNA localization. Another possible hindrance may be that mRNA in muscle is present at low copy number, even when overexpressed. mRNA *FISH* in this study has shown that RNA levels in muscle are low, and require high exposure imaging to detect (see Chapter 2). It is of note that the MS2 system has not been previously published in larval muscle. Indeed, one study mentions that *gluRIIA-MS2* (a different construct to the one presented in this thesis) did not resemble the distribution shown by mRNA *FISH* (Ganesan *et al.* 2012). In another study from a different research group, the MS2 system was used in embryonic muscle with success (van Gemert *et al.* 2009). By contacting the first author, it was discovered that the authors had attempted to reproduce their data in larval muscle, but could not. The authors concluded that by third instar excessive levels of MCP-GFP had accumulated and inhibited mRNA localization (van Gemert *et al.* 2009; personal communication). While it may be possible to balance expression of MCP-GFP and MS2-RNA so that cytoplasmic MS2-RNA signal is detectable, this was not possible within the time scale of this thesis.

In the absence of conditional manipulation of NMJ activity, and live imaging of mRNA, it is challenging to approach the mechanisms by which membrane excitability, signaling, or translation regulate mRNA levels and localization. Nevertheless these data here show that mRNA levels and localization are regulated in response to processes that constitute plasticity at the NMJ. Moreover, using the mRNA *FISH* tools that have been optimized in

this chapter, it was possible to interrogate how Syncrip regulates changes in gene expression in muscle. The hypothesis that Syncrip regulates mRNA localization and translation at the NMJ was tested.

Chapter 6: Overexpression and loss of Syncrip reveals a role in distributing mRNA and a novel distribution of eIF4E protein

Introduction

Thus far, two main observations have been made in this study. First, Syncrip is necessary for proper neuronal structure and function. Second, the localization and levels of muscle mRNAs encoding key synaptic proteins are dynamic, and change with perturbations in processes that constitute synaptic plasticity. In addition, previous data shows that Syncrip is required for mRNA localization and translational control in the oocyte (McDermott *et al.* 2012). Experiments performed on larval body wall preparations reveal that Syncrip associates with a number of transcripts encoding synaptic proteins central to neuronal structure and function, and regulates protein expression independent of changing mRNA levels (McDermott *et al.* in preparation; Hamilton *et al.* in preparation). From the data in the present and previous studies, it is hypothesized that Syncrip regulates expression of synaptic proteins required for plastic growth through mRNA localization and local translation. Optimization of an existing mRNA *FISH* protocol has improved the sensitivity to detect minute mRNP structures, possibly representing single mRNAs. It is these tiny structures that have been shown to populate the muscle cytoplasm, and in some circumstances localize *en masse* to the NMJ, in response to genetic changes in synaptic transmission, cAMP and Ca^{2+} signaling, and translation. To understand the meaning of the changes in mRNA distribution observed thus far, it is important to appreciate previous and current research into mRNA localization in muscle.

i) mRNA localization is poorly understood in muscle

As discussed in Chapter 1, relatively little is known is about mRNA localization in muscle relative to other tissues. A small number of studies however, show that mRNA localization in muscle changes with development, disease, wound regeneration, and synaptic plasticity (reviewed in Chapter 1). This suggests a key role for mRNA localization in these processes. Moreover, these data suggest that translation can be restricted to key subcellular compartments, the perinuclear region, the myoplasm periphery, the postsynapse, and the contractile machinery. Muscle cells are large, and local translation may establish the polar organization of macromolecules necessary to contract with appropriate directionality. Despite these data, there are very few recent studies on mRNA localization in muscle, and fewer still research groups dedicated to this field.

ii) Multiple subcellular regions have been proposed to house local translation in muscle

Local translation has been proposed to occur at a number of sites within muscle. Understandably, most proposed sites follow regions of concentrated mRNA. For example, the postsynapse, contraction machinery, perinuclear region, and myoplasm periphery have all been proposed as sites of local translation and ribosomes have been detected at these locations (Figure 1.3. references within). The best-studied example of the local translation in muscle is the larval postsynapse. As discussed in Chapters 1 and 4, mRNA and ribosomes have been reported to be enriched at the NMJ postsynapse (Sigrist *et al.* 2000). While other reports, including the present study, have found mRNA to be distributed throughout muscle, and not enriched at the post synapse, infrequent mRNA foci are observed at the postsynapse (This study, Currie *et al.* 1995; Karr *et al.* 2008; Ganesan *et al.* 2012). These rare postsynaptic foci could represent an active pool of mRNA that is competent for localized translation at the NMJ. Consistent with this notion, mRNA has been shown to localize to the NMJ with increased NMJ activity in Chapter 4.

Moreover, increased neuromuscular activity, through either crawling or genetic perturbation, has been shown to lead to an accumulation of the translation initiation factor eIF4E and the mRNA-binding protein PABP at the postsynapse (Sigrist *et al.* 2003; Menon *et al.* 2004 and 2009). The binding of eIF4E to the mRNA cap structure is the rate-limiting step in translation initiation under most circumstances and is a pivotal point of control (Duncan *et al.* 1987). Indeed, multiple pathways act to restrict the accessibility of eIF4E to mRNA as a mechanism for global translational regulation. As eIF4E, like mRNA, is largely distributed throughout the muscle cytoplasm under wildtype resting conditions (Ganesan *et al.* 2012; Menon et al 2004; Sigrist et al 2000 and 2003), it can be hypothesized that eIF4E enrichment at the NMJ is a prerequisite for translation. Indeed, multiple pathways act to regulate subceullar-distribution of eIF4E to mRNA as a mechanism for global translational regulation. Of these, the best studied is the sequestration of eIF4E by hypophosphorylated forms of eIF4E Binding Proteins (4E-BPs) (Pause *et al.* 1994; Gingas et al 1998.). Unphosphorylated 4E-BPs binds to eIF4E and prevents its interaction with eIF4G, thereby preventing cap-dependent translation (Haghighat *et al.* 1995). Conversely, phosphorylation of 4E-BPs, under nutrient starvation for example, releases eIF4E for assembly into the translation-competent eIF4F complex. It was previously shown while eIF4E is predominantly cytoplasmic, a sizeable fraction of eIF4E localizes to the nucleus in mammalian cells and yeast (12 – 33%) (Lejbkowicz *et al.* 1992; Land *et al.* 1994; Dostie *et al.* 2000b).

To this end, the mechanism by which Syncrip regulates translation in the muscle was investigated. If Syncrip affects mRNA localization and local translation in muscle, then it would be required for the wildtype distribution of mRNA and eIF4E protein.

The eukaryotic initiation factor 4E (eIF4E) plays a critical role in controlling protein synthesis. The binding of eIF4E to the mRNA cap structure is the rate-limiting step in

translation initiation under most circumstances and is a pivotal point of control (Duncan *et al.* 1987). Indeed, multiple pathways act to restrict the accessibility of eIF4E to mRNA as a mechanism for global translational regulation. Of these, the best studied is the sequestration of eIF4E by hypophosphorylated forms of eIF4E Binding Proteins (4E-BPs) (Pause *et al.* 1994; Gingas et al 1998.). Unphosphorylated 4E-BPs binds to eIF4E and prevents its interaction with eIF4G, thereby preventing cap-dependent translation (Haghighat *et al.* 1995). Conversely, phosphorylation of 4E-BPs, under nutrient starvation for example, releases eIF4E for assembly into the translation-competent eIF4F complex. It was previously shown while eIF4E is predominantly cytoplasmic, a sizeable fraction of eIF4E localizes to the nucleus in mammalian cells and yeast (12 – 33%) (Lejbkowicz *et al.* 1992; Land *et al.* 1994; Dostie *et al.* 2000b).

Aims of this Chapter

- **To test if Syncrip is necessary for the distribution of Syncrip-target mRNA in muscle.**
- **To test if Syncrip regulates the localization of eIF4E protein.**
- **To test if loss of Syncrip affects the distribution and shape of muscle nuclei.**

Results

i) Syncrip is necessary for proper mRNA localization in muscle

To test the hypothesis that Syncrip regulates mRNA localization in muscle, mRNA *FISH* was performed on *Syp* loss-of-function mutants. First, the distribution of *dlg* mRNA was tested. In the absence of Syncrip, *dlg* mRNA formed fewer brighter foci in the cytoplasm in all cases (Figure 6.1). In some mutant muscles, only a handful of large foci could be

detected. No increase in proximity to the NMJ was detected. To quantitatively assess the distribution of *dlg* mRNA, the intensity profile of a 30μmx30μm region was generated from *Syp* mutant and control muscles. To ensure experimental homogeneity, the region of interest was centred on the foci with the greatest intensity in each image (Figure 6.2). As shown, *Syp* mutant muscles exhibit fewer foci with a greater mRNA content relative to wildtype. As previous work has shown that *dlg* mRNA levels are unaltered in *Syp* mutants, it appears that *Syp* is required for proper distribution of *dlg* mRNA (McDermott *et al.* in preparation). In the absence of Syncrip, *dlg* mRNA traffic appears to accumulate in fewer (<10) foci. It is a possibility that these foci represent a functionally specialized species of mRNP (See Discussion). It is interesting to note that the genomic rescue muscle exhibits a wildtype distribution of mRNA foci, but still contains foci with elevated mRNA content. There are a number of potential explanations for this observation (See Discussion).

To test if Syncrip was required for wildtype distribution of other Syncrip-target mRNAs, mRNA *FISH* was performed in *Syp* mutants using *gluRIIA* antisense riboprobes. In contrast to *dlg* mRNA, loss of Syncrip did not change the distribution of *gluRIIA* mRNA (Figure 6.3). *Pumilio* mutants, like *Syncrip* mutants, exhibit elevated levels of GluRIIA protein (Menon *et al.* 2004; this study, Figure 3.6). As discussed, GluRIIA has been proposed to be translated at the NMJ postsynapse. Further, as previously shown, eIF4E mRNA is enriched at the NMJ in loss-of-function *Pumilio* mutants, which is proposed to precede local translation of GluRIIA at the NMJ. To test if loss of Syncrip lead to an accumulation of *eIF4E* mRNA at the NMJ, mRNA *FISH* was performed on *Syp* mutants (Figure 6.4). As with *gluRIIA* mRNA, loss of Syncrip did not affect the distribution of *eIF4E* mRNA. Despite evidence showing that Syncrip is present in particles containing *dlg, gluRIIA,* and *eIF4E,* Syncrip is required for localization for *dlg* mRNA only (McDermott *et al.* PhD Thesis 2009; McDermott *et al.* 2012). RNA-immunoprecipitation of native RNP complexes from larval lysate, followed by deep sequencing, has shown that *dlg* is enriched to a greater extent than *gluRIIA* and *eIF4E* mRNA (McDermott *et al.* in

preparation; Hamilton *et al.* in preparation). The close association of Syncrip and *dlg* mRNA is consistent with Syncrip being required for *dlg* distribution, but not for *gluRIIA* and *eIF4E* mRNA.

Figure 6.1. Syncrip is necessary for *dlg* mRNA localization in muscle

Figure 6.2. Loss of Syncrip changes the distribution of *dlg* mRNA from multiple small foci, to fewer foci with greater mRNA content

Figure 6.3. Loss of Syncrip does not affect *gluRIIA* mRNA distribution

Figure 6.4. Loss of Syncrip does not affect *eIF4E* mRNA distribution

During analysis of mRNA *FISH* data it was noticed that in some instances the genomic rescue line exhibited enrichment of mRNA at the NMJ postsynapse. A striking localization of *dlg, gluRIIA*, and *eIF4E* mRNA was observed at approximately 1/15 NMJs tested (Figure 6.5). While rare, these observations were reproducible. The localization of mRNA to the NMJ was never observed in wildtype or *Syp* mutant muscle. It is surprising that the genomic rescue exhibits a phenotype distinct to both mutant and wildtype muscle. There are two potential explanations for this. First, it is possible that the two Syncrip isoforms not expressed in the genomic rescue, A and H, may be required for wildtype mRNA distribution (See Discussion, Suzanne McDermott; McDermott *et al.* in preparation). This is unlikely however, as Syncrip isoforms A and H have not been detected in the third instar larva. Instead, it is possible that despite employing the endogenous promoter to drive expression of Syp in the genomic rescue line, Syp is inappropriately expressed. As the localization of mRNA to the NMJ is not observed in *Syp* loss of function mutants, the phenotype is unlikely to arise from under expression of Syncrip. As a result, it was hypothesized that overexpression of Syncrip may cause mRNA localization to the NMJ.

Figure 6.5. The Syncrip genomic rescue line exhibits *dlg*, *eIF4E* and *gluRIIA* mRNA localized in the NMJ trunk

To test if overexpression of Syncrip results in mRNA localization to the NMJ, mRNA *FISH* was performed in larvae in which expression Syp-GFP was driven by the muscle-specific driver Mhc-Gal4. Analysis of *dlg* mRNA in multiple muscles revealed that overexpression of Syp-GFP did not lead to enrichment of mRNA at the NMJ (Figure 6.6). In contrast, *dlg* mRNA levels appeared reduced, but with a wildtype distribution, both around the NMJ and throughout the muscle. Analysis of *gluRIIA* mRNA in muscle however revealed a striking increase in mRNA localization to the NMJ upon overexpression of Syp-GFP (Figure 6.7). Every muscle examined exhibited at least a weak enrichment of *gluRIIA* mRNA at the NMJ (yellow arrow). In some cases, strong enrichments of *gluRIIA* mRNA at the NMJ (arrowheads) were accompanied by a strong signal between nuclei (white arrows). The *gluRIIA* mRNA enriched at the NMJ, and parallel to muscle 6 nuclei, was distributed evenly in a haze-like distribution rather than in larger cytoplasmic foci observed in wildtype. This distribution of mRNA, likely representing smaller mRNP complexes, is similar to that observed in mutants with elevated NMJ signaling (Chapter 5). No change in eIF4E mRNA distribution was detected upon overexpression of Syp-GFP (not shown). These data correlate Syncrip expression levels with mRNA localization. The relationship between Syncrip and mRNA localization is complex however, and differs between transcripts. It is surprising that Syncrip binds multiple mRNAs encoding key synaptic proteins, but yet changes in Syncrip expression affect the localization of each to a different extent (See below and Discussion).

Figure 6.6. Overexpression of Syncrip-GFP in muscle leads to a change in the level, but not distribution, of *dlg* mRNA

Figure 6.7. Overexpression of Syncrip-GFP in muscle leads to accumulation of *gluRIIA* mRNA at the NMJ

ii) Overexpression of Syncrip in neurons affects mRNA localization

As overexpression of Syncrip in muscle lead to dramatic enrichments of mRNA at the postsynapse, it was hypothesized that overexpression of Syncrip in neurons may lead to an increase in mRNA localization in the bouton. To this end, mRNA *FISH* was performed with *dlg* antisense probes in animals overexpressing Syp-GFP in motoneurons. Overexpression of Syp-GFP in neurons produced a varied distribution of *dlg* mRNA localization (Figure 6.8). In most NMJs no presynaptic mRNA could be detected, while in 2/12 NMJs (across 3 animals per repeat) bold presynaptic signal could be detected. Contrast adjustment revealed a filament-like distribution of mRNA within a bouton (Figure 6.8, D and E, arrow). Unfortunately the mRNA *FISH* procedure quenches endogenous fluorescence, so while the neurons are expressing Syp-GFP, none can be visualized. Thus, it was not possible to assess whether these presynaptic mRNA structures contain Syncrip. While this result was reproducible across repeat experiments, time restrictions prevented further testing of the mRNA and protein composition of these presynaptic bodies.

Detection of endogenously-expressed mRNA in the bouton was a key prerogative of this study. Despite employing a range of different techniques, no presynaptic mRNA could be detected. This indicated that either the mRNA was not present, present at levels below the detection threshold, or that the mRNA *FISH* method was failing to hybridize to presynaptic mRNA. Moreover, genetic perturbations expected to concentrate mRNA in motoneurons failed to reveal presynaptic mRNA. The visualization of presynaptic *dlg* mRNA in larvae overexpressing Syp-GFP in motoneurons validates mRNA *FISH* as a method for detecting presynaptic mRNA. Moreover, these data show that mRNA is present in the third instar larval NMJ, *albeit* in non-wildtype animals. This an important step for future research into this field. As with previous observations in muscle, these data tie the expression levels of

Syncrip with mRNA localization. Deeper interpretation of this observation is inhibited by a number of issues however. First, the loss-of-function *Syp* mutants do not exhibit any change in presynaptic mRNA relative to wildtype; no mRNA can be detected in either case. It would be expected that a phenotype resulting from Syncrip overexpression would oppose that from *Syp* mutants. This is the case for neuronal structural phenotype, where loss of Syncrip leads to an increase in NMJ growth, while overexpression in muscle leads to undergrowth. It is challenging to interpret a neuronal mRNA localization phenotype resulting from Syp overexpression, when an opposing phenotype is not detected in *Syp* mutants. Second, the overexpression of a protein can lead to a number of artifacts. The role of Syncrip when expressed at higher levels may not reflect its wildtype function. That said, it should be noted that western blots performed on larvae overexpressing Syp-GFP in neurons (with the same genotype as those tested here) exhibit a very slight increase in Syncrip levels. Nevertheless, it is impossible to assess if overexpression affects Syncrip function in neurons from these experiments.

Figure 6.8. Overexpression of Syncrip-GFP in motoneurons changes the localization of *dlg* mRNA in motoneurons but not muscle

These data presented here correlate the loss and overexpression of Syncrip with a number of different distributions of mRNA. Rather than shedding light on how Syncrip may regulate translation in muscle however, these observations pose a number of complexities that demand attention (See discussion). While it is safe to conclude that changes in the levels of Syncrip correlate with changes in the distribution of *dlg* and *gluRIIA* mRNA and the levels of their respective proteins, how these two events are linked remains elusive. If localization of mRNA to the NMJ were a prerequisite for translation of *dlg* and *gluRIIA* then mRNA would be expected to be NMJ-localized in the third instar larvae, to facilitate the growing new boutons and synapses. Instead the loss and overexpression of Syncrip is correlated with opposing localization phenotypes for two Syncrip-target mRNAs. As a result, it is worth considering that Syncrip may correlate with, but not cause, changes in mRNA localization. Instead, Syncrip may regulate translation of Dlg and other transcripts through a different mechanism. To this end, the role Syncrip plays in regulating translation, rather than mRNA localization became a focus.

iii) Overexpression and Loss of Syncrip reveals a novel distribution of eIF4E protein in muscle

As discussed previously, the localization of eIF4E protein has been used in other studies as a proxy for translation sites. For example, in *Pumilio* mutants eiF4E bodies accumulate at the NMJ, indicating upregulated translation at the postsynapse (Menon et al 2004). To test the anti-eIF4E antibody, eIF4E immunostaining was performed in *Pumilio* mutants to reproduce this observation. Inspection of eIF4E distribution revealed a distribution throughout the muscle, with a slight enrichment at the NMJ in wildtype animals (Figure 6.9, arrows). In *Pumilio* loss-of-function mutants accumulation of eIF4E protein at the NMJ was robust and dramatic (Figure 6.9, arrowhead). These data are consistent with previous studies and indicate that eIF4E immunostaining is reproducible (Sigrist *et al.* 2003; Menon *et al.* 2004; Ganesan *et al.* 2012).

Figure 6.9. Loss of Pumilio leads to accumulation of eIF4E protein at the NMJ

Loss of Syncrip leads to a redistribution of *dlg* mRNA to fewer foci, while overexpression of Syncrip leads to an increase of *gluRIIA* mRNA at the NMJ. If these redistributions of mRNA represent increased translation at specific subcellular locations, eIF4E protein would be expected to label translation sites. To this end, the distribution of eIF4E was tested in animals with both reduced and increased levels of Syncrip. First, to test if loss of Syncrip leads to a change in eIF4E protein, eIF4E immunostaining was performed in *Syp* loss-of-function mutants. Surprisingly, loss of Syncrip correlated with a nuclear enrichment of eIF4E protein. The distribution of cytoplasmic eIF4E appeared unchanged in the absence of Syncrip, though there appeared to be less eIF4E in the cytoplasm, particularly around the nuclei (Figure 6.10). Approximately half of muscles examined showed a wildtype eIF4E distribution, while half exhibited a clear accumulation of eIF4E in every nucleus of both muscle 6 and 7. 1/21 muscles tested revealed strong accumulation in some muscle nuclei, but not others.

Close inspection of the eIF4E distribution in *Syp* mutants revealed that the eIF4E protein accumulated in a region of the nuclei labeled least by DAPI, likely correlating with the nucleolus. The genomic rescue line exhibited a similarly varied distribution of eIF4E protein as the loss-of-function *Syp* mutants. There is a precedent for RBPs functioning in the nucleolus, and in particular those involved in pre-mRNA splicing, though no study could be found that reports nucleolar eIF4E (See discussion). To further investigate this observation, the distribution of eIF4E protein was tested in animals overexpressing Syp-GFP in muscle. While, no enrichment of eIF4E was observed at the NMJ, inspection of nuclei revealed a striking pattern of eIF4E around the perinuclear region (Figure 6.11). eIF4E protein is held in filamentous structures surrounding the nucleus, with less protein in the cytoplasm relative to the wildtype control. It is interesting that in muscle lacking Syncrip, eIF4E is localized to the nucleolus, while in muscles overexpressing Syncrip, eIF4E is restricted to the perinuclear region. This implies that Syncrip has a role, either

directly or indirectly, in regulating eIF4E localization (Figure 6.12 A-C). To test the frequency of nucleolar eIF4E accumulation, the number of nuclei containing eIF4E was quantified (Figure 6.12 D). While no nucleolar eIF4E was detected in wildtype muscle, both *Syp* mutants and the genomic rescue line exhibited significant enrichment in nucleolar eIF4E. Syncrip is known to be present in mRNPs with multiple different mRNAs in both larvae and egg chambers, most likely through direct binding (McDermott *et al.* 2012). How the loss of Syncrip leads to a nuclear accumulation of eIF4E is unknown, though a number of possibilities exist (See Discussion). The nucleus is known to have a number of roles in regulating gene expression independent of transcription and mRNA export. For example, sequestration of proteins to the nucleolus is an observed mechanism of regulating protein expression, and the perinucleus is a prominent region of local translation in muscle cells, particularly in development (See Discussion; Chapter 7; reviewed in Sirri *et al.* 2008 and Emmott *et al.* 2009; Lawrence and Singer 1988). Owing to the localization of eIF4E to the nuclei upon changes in Syncrip levels, attention was focused on the nuclei in *Syp* mutant muscles.

Figure 6.10. Loss of Syncrip leads to accumulation of eIF4E protein in the nucleus

Figure 6.11. Overexpression of Syncrip-GFP in muscle leads to an accumulation of eIF4E protein around the outside of the nuclei in a complex fibrous-like structure

Figure 6.12. Overexpression and loss of Syncrip reveal novel eIF4E distributions

iv) Loss of Syncrip leads to changes in nuclei shape and distribution

Close inspection of the nuclei revealed that in *Syp* loss-of-function mutants nuclei in both muscle 6 and 7 exhibited abnormal shape and distribution. To investigate this phenotype, nuclei with abnormal shape were quantified in muscles 6 and 7 (per hemisegment) in *Syp* mutant animals, and animals overexpressing Syncrip (Figure 6.13). Quantitative assessment revealed that animals lacking Syncrip, but not wildtype or genomic rescue controls, exhibit abnormally shaped nuclei. There was no detectable correlation between eIF4E content and nucleus shape. Nucleolar eIF4E enrichment was observed in nuclei with abnormal and wildtype shapes with apparent equal frequency. Animals in which Syp-GFP was overexpressed in muscle or neuron showed wildtype nuclear shapes. To quantitatively assess the abnormal distribution of nuclei in *Syp* mutants, the clustering of nuclei was counted in muscles 6 and 7. In wildtype the nuclei of muscles 6 and 7 are evenly and linearly spread throughout the muscle in columns. Wildtype muscle nuclei never touch one another and are typically separated by ~10-30μm. Each touching interface between nuclei was counted in *Syp* mutants. For example, in Figure 6.14, panel A (wt^{OR}) shows no touching nuclei, whereas panel B ($Syp^{e286/e286}$) shows five touching interfaces shared between nuclei, often resulting from clusters of multiple nuclei. While no touching nuclei were observed in wildtype muscles, *Syp* mutants and the genomic rescue control exhibited multiple clusters of nuclei (Figure 6.14). Intriguingly, nuclei with abnormal shape, and positioning within a muscle, have been correlated with muscular dystrophies in humans. While Syncrip is a known potential therapeutic target for spinal muscular atrophy, it is through interaction with SMN in motoneurons that Syncrip is thought to contribute to SMA, rather than through activity in muscle. The data presented here however correlates loss of Syncrip with direct effects in the muscle (See discussion).

Figure 6.13. Loss of Syncrip leads to abnormally shaped nuclei

Figure 6.14. Nuclei positioning defect in *Syp* mutants and *Syp* genomic rescue

Discussion

i) Syncrip is necessary for wildtype mRNA distribution in muscle

In the absence of Syncrip, mRNA traffic appears to accumulate in fewer foci (5-10) relative to wildtype controls (>100). It is a possibility that these foci represent a functionally specialized species of mRNP complex. Loss of Syncrip increased Dlg levels, and redistributes the mRNA to particular foci (McDermott *et al.* in preparation; this study). The life cycle of a mRNA is known to involve multiple remodeling steps while changing association with a host of different RBPs. Trafficking mRNA between different mRNP complexes containing different mRNPs could facilitate this in muscle. The translational competence, stability, etc, of a given mRNA could be defined by the foci, or mRNP body, in which it is held. Indeed, there is a precedent for this form of mRNA regulation in the oocyte, in which Syncrip regulates mRNA localization (McDermott *et al.* 2012). Under wildtype conditions, the Syncrip-target mRNA *gurken*, is transported from the posterior, to the dorso-anterior corner in the stage 8 oocyte. In the dorso-anterior corner, *grk* mRNA is anchored in mRNP structures, named sponge bodies, which contribute to regulating Gurken translation (Delanoue *et al.* 2007; Weil and Parton *et al.* in preparation). In the absence of Syncrip, *grk* mRNA is not solely restricted to the sponge bodies in the dorso-anterior. This misdistribution of *grk* mRNA correlates with ectopic expression of Gurken protein throughout the oocyte (McDermott *et al.* 2012). The wildtype distribution of mRNA in foci of varying size and distribution reported here for *dlg, shaker, fasII,* and *eIF4E* mRNA is consistent with previous data (Currie *et al.* 1994; Karr *et al.* 2012; Ganesan *et al.* 2012). While the distribution of mRNAs encoding different components of glutamate receptors changes with development, nothing is known about the role or composition of the foci that house muscle mRNA. It has been shown here that the distribution of mRNA in muscle cells is responsive to changes in synaptic transmission, secondary messenger

signaling, and translation, all of which contribute to synaptic plasticity. Further, in the absence of Syncrip, mRNA appears funneled into a few mRNP foci. While interesting, interpreting the redistribution of mRNA in these examples lacks meaning unless the compositions of the different mRNP foci are known. If different mRNP foci are functionally distinct, then it would be expected that their composition would differ. For future work, mRNA *FISH* should be performed with immunofluorescent staining to assess the composition of mRNP foci. Preliminary work has begun with a focus on RBPs known to associate with Syncrip-mRNAs in the oocyte, such as Me31B.

ii) The Syp genomic rescue line does not recapitulate wildtype mRNA localization

The *Syp* genomic rescue line rescues the structural and electrophysiological defects observed in *Syp* mutants, but does not fully rescue the changes observed in mRNA localization in *Syp* mutants. Furthermore, specific phenotypes have been observed in the *Syp* genomic rescue line that are not present in animals lacking, or overexpressing Syncrip. As a result, it may be possible that the *Syp* genomic rescue line contains a mutation, either in the transgenic *Syp* sequence or elsewhere, that may contribute to these phenotypes. Work is currently underway to 'clean up' this stock and to sequence the Syncrip insertion to search for mutation.

iii) Overexpression and Loss of Syncrip leads to opposing localization patterns for dlg and gluRIIA mRNAs

mRNA *FISH* in animals overexpressing Syp-GFP revealed that overexpression of Syp was sufficient to cause localization of *gluRIIA* to the NMJ, but not *dlg* or *eIF4E* mRNA. It is surprising that while loss of Syncrip affected the distribution of *dlg,* but not *gluRIIA* mRNA, the opposite is true for the overexpression of Syp-GFP. This could imply that Syncrip has different roles with different transcripts, resulting in different localization patterns. This is

consistent with Syncrip's role in the oocyte, where Syncrip is required for appropriate localization of two transcripts, gurken and oskar, that have distinct distributions.

Rather than shedding light on how Syncrip may regulate translation in muscle, these observations pose a number of complexities that demand attention. For example, overexpression of Syp-GFP and loss of function *syp* mutants both exhibit similar distributions of *dlg* mRNA, i.e. fewer foci in the muscle relative to wildtype. In the oocyte too, McDermott *et al.* have noted that both the overexpression and loss of Syncrip result in similar *grk* mRNA localization phenotypes. It was proposed that a certain stoichiometry is necessary in the mRNP complexes in which Syncrip functions (McDermott et al 2012). As a result, both overexpression, and loss, of Syncrip may inhibit proper mRNA localization and translation. Though this proposal is possible, it is challenging to interrogate further. In addition, a key observation is that the changes in *dlg* mRNA localization observed in animals overexpressing or lacking Syp do not correlate with any change in protein distribution. While Syncrip is necessary for repressing Dlg expression, and changes in Syncrip levels correlate with changes in *dlg* mRNA distribution, the localization and levels of Dlg protein are independent. As a result, it is worth considering that Syncrip may correlate with, but not cause, changes in mRNA localization. Instead, Syncrip may regulate translation of *dlg* and other transcripts through a different mechanism.

iv) Overexpression and loss of Syncrip reveals a novel distribution of eIF4E protein

The eukaryotic initiation factor 4E (eIF4E) plays a critical role in controlling protein synthesis. The binding of eIF4E to the mRNA cap structure is the rate-limiting step in translation initiation under most circumstances and is a pivotal point of control (Duncan *et al.* 1987). Indeed, multiple pathways act to restrict the accessibility of eIF4E to mRNA as a mechanism for global translational regulation. Of these, the best studied is the

sequestration of eIF4E by hypophosphorylated forms of eIF4E Binding Proteins (4E-BPs) (Pause *et al.* 1994; Gingas et al 1998.). Unphosphorylated 4E-BPs binds to eIF4E and prevents its interaction with eIF4G, thereby preventing cap-dependent translation (Haghighat *et al.* 1995). Conversely, phosphorylation of 4E-BPs, under nutrient starvation for example, releases eIF4E for assembly into the translation-competent eIF4F complex. It was previously shown while eIF4E is predominantly cytoplasmic, a sizeable fraction of eIF4E localizes to the nucleus in mammalian cells and yeast (12 – 33%) (Lejbkowicz *et al.* 1992; Land *et al.* 1994; Dostie *et al.* 2000b). This led to a number of speculations into the role of nuclear eIF4E. One popular proposal is that eIF4E contributes to mRNA splicing and nuclear export. Indeed, eIF4E enhances mRNA export and colocalizes with some splicing factors in cultured human cells (Hamm and Mattaj 1990; Dostie *et al.* 2000; Rousseau *et al.* 1996; Topisirovic, *et al.* 2003). However, no study could be found that reports the localization of eIF4E to the nucleolus.

In the absence of Syncrip, eIF4E appears to be enriched in the nucleolus, which is thought to be devoid of splicing machinery or polymerase II transcripts (though see Bubulya *et al.* 2004 for evidence that splicing factors collect around the nucleolus). Also, while eIF4E is housed in foci in mammalian nuclei, representing functional mRNPs, in *Syp* mutants eIF4E appears to occupy the whole nucleolus uniformly. It is tempting to speculate that rather than possessing nucleolar activity, eIF4E is instead sequestered in the nucleolus, to limit cytoplasmic translation. Nucleolus sequestration is an observed phenomenon for regulating protein activity in the cytoplasm and has been shown to be essential in fundamental processes such as cell cycle progression and DNA damage response (Shou *et al.* 1999; Wong *et al.* 2002; Bernadi *et al.* 2004; reviewed in Sirri *et al.* 2008 and Emmott *et al.* 2009). Intriguingly, the data in this study is not the first evidence to tie Syncrip in with processes restricted to the nucleolus. One interesting observation has been that Syncrip is involved in mouse Hepatitis Virus RNA synthesis (Choi *et al.* 2004). In cultured mouse cells, the synthesis of the viral genome proceeded more slowly

in the absence of Syncrip. Intriguingly, localization to the nucleolus is a common feature of coronavirus proteins, including mouse Hepatitis Virus, and a whole host of human viruses. It is fascinating to consider that nucleolar sequestration of proteins by Syncrip may be an integral part of a viral lifecycle. With further research, Syncrip could become a potential therapeutic target for viral infection.

While robust and reproducible, the observed sensitivity of eIF4E localization to Syncrip levels is simply too preliminary to make conclusions from. Nevertheless, the data presented here tentatively suggest that Syncrip could play a role in linking translational regulation and eIF4E localization. Coupled with the fact that the *Drosophila* larva is free from many artefacts that complicate analysis of cultured cells, the powerful genetic and experimental tools available to the *Drosophila* researcher may allow precise dissection of the mechanism by which eIF4E and Syncrip interact. A tentative hypothesis is that when the levels of Syncrip are perturbed in muscle, so is the level of translation of Syncrip-target mRNAs. As a result, the sequestration, or mobilization of eIF4E to the perinucleus, could act to maintain muscle homeostasis by reducing or increasing translation respectively (See Chapter 6). Before a firm hypothesis can be formed however, more needs to be known about the mechanisms that link Syncrip and eIF4E to this novel localization pathway.

v) Loss of Syncrip correlates with abnormal nuclei morphology and positioning

Loss of Syncrip correlated with abnormal nuclei shape and positioning. The genomic rescue line rescued the nuclei shape to that of wildtype, but not the distribution. It is not clear why this is the case, though throughout this study some phenotypes observed in *Syp* mutants have been rescued to wildtype, while others have not (See Chapter 7). It is interesting to note that changes in nuclei shape have been long correlated with various muscle diseases. Cultured muscle cells that exhibit one such myopathy, Emery-Drifuss

muscular dystrophy, have been shown to have abnormal nuclear shape and impaired mechanical properties (Lammerding *et al.* 2005). Despite the close association between nuclei shape and certain myopathies, including those caused by mutations in lamins, little is known about how, or if, changes in nuclear shape can cause disease. Interestingly however, recent studies have started to provide insight into how nuclear shape in muscle can reflect function. First, a recent study ties laminopathies with mRNA export into the cytoplasm (Speese *et al.* 2012). Changes in nuclear shape, which are regulated by lamins, are shown to be necessary for the export of large mRNP complexes containing synaptic mRNAs. In addition, another recent study indicates that the changes in gene expression that cause muscular atrophy correlate with conditional changes in nuclear structure. A screen revealed that a DNA-binding protein known to be involved in DNA-damage responses, named Gadd45a, was central to muscular atrophy in mice (Ebert et al 2012). Gadd45a was both necessary and sufficient for muscle atrophy induced by immobilization, fasting, and deneveration. Moreover, overexpression of Gadd45a lead to changes in nuclear shape, which correlated with nuclear reprogramming and changes in mRNA transcription. Gadd45 is conserved in *Drosophila,* and changes in Gadd45 expression levels in the germline affect the dorsal-ventral axes specification of the egg chamber, comparable to that of *Syp* mutants (Peretz *et al.* 2007). Moreover, overexpression of Gadd45 leads to the same mislocalization of *grk* mRNA as overexpression of Syncrip (Peretz *et al.* 2007). It is a fascinating possibility that Syncrip and Gadd45 are functionally linked. Inspection of the list of mRNAs that precipitate with Syncrip however does not reveal *gadd45* mRNA (*CG11086*). It is interesting to consider that mutants in *Syp* and *Gadd45*, may both in part exert mislocalization of *grk* mRNA through changes in nuclear, rather than cytoplasmic, mRNA metabolism. How the loss of Syncrip causes changes in nuclear shape is unclear. Moreover, how the change in nuclear shape may contribute to muscle function in larvae is unknown. The progress made in the literature however indicates that Syncrip could well be required for proper muscle function. Further work is underway to examine this.

In addition to nuclear shape, nuclear positioning is also central to muscle health, and defects in nuclei positioning are known to correlate with myopathies. Indeed, nuclear positioning is a dynamic process that is central to many cellular processes, from axes specification and development, to wound healing and cell migration (Reviewed in Dupin *et al.* 2011; but see Zhao *et al.* 2012 for novel mechanistic insights). In muscle, nuclear position is particularly important, as evidenced by a number of major studies. First, the discovery of the centronuclear myopathies (CNMs) has linked nuclear positioning to muscle disease. Congenital CNMs is an X-linked myopathy that affects approximately 1/50,000 males, most of which will die in infancy from difficulty with breathing. The identifying characteristic of CNMs is the abnormal distribution of nuclei in muscle fibres (Spiro *et al.* 1966; Sher *et al.* 1967; Sher *et al.* 1967; Reviewed in Romero *et al.* 2010). While 80% of CNMs have been genetically correlated with mutations in one gene, *MTM1*, encoding a putative conserved tyrosine phosphatase (Laporte *et al.* 1996), little is known about how MTM1 contributes to poorly positioned nuclei. Nevertheless, other studies have confirmed the importance of nuclear position in muscle function. As discussed in Chapter 1, the proper positioning of nuclei proximal to the mammalian neuromuscular junction is necessary for restricting protein expression at the synapse (Apel *et al.* 2000). In addition, a recent study has confirmed that nuclear positioning is central to skeletal muscle function in cultures mammalian muscle with supporting evidence in *Drosophila* larvae (Metzger *et al.* 2012). The authors identify Ensconsin and Kinesin Heavy Chain as necessary for nuclear positioning in muscle (neither mRNA associates with Syncrip). Moreover, animals with mislocalized nuclei were shown to crawl at a slower rate relative to wildtype. To investigate if this is the case in Syncrip, open access software for the tracking of larval movement has been obtained (Marin *et al.* 2012). To investigate this properly however, it is necessary to generate a line in which the positioning of muscle nuclei is rescued in a *Syp* mutant background. To this end, steps are being taken to construct a 'muscle-rescue' line for Syncrip.

Previous studies have provided a number of genes that are frequently mutated in myopathies, including those resulting in abnormally shaped or distributed nuclei. To investigate if Syncrip may regulate the expression of these genes to produce the phenotype observed here, the most statistically significant genes associated with myopathies with abnormal nuclei shape were taken and their *Drosophila* homologues were catalogued. Each homologue was then searched for in the list of mRNAs shown to associate with Syncrip by immunoprecipitation followed by QRT-PCR, previously performed by Suzanne McDermott and analyzed by Russell Hamilton (McDermott *et al.* in preparation; Hamilton *et al.* in preparation). In no case however did Syncrip associate with a *Drosophila* myopathy homologue mRNA (Table 3.4). As a result it is unlikely that loss of Syncrip causes changes in nuclei shape and distribution through direct regulation of myopathy genes. Nevertheless, though preliminary, the data presented here, taken together with previous studies showing a link between muscle nuclei shape and distribution with muscle function, offer a tempting hypothesis that the loss of Syncrip leads to myopathy in larvae. It is of note that adult flies deficient in Syncrip fail to eclose and are trapped as pharate adults, a phenotype commonly observed in mutants with aberrant muscle function. Further investigation of this possibility may show Syncrip to be a novel gene myopathy, and if related to nucleolus-sequestration of eIF4E, a novel mechanism of this disease also.

Chapter 7: Discussion and Future Perspectives

Discussion and Future Perspectives

This study has provided interesting insights into mRNA localization and the mechanism of translation regulation in muscle. While these data are too preliminary to build a complete picture of the role of Syncrip in mRNA localization and translation at the NMJ, this study provides novel observations and improved techniques that will further future research into this area.

Aims of this Chapter:

- **To briefly discuss the general conclusions of this study**
- **To approach the limitations of these conclusions**
- **To propose future experiments to answer key remaining questions**

General conclusions:

i) Syncrip is necessary for NMJ structure and function.

It has been shown here that Syncrip is required for the proper morphology of the larval NMJ. Moreover, the loss of Syncrip causes a decrease in the vesicle release probability in the neuron, which correlates with a decrease in the levels of the key vesicle release component Synaptotagmin1. As discussed in Chapter 3, other mRNA binding proteins with diverse functions have been known to exert control over both the shape and efficacy of the NMJ (Tables 3.1 and 3.2). Syncrip is known to be involved in multiple aspects of mRNA metabolism as well binding synaptotagmins, and has been hypothesized to be involved in local translation in the nervous system (discussed in Chapter 1 and 4). Using the data from this study, and the highly tractable NMJ as a model system, it may be

possible to examine exactly how Syncrip acts to regulate neuronal structure and function (see remaining problems and future experiments below).

ii) mRNAs encoding synaptic proteins are distributed throughout muscle, and rarely at synapses.

As discussed in Chapters 1 and 5, it is well accepted that local translation occurs at the NMJ postsynapse. It is surprising then, to find that mRNA encoding the key synaptic proteins Dlg, FasII, GluRIIA, and Shaker are distributed throughout muscle, and rarely present at boutons, rather than localized to boutons as previously reported (*gluRIIA,* Sigrist *et al.* 2000). While no explanation for the discrepancy between this study and Sigrist *et al.* 2000 is given here, it is important to consider that mRNA localization may be a highly dynamic process in muscle, and that differences in animal rearing and activity may produce distinct localization patterns (discussed in Chapter 5).

iii) mRNA localization in muscle responds to changes in synaptic plasticity, including changes in Syncrip expression.

This study shows that the localization of mRNA encoding postsynaptic proteins changes with a range of perturbations to plasticity. These observations lead to a few tentative conclusions on mRNA localization in muscle (Summarized in Figure 7.1):

1. In wildtype muscle, mRNAs encoding a number of key synaptic proteins are distributed throughout the muscle and housed in foci of varying size.

2. mRNA localization is sensitive to perturbations in synaptic plasticity. In particular, mRNA appears to be released from foci upon elevated synaptic transmission and signaling, to form a ‘haze’ of small particles.

3. In some mutants, particularly those with perturbations in translational regulation, the haze-like distribution of mRNA can localize to the NMJ postsynapse.

4. Different transcripts can exhibit distinct localization patterns in the same genetic background.

5. Localization of mRNA to the NMJ can correlate with increased expression of the cognate protein, however, localization of mRNA to the synapse appears to not be sufficient for increased translation.

The functional relevance of these distributions is not fully understood. Crucially, the composition of different mRNA foci must be elucidated. The accumulation of mRNA into one type of foci, or the release of mRNA from another, may be interpreted with more meaning if the functional nature of these foci is better understood. While it is challenging to make more detailed conclusions, the data presented here make an important first step in understanding how mRNA localization may contribute to synaptic plasticity in muscle.

iv) Overexpression and loss of Syncrip reveals eIF4E distributed between the nucleolus and perinuclear region.

As discussed in Chapter 6, the eukaryotic initiation factor 4E (eIF4E) plays a critical role in controlling protein synthesis and is found in the nucleus and cytoplasm. Here it appears that the loss of Syncrip may correlate with localization of eIF4E to the nucleolus. While it is tempting to speculate that Syncrip could play a role in linking translational regulation and eIF4E localization (discussed in Chapter 6), a clear limitation of this data set, however, is that the genomic rescue line does not rescue the eIF4E localization phenotype. Genetic experiments to isolate the cause of this phenotype are necessary before these data can be fully interpreted (see remaining problems and future experiments below).

Remaining problems and future experiments:

While a number of conclusions can be made from the data presented here, new questions emerge that must be answered to understand how Syncrip regulates NMJ structure and function. These unanswered questions and problems limit the conclusions of this thesis. The most salient of these issues, and the specific experiments that will be performed to approach them, are discussed here.

i) The tissue-specific roles of Syncrip remain unknown

While it is clear that Syncrip is necessary for neuron structure and function, how exactly Syncrip contributes to these processes remains unclear. One obstacle to dissecting the cause of these phenotypes is that Syncrip likely plays a role in both neuron and muscle to regulate NMJ structure and function (Discussed in Chapters 3 and 5).

To this end, neuron- and muscle-specific rescue lines are in construction. Tissue-specific rescue lines require the Gal4-driven expression of the Syncrip protein in a loss-of-function mutant. These are commonplace tools in the field and have been used with success in elucidating the roles of other RBPs at the NMJ (Menon *et al.* 2004). These lines will allow key phenotypes observed in *Syp* mutants, such as perturbed NMJ structure and vesicle release, to be measured in animals in which Syncrip is only deficient in neuron or muscle, rather than both. In addition, combining the MiMIC Syncrip-eGFP line with anti-GFP RNAi driven in neuron or muscle, will lead to tissue-specific knockdown of Syncrip. The anti-GFP RNAi is predicted to be highly specific and does not affect NMJ structure or function when expressed alone (personal communication, Hugo Bellen). Taken together, these different approaches will shed light on how Syncrip functions in neuron and muscle.

ii) The mechanism by which Syncrip contributes to vesicle release is unknown.

Synaptic transmission is a complex multi-step process. To investigate how Syncrip contributes to vesicle release, a number of different experiments are planned using a range of techniques. First the extent to which loss of Synaptotagmin1 contributes to reduced transmission in *Syp* mutants will be tested. UAS-Syt1-GFP and UAS-Syt-Wt will be expressed in neurons lacking wildtype Syncrip, and electrical recordings will reveal if expression of Synaptotagmin is sufficient to rescue the defective synaptic transmission (Zhang et al. 2002; Paddock et al. 2011). As discussed in Chapter 3, it is possible that loss of Synaptotagmin is not sufficient alone to lead to the electrophysiology of *Syp* mutant animals. As a result, other experiments will be performed in parallel to shed light on how Syncrip regulates vesicle release. For example, electron microscopy is currently being used to investigate the ultrastructures of *Syp* mutant presynapses. Electron microscopy of boutons can reveal the number, size, and distribution of vesicles, the structure of active zones, and the development of the SSR, all of which contribute to synaptic transmission. It is of particular interest to test whether *Syp* mutant boutons contain fewer glutamate vesicles, smaller glutamate vesicles, or a perturbed distribution of glutamate vesicles. These phenotypes have been linked with decrease in synaptic transmission in other studies (Poskanzer *et al.* 2006; reviewed in Denker *et al.* 2009). In addition, it is possible that the reduction in vesicle release in *Syp* boutons is caused by a decrease in the number of active zones relative to wildtype. To this end the anti-Bruchpilot antibody will be employed to visualize active zones, and allow the quantification of active zones per bouton. Another known cause of reduced vesicle release is a decrease in the number of mitochondria in the NMJ presynapse (Verstreken *et al.* 2005). To quantify the number of mitochondria in the presynapse of *Syp* mutant boutons relative to wildtype, both antibodies (anti-ATP synthase-β), and genetically-encoded fluorophores (UAS-mito-GFP), that label mitochondria have been obtained (Vos *et al.* 2012; Cox and Spralding 2003).

Taken together, the experiments proposed here will examine how loss of Syncrip leads to decreased synaptic transmission, and while focus is given to Synaptotagmin as a key regulator of this phenotype, other mechanisms will be tested in parallel.

iii) Studying dynamic processes in mutants is challenging

In this thesis the study of Syncrip function, and the processes that contribute to synaptic plasticity, have been restricted to analysis of fixed tissue in mutants. As discussed in Chapter 5, investigating the phenotypes of mutants can only shed so much light on protein function, particularly when the phenotypes of interest (i.e. plastic growth of the NMJ and synaptic transmission) are dynamic processes. Using MiMIC lines that are currently in production however, it may be possible to acutely inactivate Syncrip with temporal specificity. In addition to eGFP, the CG17838 MiMIC gene tag also includes two protein domains that can be used to conditionally ablate Syncrip protein. First, each Syp isoform is tagged with a specific protease cleavage site, targeted byt the Tobacco Etch Virus (TEV) protease. Conditional expression of the TEV protease, driven either by Gal4 expression or injection of *TEV* mRNA into muscle, will lead to the specific degradation of the Syncrip protein. This technique has been demonstrated in *Drosophila* (Pauli *et al.* 2010). Second, all Syp isoforms will be labeled by FlAsH-FALI tag. A FlAsH-FALI tag is a tetracysteine motif that binds the membrane-permeable fluorescein derivative 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FlAsH). Upon exposure to 488nm light, FlAsH-FALI tags have been shown to instantly inactivate the protein to which they are tagged. Fluorophore-assisted Light Inactivation (FALI) of FlAsH tags is called FlAsH-FALI, and has been demonstrated at the *Drosophila* larval NMJ fillet prep (Marek and Davis 2002). It is a key future experiment to use TEV cleavage and FlAsH-FALI to acutely inactivate Syncrip, and so to characterize its role in vesicle release in the developed NMJ. By taking a suite of electrophysiological recordings before and after acute inactivation of Syncrip-FlAsH-FALI, information can be gathered on the somatic role of Syncrip in synaptic

transmission.

iv) The genomic rescue line does not rescue all phenotypes, and exhibits incongruous phenotypes.

The genomic rescue line does not rescue all phenotypes exhibited in *Syp* mutants. Moreover, there are some mRNA localization phenotypes that are only observed in the genomic rescue line, and not in animals lacking or overexpressing Syncrip (discussed in Chapter 5). There are a number of different explanations for this, though two simple explanations are most likely. First, it is possible that the genomic rescue line contains a mutation in the transgenic copy of Syncrip. As a result, the mutant Syncrip protein is able to rescue some phenotypes (such as NMJ structure and function) but not others (such as nuclear clustering in muscle and mRNA localization). If this were true, then the mutant variant of Syncrip may be responsible for the NMJ-localized mRNAs observed only in the genomic rescue. Alternatively, the Syncrip rescue line may contain a mutation in another gene, which is directly responsible for the phenotypes that are not rescued. This mutation could be on the syp^{e286} chromosome, as some phenotypes (such as nucleolar accumulation of eIF4E) are stronger in syp^{e286}/syp^{e286} than in syp^{e286}/syp^{Df124}(Figure 6.12). Of course, these two possibilities are not mutually exclusive. As a result, standard genetic techniques are being used to 'clean up' the syp^{e286} and syp^{Df124} chromosomes and sequence the transgenic copy of Syncrip encoded in the genomic rescue line.

v) It was not possible to detect presynaptic mRNA at the larval NMJ,

Despite employing a range of different techniques, both in live and fixed cells, it was possible to detect mRNA at the NMJ presynapse. As discussed in Chapter 4, studying mRNA localization in neurons is challenging for a number of reasons. This is a key limitation in investigating mRNA localization and translation at the NMJ. Owing to the technical difficulties encountered, visualizing neuronal mRNAs will not be a priority for

future research. It is, of course, entirely possible that mRNA does not localize to bouton presynapse, but instead protein trafficking from the cell body is sufficient to maintain plastic growth. This possibility is neither excluded by the data here, nor demonstrated to be true. If mRNA is present at the presynapse, then a different approach will be required for detection.

Concluding remarks

This study has approached how mRNA localization may contribute to synaptic plasticity, a phenomenon central to memory and learning. mRNA localization in neuron and muscle is central the functions of these two tissues, and the *Drosophila* larval NMJ has proved a useful model for investigating these processes in this thesis. While providing preliminary insight into how gene expression may be regulated at the NMJ, these observations have raised more questions than answers. To further understand how mRNA localization contributes to synaptic plasticity, tools to detect mRNA in living neuron and muscle must be developed. Similarly, further experiments are required to dissect exactly how Syncrip regulates mRNA localization in muscle, and vesicle release in neuron. Nevertheless, this study is key first step in understanding mRNA localization and local translation at the neuromuscular junction, and will provide a spring board for further research into these fundamental and important processes.

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