



Actin Regulation in Activity-Dependent Synaptic Plasticity at the *Drosophila* Neuromuscular Junction

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Abstract

Synaptic plasticity is the process by which synaptic connections are continuously modified and is the molecular basis of learning and memory. These modifications require new protein synthesis and cytoskeletal rearrangements at or near the synapse. Syncrip, an mRNA-binding protein, is conserved from *Drosophila* to vertebrates and is found to regulate mRNAs encoding proteins required in synaptic plasticity. Many of these proteins have functions in actin-binding, actin cytoskeleton organization, or other actin-related processes, including Msp300, which was recently described in detail by the Davis lab. Here, we investigate two other targets of Syncrip, Basigin and Cofilin (Twinstar), among the most highly expressed of these genes in the *Drosophila* nervous system.

By studying the activity-dependent synaptic plasticity, the change in neuronal connectivity as a result of activity, in the *Drosophila* neuromuscular junction, we showed that the loss of Cofilin in the presynapse leads to a decrease in activity-dependent synaptic plasticity, whereas the loss of Basigin in the postsynapse promotes plasticity. Deficiency of Basigin in the presynapse causes growth of larger boutons during development, while its effect during plasticity has yet to be determined. Furthermore, activity-induced expression of Basigin transcriptional level while the regulation of Cofilin takes place at posttranscriptional level. Basigin is present around some immature boutons. These data suggest that Basigin and Cofilin are involved in activity-dependent synaptic plasticity and have distinct roles in the pre- or postsynaptic compartments. Basigin could also play a part in the maturation of boutons. Our results add to the growing number of actin regulators that are downstream of Syncrip and have a role in neuronal plasticity.

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Declaration of Authorship

I hereby declare that this thesis is my own work, and all experiments described were performed by myself. Exceptions are detailed below:

- Syncrip iCLIP in *Drosophila* data was generated by Jeff Lee
- smFISH probes used to detect *yfp* mRNA transcripts were designed and synthesized by Dalia Gala

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List of Abbreviations

ABP . . . . . Actin-binding protein

Aβ . . . . . Amyloid β

AD . . . . . Alzheimer's disease

ADF . . . . . Actin-depolymerizing factor

ALS . . . . . Amyotrophic lateral sclerosis

AMPA . . . . . α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Arc . . . . . Activity-regulated cytoskeleton-associated protein

AZ . . . . . Active zone

BMP . . . . . Bone Morphogenic Protein

Bsg . . . . . Basigin

CAM . . . . . Cell-adhesion molecule

cAMP . . . . . cyclic adenosine monophosphate

CaMKII . . . . . Calcium/Calmodulin-dependent Kinase II

CDS . . . . . Coding sequence

Cfl . . . . . Cofilin

cGMP . . . . . cyclic guanosine monophosphate

CNS . . . . . Central nervous system

CPEB . . . . . cytoplasmic polyadenylation element binding protein

CREB . . . . . cAMP response element binding protein

DAPI . . . . . 4', 6-diamidino-2-phenylindole

DEPC . . . . . diethylpyrocarbonate

Dlg . . . . . Disc large

DM . . . . . Myotonic dystrophy

DMSO . . . . . deoxyribonucleic

ECM . . . . . Extracellular matrix

EDTA . . . . . Ethylenediaminetetraacetic acid

eIF4E . . . . . eukaryotic translation initiation factor 4E

EMMPRIN . . . . extracellular matrix metalloproteinase inducer

EPSP . . . . . excitatory postsynaptic potentials

F-actin . . . . . Fibrous actin

FMRP . . . . . Fragile X mental retardation

FLP . . . . . flippase

FRT . . . . . FLP recombinase target

G-actin . . . . . Globular actin

GFP . . . . . Green fluorescent protein

GluR . . . . . Glutamate receptor

GPCR . . . . . G-protein-coupled receptor

HL3 . . . . . Hemolymph-like 3 saline

hnRNP . . . . . heterogeneous nuclear ribonucleoprotein

hnRNPQ . . . . . heterogeneous nuclear ribonucleoprotein Q

HRP . . . . . Horseradish peroxidase

iCLIP . . . . . Individual nucleotide resolution crosslinking and immunoprecipitation

IE . . . . . immediate-early

IF . . . . . immunofluorescence

iGluR . . . . . Ionotropic glutamate receptor

Imp . . . . . IGF-II mRNA-binding protein

LE . . . . . Localization element

LIMK . . . . . LIM kinase

LTD . . . . . Long-term depression

LTP . . . . . Long-term potentiation

MAGUK . . . . . membrane-associated guanylate kinase

MAP . . . . . Microtubule-associated protein

MBNL . . . . . Muscleblind-like

MCT . . . . . monocarboxylate transporter

mDia1 . . . . . mammalian homolog of Drosophila diaphanous 1

MF . . . . . microfilament

mGluR . . . . . Metabotropic glutamate receptor

Mhc . . . . . Myosin heavy chain

miRNA . . . . . microRNA

MMP . . . . . matrix metalloproteinase

mRNA . . . . . Messenger RNA

Msp300 . . . . . Muscle-specific protein 300

MT . . . . . microtubule

NB . . . . . neuroblast

NM . . . . . Nemaline myopathy

NMJ . . . . . Neuromuscular junction

NT . . . . . Neurotransmitter

OrR . . . . . OregonR

Pak1 . . . . . p21-activated kinase

PBS . . . . . Phosphate-buffered saline

PBSTX . . . . . Phosphate-buffered saline with Triton-X

PFA . . . . . Paraformaldehyde

PH . . . . . periventricular heterotopia

PPV . . . . . Porcine Parvovirus

PRP . . . . . Plasticity-related protein

PSD . . . . . Postsynaptic density

RBD . . . . . RNA-binding domain

RBFOX1 . . . . . RNA Binding Fox-1 Homolog-1

RBP . . . . . RNA-binding protein

RISC . . . . . RNA-induced silencing complex

RH5 . . . . . PfRH5

RNA . . . . . Ribonucleic acid

RNAi . . . . . RNA interference

RNP . . . . . ribonucleoprotein

RPM . . . . . Reads per million

RT . . . . . Room temperature

RVC . . . . . Ribonucleoside vanadyl complex

SIH . . . . . Starvation-induced hyperactivity

SiR . . . . . Silicon rhodamine

SMA . . . . . Spinal muscular atropy

smFISH . . . . . Single molecule RNA fluorescent in situ hybridisation

SMN . . . . . Survival motor neuron

SSC . . . . . Saline-sodium citrate

SSR . . . . . Subsynaptic reticulum

Stau1 . . . . . Staufen 1

Stau2 . . . . . Staufen 2

STC . . . . . Synaptic tag and capture

SYNCRIP . . . . . Synaptotagmin binding cytoplasmic RNA interacting protein

Syp . . . . . Syncrip

TPM . . . . . Transcripts per million

Tsr . . . . . Twinstar (cofilin)

UAS . . . . . Upstream activating sequence

UTR . . . . . Untranslated region

VNC . . . . . Ventral nerve cord

ZBP1 . . . . . zipcode binding protein 1

1 | Introduction

Actin is a major component of the cytoskeleton and is involved in many biological processes, including muscle contraction, cell movement, and activity-dependent synaptic formation (W.-H. Lin & Webb, 2009). The dynamic actin remodelling is a crucial part of most cellular activities. Actin in cells can take two forms: the monomeric globular actin (G-actin) and polymeric fibrous actin (F-actin). The rapid transition between these two forms during cellular processes is known to be regulated by actin-binding proteins (ABPs). mRNAs encoding ABPs and actin-related proteins are commonly detected at the periphery of neurons (Kugelgen & Chekulaeva, 2020), but how these mRNAs are localized and how their translation is regulated at the synapse remain unclear.

F-actin is present in both presynaptic (axon) and postsynaptic (dendrite) compartments and plays a critical role in activity-dependent synaptic plasticity (Papandréou & Leterrier, 2018). Synaptic plasticity is widely considered as the cellular basis of learning and memory. Long-term synaptic plasticity is dependent on new protein synthesis. The synapse is located far from the cell body (Villareal et al., 2007). Local protein synthesis allows modulation of synaptic strength in a rapid and synapse-specific manner independent of the cell body (K. C. Martin et al., 2000). The stability, localization, and translation of mRNAs are regulated by different subsets of RNA-binding proteins (RBPs) (R. Ohashi & Shiina, 2020). This thesis aims to examine the localization and transcription of two actin-binding and actin-related genes regulated by RBPs during activity-dependent synaptic plasticity.

In this chapter, I will provide general introductions to synaptic plasticity, actin, RBP regulation of translation at the synapse, and *Drosophila* larval NMJ as a

model system. I will then introduce Syncrip (Syp), an RBP, as well as Basigin (Bsg) and Cofilin (Tsr), two actin-related proteins regulated by Syp, which will be the focus of this thesis. Finally, I will discuss the implications of this study in diseases and other biological contexts.

1.1 Synaptic Plasticity

Synaptic plasticity is the activity-dependent change in synaptic strength (Citri & Malenka, 2008). The correlation between synaptic modifications and memory was hypothesized over 100 years ago by Santiago Ramon y Cajal, a Spanish Nobel laureate. The psychologist Donald Hebb then better characterized it, suggesting that the connection between two neurons can be strengthened if they consistently fire together. In the early 1970s, the experiments by Kandel's group showed the connections between behavioural learning and increase in excitatory postsynaptic potentials (EPSPs) in *Aplysia californica* (Pinsker et al., 1970; Castellucci et al., 1970), while the Bliss' group found evidence for long-lasting potentiation caused by high-frequency stimulation in the hippocampus (Bliss & Lømo, 1973). The phenomenon of activity-dependent strengthening of synaptic transmission can last for many hours to several days and is thus termed long-term potentiation (LTP). Since then, more studies have been done on the cellular and molecular mechanisms that enable this process. In contrast to short-term potentiation, LTP involves both transcription and translation because studies indicate that long-term memory formation is inhibited by applying transcription or translation inhibitors (Costa-Mattioli et al., 2009).

It is well established that there are multiple forms of LTP, and most synapses that express LTP also exhibit its reverse process, long-term depression (LTD). The mechanisms of LTP and LTD may involve modifications to either or both pre-

and postsynaptic subcellular compartments. The axon terminal contains a region called the active zone (AZ) that mediates neurotransmitter release. Presynaptic plasticity generally involves changes in neurotransmitter release. The corresponding structure in the dendrite terminal is the postsynaptic density (PSD), which consists of neurotransmitter receptors. Postsynaptic plasticity typically involves modifications in the size and sensitivity of the PSD (Luo, 2016). Both the AZ and the PSD are composed of dense networks of scaffolding proteins, which are in turn associated with the actin cytoskeleton.

1.1.1 mRNA localization and synaptic plasticity

Neurons go through morphological changes during development as well as in response to stimuli. Evidence shows that local translation of RNA in neurites underlies synaptic formation, long-term synaptic plasticity and axon guidance during development (D. Wang et al., 2010). Neurons are highly polarized cells with elaborated branch structures. Axon terminals of neurons can be a long way from the cell body. For instance, human sciatic nerve axons are generally over one meter in length, while the recurrent laryngeal nerve in giraffes is thought to be up to five meters long. A mature neuron can form connections with multiple other cells, and these connections are subject to changes when learning occurs. These features of neurons demand local protein synthesis of pre-localized mRNAs at the neurites because de novo transcription, translation, and protein trafficking simply would not meet the physiological needs of rapidly changing synapses. Steward and Levy first proposed the local translation model in 1982 in their study of localization of polyribosomes in dendritic spines in the mouse dentate gyrus (Steward & Levy, 1982). Similar mechanisms have been observed in many other systems, such as giant squid photoreceptor neurons and *Aplysia* neuronal culture (Gioio et al., 2004; Lyles et al., 2006). Systemic, unbiased

screens identified a large number of subcellularly localized mRNAs in vertebrate neurons (Eberwine et al., 2002; Moccia et al., 2003; Poon et al., 2006; Suzuki et al., 2007).

Local mRNA translation has been a research focus for many in the past four decades, owing to its significance in synaptic plasticity and neuronal pathologies. An early study identified mRNA localization of protein F1 in the presynapse, a membrane-bound protein kinase C substrate enriched at the synapse. Protein F1 is associated with axon growth as well as synaptic plasticity in adults (Rosenthal et al., 1987). A key study revealed the necessity of local protein synthesis in synaptic plasticity in the rat hippocampus. In this study, the synaptic regions were isolated with microlesion and found that inhibition of local protein synthesis led to attenuation of neurotrophin-induced synaptic plasticity (Kang & Schuman, 1996). A neuronal isoform of *Aplysia* cytoplasmic polyadenylation element binding protein (CPEB), a protein that regulates local mRNA translation, was found to behave in a prion-like manner in stimulated synapses. Its expression is activity-dependent, and it is required for the maintenance of LTP (Si, Giustetto, et al., 2003; Si, Lindquist, & Kandel, 2003). Another important dendrite-localized mRNA encodes an activity-regulated cytoskeleton-associated protein (Arc). *Arc* mRNA is directed to dendrites of synapses that have experienced certain activity patterns. Arc protein is then translated and assembled into the postsynaptic junction. The local translation of *arc* mRNA is found to be essential to the stabilizing LTP and memory formation (Steward & Worley, 2002). In *Drosophila*, the association of local protein synthesis and synaptic plasticity has also been demonstrated. A study found that Calcium/Calmodulin-dependent Kinase II (CaMKII) mRNA is transported to postsynaptic sites in response to neural activity, where it is then translated. The study also found that mRNA transport and local protein synthesis are regulated

by components of the RNA interference (RISC) pathway (Ashraf et al., 2006). While most studies provided evidence for mRNA localization in synaptic plasticity in dendrites, a few studies also show mRNA localization in axons. Notably, *sensorin* mRNA, encoding a neuropeptide particularly concentrated at presynaptic sites, is found to be required for synaptic formation between sensory and motor neurons in *Aplysia*. Reducing *sensorin* mRNA at the presynapse could block synapse formation (Lyles et al., 2006). A later study found *sensorin* mRNA local translation occurs in serotonin-induced synaptic plasticity as well. This study investigated the long-term plasticity at the *Aplysia* sensory-motor synapse using live-cell imaging of photoconvertible fluorescent proteins. It also discovered that local translation is restricted to stimulated synapses as well as its stimulus-specificity and transcript-specificity (D. Wang et al., 2010).

1.2 Actin and Actin-binding Proteins

Actin is an important structural protein that is present in essentially all eukaryotic cells. It is most known for its involvement in contractility in muscle as well as during cell division. However, actin also plays an indispensable role in the nervous system. Actin microfilaments (MFs) are abundant in neurons and glia and are found to be enriched in presynaptic terminals, dendrite spines and growth cones (Kirkpatrick & Brady, 1999). Actin MFs sometimes are cross-linked and can form structures including bundles and membrane-bound networks, which maintain membrane protein distribution, determine cell morphologies and facilitate protein sorting into the axonal and dendritic compartments (Beck & Nelson, 1996). Other times, actin MFs act like rails for myosin, the motor protein that converts chemical energy to mechanical energy. The mechanical energy generated is used in neuronal processes, such as growth cone motility,

activity-dependent motility of dendritic spines and vesicle transport along axons and dendrites (Ahmed & Saif, 2014). More importantly, an interplay between activity-dependent synaptic plasticity and actin cytoskeleton remodelling both pre- and postsynaptically has been demonstrated (Szabó et al., 2016). A number of ABPs, including CaMKIIβ, cyclic adenosine monophosphate (cAMP), and cyclic guanosine monophosphate (cGMP), regulate in spine structural potentiation (Borovac et al., 2018). Actin cytoskeleton and ABPs are involved in maintaining stable spine morphology, which is required for maintaining long-term memory (Basu & Lamprecht, 2018).

Actin assembly and disassembly are spatially and temporally regulated by many different ABPs. Actin polymerization consists of three phases: nucleation, elongation and equilibration. The ABPs in the nervous system allow the formation of a variety of structures. The Arp2/3 complex binds to F-actin and creates a nucleation site. It plays a role in axon branching and dendritic spine maturation and enlargement. Actin severing protein, such as actin-depolymerizing factor (ADF) and cofilin-1, accelerates the turnover of actin and is involved in growth cone motility, dendritic spine formation and long-term synaptic plasticity (Noguchi et al., 2016). Formin, the mammalian homolog of *Drosophila* diaphanous 1 (mDia1), binds to the barbed end of F-actin and acts as an unbranched nucleator. It is essential to axon elongation (Arakawa et al., 2003).

1.3 RNA-binding protein regulation of local translation at the synapse

In eukaryotic cells, RBPs play vital roles in the posttranscriptional regulation of gene expression and contribute much to the overall gene expression. RBPs have functions in splicing regulation, mRNA transport, localization, and modulation of mRNA translation and decay (Oliveira et al., 2017). Many RBPs are composed of multiple repeats of RNA-binding domains (RBDs). By arranging these repeats in various ways, RBPs form ribonucleoprotein (RNP) complexes that bind to nascent RNA transcripts (Lunde et al., 2007). Recent genome-wide studies discovered a number of RBPs that lack discernible RBDs. This suggests the existence of alternative modes of RNA binding (Hentze et al., 2018).

RBPs are crucial in neurons because they determine if an mRNA gets transported along the axon or dendrites to the synapse, when it is translated, and when it is degraded (Lenzken et al., 2014). Evidence suggests posttranscriptional regulation is necessary for synaptic plasticity, and local translation is required at the synapse (Ule & Darnell, 2006). This phenomenon has been proven via the detection of local protein synthesis using approaches, including physically isolating the synapse from the rest of the cell body, local perfusion of translation inhibitors, and use of photoconvertible or bleachable fluorescent reporters (Holt et al., 2019; Younts et al., 2016). To regulate the spatiotemporal protein synthesis, trans-acting RBPs bind to mRNAs and mediate their transport and translation in response to synaptic activity (Klein et al., 2016). Researchers have identified groups of RBPs that modulate translation and localization of functionally related mRNAs, such as zipcode binding protein 1 (ZBP1) and Fragile X mental retardation protein (FMRP) (A. C. Lin & Holt, 2007; Bassell & Warren, 2008).

This finding suggests that mRNA translation and localization are closely linked. In addition, studies have shown different sets of RBPs are responsible for distinct types of synaptic plasticity. For example, the Staufen 1 (Stau1) is essential in regulating the transport of mRNAs in dendrites during late-phase LTP, whereas Staufen 2 (Stau2) protein is responsible in mGluR-induced protein synthesis-dependent LTD (Lebeau, DesGroseillers, et al., 2011; Lebeau, Miller, et al., 2011). Notably, Arc mRNA, localized in dendrites, is involved in both LTP and LTD (Yilmaz-Rastoder et al., 2011). There exists evidence indicating that each form of synaptic plasticity may activate distinct signalling cascades that involve different signalling receptors, whose targets are RBPs that will regulate translation at the synapse (Fernandez-Moya et al., 2014).

A number of posttranscriptional regulatory mechanisms are needed to allow rapid alterations of the levels of copious proteins during synaptic plasticity. For instance, in rat hippocampal neurons, over 2500 unique mRNAs are present in the synaptic region (Cajigas et al., 2012). A number of RBPs are required to ensure these mRNAs are only translated when appropriate. mRNA 3' untranslated region (UTR) contains cis-acting elements, known as the localization elements (LEs). Several dendrite-specific LEs that also mediate synaptic plasticity have been identified, for example in AMPA receptor, Microtubule-associated protein (MAP) 2, and CaMKIIαmRNAs (La Via et al., 2013; Blichenberg et al., 1999; Mori et al., 2000). While LEs encode where the transcripts may be directed in the neuron, a system is needed to transport the mRNAs effectively. Such a mechanism involves RNP granules, the MT network, and associated motor proteins. RNP granules consist of mRNA-RBP complexes, which are believed to form cis-trans interactions between RBPs and target LEs, ensuring specificity while simultaneously repressing the translation of the mRNA being transported (Hutten et al., 2014). Therefore, RNPs are often referred to as

trans-acting factors. Although it is still unknown how specific RNP granules are transported to the synapse, several models have been proposed. Two of the most well-adopted are the "synaptic tag and capture (STC) model" and the "sushi belt model" (Figure 1.1). The traditional synaptic tag and capture model describes that synaptic stimuli generate a local synaptic tag to signal protein uptake. The stimuli also induces the expression of plasticity-related proteins (PRPs). Then, the PRPs are transported into the dendritic region and captured by tagged synapses. Thus, the stabilization of synaptic strength is achieved (Frey & Morris, 1997). However, this version of the STC models has many limitations, for example it does not explain dendritic protein synthesis, which is supported by ample evidence. Therefore, in revised STC model, PRP mRNAs are required to be diffusible in the dendrite and are anchored to the tagged synapse (Redondo & Morris, 2010). In comparison, the sushi belt model proposed that the RNPs constitutively patrol multiple synapses and the synapse that is undergoing activity-dependent tagging recruits a neuronal RNP (Doyle & Kiebler, 2011). This model can be used to explain the bidirectional transport mRNAs in the dendrite, which has been experimentally observed. However, computational and mathematical modeling revealed that this model faces tradeoffs between speed, accuracy, and efficiency in delivering mRNAs to the synapse that require mRNA uptake (Williams et al., 2016). While both theories are supported by some experimental evidence, the true molecular mechanism underlying dendritic transport is still unclear based on our current understanding. Another important facet of posttranscriptional regulation involves microRNA (miRNA), a class of small non-coding RNA. miRNA functions in suppressing translation and facilitating degradation of mRNAs by employing complementary base pairing and forming the RNA-induced silencing complex (RISC). Although miRNA molecules are believed to be generally stable, in neurons, miRNAs are subject to

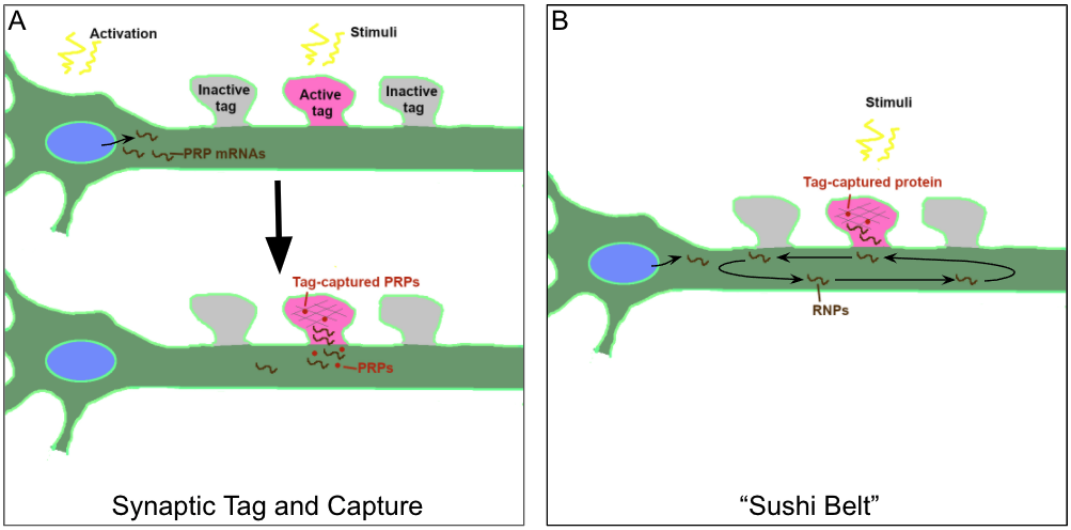


Figure 1.1: **A schematic of "synaptic tag and capture" and "sushi belt" models**
A: In the STC model, synaptic stimuli develop an active tag within the activated synapse. This tag distinguishes stimulated synapse from non-stimulated synapses. The stimuli also induces transcription of PRP in the soma. During the late phase of LTP, the activity-induced PRP mRNAs are transported and anchored to the actived synapse, where the PRPs are translated and captured. B: In the sushi belt model, RNAs are transcribed and processed in the nucleus of a neuron before they are exported to the cytoplasm. In the cytoplasm, RNP granules are formed and transported along the MT cytoskeleton, circling a number of synapses like sushi on conveyor belt. When one synapse becomes activated from stimuli, RNPs are recruited to the synapse. The RNAs from the RNPs are released and translated.

rapid turnover dependent on neuronal activity. The expression of miR-132, the most studied miRNA in the context of synaptic function, is proven to be modulated by a variety of transcription factors involved in plasticity, including cAMP response element binding protein (CREB) and BDNF. In turn, miR-132 regulates plasticity-related transcripts, such as p250GAP, glutamate receptor (GluR) subunits, NR2A, and NR2B (Wayman et al., 2008; Kawashima et al., 2010). The regulation of neuronal miRNAs by FMRP also has an effect on dendritic morphology (Edbauer et al., 2010).

1.4 *Drosophila* NMJ as a model system

1.4.1 *Drosophila* as a model organism

Drosophila melanogaster, also known as the fruit fly, is a popular model organism in biomedical sciences. They are commonly used due to their small size, minimal requirements, low cost and fast generation time. In recent years, the addition of numerous molecular tools along with the highly annotated genome and excellent genetic tools in *Drosophila* has made them the ideal organism for answering questions in gene expression, cell biology, developmental biology and neurobiology.

Drosophila may not look like mammals, but many of the biological mechanisms and pathways are conserved across vertebrates and invertebrates. The *Drosophila* genome is 60% homologous to that of humans, and approximately 65% of the genes related to human diseases have homologues in *Drosophila* (Ugur et al., 2016). Together with the advantages highlighted above, *Drosophila* research continues to provide valuable insights to research in humans and other mammals.

Specifically in the nervous system, although the anatomies of the brain and nervous systems are very different in *Drosophila* and humans, they share a large number of conserved genetic, cellular, electrophysiological and chemical properties. The fly brain is estimated to have 90,000 neurons, many fewer than in the human brain (Venken et al., 2011). However, the *Drosophila* nervous system is a complex system that contains numerous neural cell types. For instance, in the *Drosophila* visual system, around 115 different types of neurons were identified, which is close to the number that has been estimated in human eyes (Venken et al., 2011). Because of the genetic and cellular similarities between *Drosophila* and human, *Drosophila* has been used to model some aspects of human brain

diseases. Nonetheless, researchers have to bear in mind the divergence between vertebrate and invertebrate anatomies (Jeibmann & Paulus, 2009).

1.4.2 *Drosophila* Genetics

In this section, I will cover some of the genetic tools in *Drosophila* that were used to design mating schemes or to generate fly stocks that were used in this project. Over the past centuries, scientists have constructed unique genetic tools in *Drosophila* to make the crossing schemes more reliable and less ambiguous by controlling the recombination effects during meiosis. Interestingly, male *Drosophila* are achiasmatic, which means there is effectively a complete absence of recombination in male flies (Hawley, 2002). Although recombination is still present in female *Drosophila*, recognizable dominant and recessive markers have made chromosomal transmission to progeny more trackable. Recognizable markers are mutations affecting phenotypes, such as eye colour, eye shape, wing shape, bristle colour and bristle shape. They are often used to mark the chromosomes one tries to follow or lose in genetic crossings.

Balancer chromosomes were used to suppress recombination. They were first described by Hermann Muller in 1918, where the term "balanced" referred to enforced heterozygosity in a stock due to a recessive lethal mutation and a crossover suppressor on one chromosome (Miller et al., 2019). Balancer chromosomes are chromosomes that contain multiple inversions and are rearranged. Balancer chromosomes can suppress recombination because the product of any recombination will be lethal due to duplication or deletions of chromosome fragments. In addition, balancer chromosomes are lethal when homozygous in most cases.

Another common chromosomal mutation in *Drosophila* that leads to powerful

genetic tools is deletion. Chromosomal deletions are used extensively in *Drosophila* research for 1) deletions that fail to complement loss-of-function mutations can help map the mutations to specific chromosomal regions, and 2) reducing the copy number of a gene can enhance or suppress mutant phenotypes or modify the expression of a related gene (Cook et al., 2012). A number of deletion projects used FLP-FRT recombination, which is a site-directed recombination technology. Flippase (FLP) recombination targets (FRTs) are short asymmetric sequences, usually carried on transposable elements, that are recognized by FLP and recombined directionally (Golic & Golic, 1996). Using this FLP-mediated approach to make deletions is much more efficient than relying on transposable elements alone (Parks et al., 2004). However, transposable elements/transposons are still most frequently used to generate transgenic flies.

A transposon is a DNA sequence that encodes a transposase and is flanked by inverted repeats, allowing the sequence to change its location within the genome via excision and reintegration or replication. A commonly used class of transposable elements is the P-element. P-element has a broad range of usage, including gene disruption through imprecise excision, gene tagging and inducing gene expression (Bachmann & Knust, 2008).

Moreover, thousands of UAS/GAL4 lines became available, along with the application of a temperature-sensitive GAL80 protein in *Drosophila* (Pfeiffer et al., 2008; T. Lee & Luo, 1999). Together, these systems allow tissue-specific and temporal-specific gene expression or ablation when studying a variety of processes in *Drosophila*. Another major development is the application of low toxicity genetically encoded fluorescent markers, such as the green fluorescent protein (GFP). This allows visualization of precise localization of specific proteins in living cells, which makes observation of cellular activities, including

neuronal activities, possible. Finally, the availability of optogenetic tools and the use of warmth-activated cation channel, TRPA1, allowed less invasive approaches possible when manipulating neuronal and other activities in *Drosophila* (Pulver et al., 2009).

1.4.3 Introduction to the *Drosophila* NMJ

The *Drosophila* larval neuromuscular system is a simple system, and the neuromuscular junctions (NMJs) have been extensively studied. The use of larval body wall muscles and its NMJ as a model system was first developed in the mid to late 1970s, led by authors, such as Jan and Jan, as well as Magazanik and Vyskocil (Jan & Jan, 1976; Vyskochil & Magazanik, 1978). In a series of papers, these groups first described the physiological and pharmacological properties of the larval NMJ. In addition, they pioneered the first genetic analysis of synaptic transmission and short-term plasticity. Following these findings, other research groups explored the use of NMJ in investigating the effects of mutations in ion channels on synaptic transmission (Ganetzky & Wu, 1982). Subsequently, the larval NMJ was made accessible to study synaptic structure and development by the Keshishian lab (Anderson et al., 1988; Halpern et al., 1991). Around the same time, a study revealed the key finding that the development of the NMJ synapse could be regulated by electrical stimuli, and therefore, it uses a similar regulatory mechanism as synapses in more complex organisms (Budnik et al., 1990). Since then, *Drosophila* NMJ has become an unparalleled genetic model in crucial fields in neuroscience, including neurogenesis, synaptic plasticity, synaptic transmission and cell signalling (Bellen et al., 2010).

The persistent popularity of *Drosophila* NMJ as an experimental tool can be attributed to a few properties. First, they are highly tractable and allow access to body wall muscles and motoneurons that innervate them in both microscopy and

electrophysiology. The larval body wall sample preparation can take as little as 5 minutes. Second, the larval body wall muscles are similar across individuals in terms of their physiology and structures (Keshishian et al., 1996). These uniform patterns of muscles and motoneurons allow easy and quick identification of specific muscles and specific hemisegments. Third, the larval NMJ model system plays an important role in the field of neuroscience because its electrophysiological properties can be roughly measured by quantifying individual synapses. At the NMJ, the synapses are contained in large, discernible varicosities called boutons. The size and complexity of neuromuscular endings are regulated by activity and development (Keshishian et al., 1993; Budnik et al., 1990). Therefore, the change of the synapse can be approximated by counting boutons. The discovery of activity dependence of motoneuron growth shows that the NMJ could be used as a model system to study synaptic plasticity using morphological changes. Finally, the similar physiological properties and molecular compositions shared by *Drosophila* NMJ and mammalian glutamatergic synapses make the larval NMJ a good model for studying fundamental questions about the synapse (Keshishian et al., 1996).

1.4.4 Structure and Development of *Drosophila* NMJ

Generally, the studies of larval NMJs focus on the abdominal segments, which consist of arrays of overlapping, striated muscle fibres attached to the inner surface of the body wall. These muscles are innervated by motoneurons. A third-instar *Drosophila* larva has 30 skeletal, contractile muscles, which are innervated by approximately 32 motoneurons located in the ventral ganglion in each hemisegment, repeated in each abdominal segment (Ruiz-Cañada & Budnik, 2006a). In general, each muscle fibre is innervated by two motoneurons. The only exception is muscle 4, which is monosynaptic (Figure 1.2).

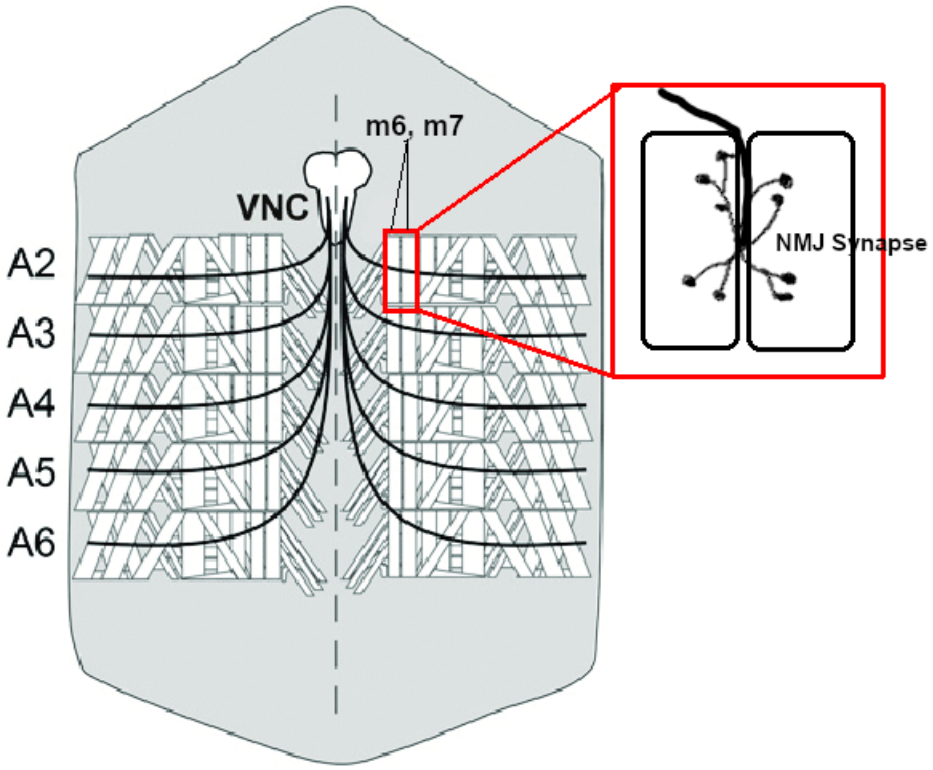


Figure 1.2: **A schematic representation of *Drosophila* nervous system** Adapted from (Perez-Moreno & O'Kane, 2019) Figure 1A. *Drosophila* ventral nerve cord (VNC) receives sensory inputs from the brain and transforms these signals into locomotive inputs for the muscles. On the right, a scheme shows an NMJ synapse between muscles 6 and 7, and it is innervated by motoneurons.

The motoneurons can be classified into three types based on the types of boutons they form, out of which type-I neurons are the most abundant and are divided into two subclasses: type-I big (type-Ib) and type-I small (type-Is) (Figure 1.3). In a type-I synapse, type-Ib boutons range in size from 2.5-5 µm, while type-Is boutons vary from 1.5-3 µm in size (Atwood et al., 1993). Type-I neurons primarily release glutamate as an excitatory neurotransmitter (NT) (Jan & Jan, 1976; Johansen et al., 1989). In addition to glutamate-filled vesicles, type-I boutons also contain mitochondria and endosomes (Atwood et al., 1993; Jia et al., 1993). Type-II and type-III neurons release octopamine or other peptides, although they also release glutamate (Ruiz-Cañada & Budnik, 2006a). Morphologically, one discerning feature between type-I and the other types of boutons is that type-I boutons are surrounded by an infold membranous structure called subsynaptic reticulum (SSR). The SSR contains scaffolding proteins, postsynaptic complexes, GluRs, and GluR translation sites (Atwood et al., 1993; S. Sigrist et al., 2000). Type-Ib boutons are surrounded by significantly more SSR compared to type-Is boutons. When labelled with an SSR marker, such as scaffolding protein Disc large (Dlg), the various types and subtypes of boutons can be readily differentiated. Ultrastructural studies showed that type-I terminals could be distinguished from extrasynaptic junctional areas by exhibiting presynaptic electron-dense structures, referred to as the T-bar ribbons (Atwood et al., 1993; Jia et al., 1993). The T-bar ribbons contain AZs, as they are shown to tether vesicles, facilitate vesicle release, and are closely associated with clusters of calcium channels (Reiff et al., 2002; Parsons & Sterling, 2003; Kawasaki et al., 2000).

During development, motoneurons are specified and generated from neuroblasts (NBs) (Landgraf et al., 1997). Upon contact of the axonal growth cone with its target muscle, the motoneuron is matched to their correct pre- and postsynaptic

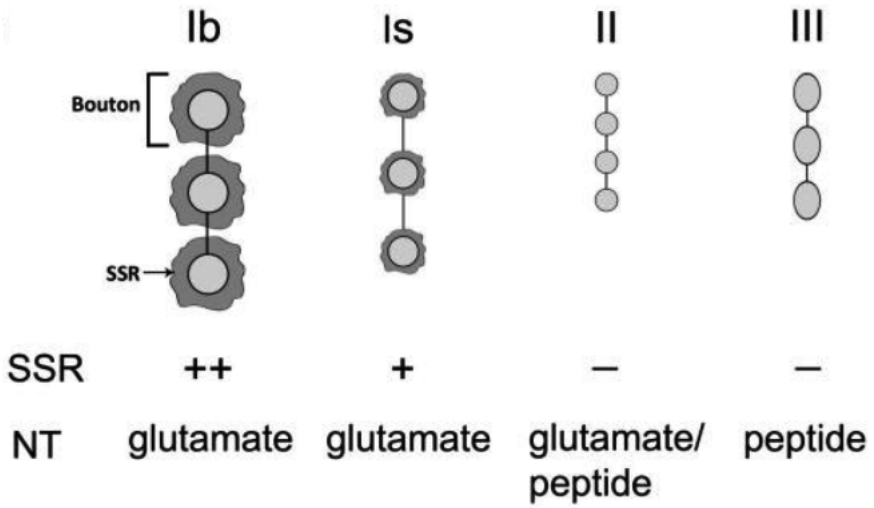


Figure 1.3: **A schematic of types of boutons**
Adapted from (Menon et al., 2013) Figure 3B. Type-Ib and Type-Is boutons are surrounded by SSR and release glutamate as an neurotransmitter. Type-II and Type-III boutons do not have SSR and release other peptides.

partners by various cell-adhesion molecules (CAMs) (Südhof, 2018). Specifically, in type-I NMJ synapses, postsynaptic GluRs and Dlg, a postsynaptic scaffolding protein, begin to cluster rapidly at the contact site (Broadie & Bate, 1993; K. Chen & Featherstone, 2005). The growth cone then differentiates into a presynaptic terminal. The axon terminals form periodic enlargements known as boutons, which consist of AZs. Each presynaptic terminal then becomes surrounded by SSR.

Synaptic refinement also occurs at the NMJs, which involves an initial overgrowth of synaptic connections and later selectively elimination of off-target contacts (Carrillo et al., 2010). This process in *Drosophila* NMJ is similar to that in the mammalian visual system: the removal of misplaced contacts in late-stage embryonic development requires an activity-dependent mechanism. The activity involves not only sensory stimuli but low-frequency voltage oscillation in the *Drosophila* embryo as well (Carrillo et al., 2010). These oscillations have been

observed in motoneurons during late embryogenesis (Crisp et al., 2008).

1.4.5 The *Drosophila* NMJ Is Glutamatergic

The glutamatergic nature of *Drosophila* NMJ is one of the reasons that has made it a popular model system. The larval NMJ type-I synapses use ionotropic GluRs to modulate postsynaptic response to neurotransmitters. These GluRs are homologous to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type GluRs in the mammalian brain (Littleton & Ganetzky, 2000). *Drosophila* NMJ also has postsynaptic scaffolds that resemble mammalian PSDs, and it is shown that 95.8% of mammalian PSD proteins have *Drosophila* homologs (Liebl & Featherstone, 2008). These similarities allow *Drosophila* NMJ to become an elegant genetic model for studying excitatory synapses in the mammalian central nervous system (CNS).

GluRs can be divided into two subclasses: ionotropic and metabotropic. Ionotropic GluRs (iGluRs) are ligand-gated channels that open when bound to glutamate. These cation channels depolarize and excite postsynaptic cells when opened. Metabotropic GluRs (mGluRs) are members of G-protein-coupled receptors (GPCRs). Unlike iGluRs, mGluRs are not ion channels. Instead, they activate biochemical cascades and modulate a number of second messenger signalling pathways on binding glutamate. Both classes of GluRs have functions in the *Drosophila* NMJ: iGluRs are responsible for fast neurotransmission leading to the depolarization of postsynaptic muscle in the NMJ (Jan & Jan, 1976); while mGluRs are involved in modulating the efficacy of short-term plasticity (Bogdanik, 2004). Most of the studies in *Drosophila* were on iGluRs, so this is what we will focus on here.

The AMPA/kainate type receptors are the most extensively characterized in

Drosophila. Five subunits contributing to GluRs at the NMJ have been identified, three of which (DGluRIII, DGluRIID, DGluRIIE) are present in all synapses and are essential to GluR formation and function. Each GluR also contains a fourth subunit, which could be either DGluRIIA (IIA) or DGluRIIB (IIB). These two muscle receptors differ in their physiological properties, which lead to different synaptic responses (DiAntonio et al., 1999). IIA and IIB are redundant for viability, but at least one of them is needed in order to form a functional GluR. Both IIA and IIB are localized opposite to distinct motoneurons, but they do not perfectly colocalize. Their localizations are determined by DGluRIII, with IIA clustering into a puncta and IIB forming a ring around it (Marrus, 2004).

During development, innervation is required for the formation of iGluR clusters (Broadie & Bate, 1993). Not only GluRs are important in the development and function of the synapse, but also synaptic plasticity. Local protein translation is found to be a key mechanism for plasticity at the synapse. A study showed that, in excitability mutants, there's an increase in the level of IIA protein, but not mRNA in the SSR, where DGluRIIA mRNAs localize (S. Sigrist et al., 2000). Studies by the same research group later demonstrated that DGluRIIA is both necessary and sufficient for the morphological and functional growth of the synapse (S. Sigrist et al., 2000; S. J. Sigrist et al., 2003). GluR activity also has a significant role in determining the plasticity of NMJ synapses. Reducing the responsiveness of GluR by using mutants or inhibiting the DGluRIIA subunit leads to a reduction in quantal size (Davis et al., 1998) and a compensatory response in the nerve (Paradis et al., 2001). Homeostatic mechanisms also position iGluRs opposite to AZs that are larger and have a higher probability of vesicle release when postsynaptic receptors are limited (Marrus, 2004). Thus, GluRs are both regulated by, and themselves regulate synaptic plasticity, affecting the size and strengths of the NMJ synapse.

1.4.6 Cytoskeleton at the NMJ synapse

The cytoskeleton has a key role in cell polarity and membrane dynamics. The coordinated reorganization of cytoskeletal network, especially actin and microtubules (MTs), is crucial in synaptic development, nerve branching, and new bouton formation. After a synapse is formed, the pre- and postsynaptic cytoskeletons are regulated simultaneously by signals received from altering synaptic strength, changes in neuronal activity levels, and the release of growth factors. Little is known about the mechanisms underlying developmental and plastic modifications of the synapse (Ruiz-Cañada & Budnik, 2006b). The glutamatergic *Drosophila* larval NMJ allows researchers to study the molecular mechanisms of synaptic growth using genetic approaches.

The enlargement of the larval NMJ synapse can be visualized by new bouton formation. The comparatively large size and accessibility of synaptic boutons made larval NMJ a tractable model to study the cytoskeletal organization and regulation during synaptic expansion. A study using live-imaging first described the three different modes of synaptic growth (Zito et al., 1999). In this study, a chimeric transmembrane protein with a GFP marker was used to target the SSR. New boutons are shown to form either between established boutons or at the end of a branch of the NMJ arbour via one of three modes: budding of a mature bouton, symmetric division of a bouton, and de novo emergence of a bouton from neurite enlargement (Menon et al., 2013).

At the *Drosophila* NMJ, MTs are present in the axon terminals, visualized by antibody staining. MTs and Futsch, a *Drosophila* homolog of vertebrate MAP1B, are involved in presynaptic bouton division. Futsch is associated with MT stabilization and bundling. Ultrastructure analysis revealed that MTs are present in presynaptic boutons, where they organize as loops that form a

thread-like structure (Atwood et al., 1993; Roos et al., 2000). During bouton division, the MT/Futsch loops undergo dynamic reorganizations where the MT/Futsch bundles split up and form a bundle across the center of the main MT/Futsch loop. The newly formed bundle is believed to predict where the bouton will be divided (Roos et al., 2000). This mechanism is involved in activity-dependent synaptic plasticity. Futsch, along with other regulators of MTs, such as Wingless and Shaggy, is involved in synaptic plasticity and regulates rearrangements of presynaptic terminal structures in an activity-dependent manner (Ataman et al., 2008).

Actin, another main component of the cytoskeleton, also has indispensable roles at the synapse. F-actin, decorated with actin interacting proteins, not only forms cross-links between F-actin but also bridges actin to MTs, the plasma membrane, and other cellular compartments. Spectrin, an important cytoskeletal protein, is expressed in both pre- and postsynaptic compartments. There are two types of Spectrin: α-Spectrin and β-Spectrin. In *Drosophila*, α-Spectrin is encoded by one gene while β-Spectrin isoforms are encoded by two, a conventional β-Spectrin and a larger form. Both α- and β-Spectrins are present at the NMJ (Pielage et al., 2005). In the motoneurons, α- and β-Spectrins colocalize in the axon. α-Spectrin has also been detected in synaptic boutons. α- and β-Spectrins are not required in early synaptic developments, but lacking Spectrins in later stage leads to impaired neurotransmitter release and synapse retraction (Featherstone et al., 2001; Pielage et al., 2005). In the postsynaptic compartment, α- and β-Spectrin form a thick layer that surrounds presynaptic boutons (Featherstone et al., 2001; Pielage et al., 2005). Interestingly, the actin/Spectrin network is present in a region where most postsynaptic MTs are not present (Ruiz-Canada et al., 2004).

Although there have been few studies on the mechanisms of GluR clusters at *Drosophila* NMJs, extensive mammalian studies have shown actin and Spectrin

are involved in this process. GluRs cluster at postsynaptic sites through interactions between the actin cytoskeleton and scaffold proteins of the membrane-associated guanylate kinase (MAGUKs) (Bredt & Nicoll, 2003; Shirao & Sekino, 2001). In *Drosophila*, several genes involved in GluR compositions have been identified. Coracle, a 4.1-type protein, stabilizes GluRIIA by anchoring it to postsynaptic actin/Spectrin cytoskeleton (K. Chen et al., 2005). Neto, a neurophilin and tolloid-like protein, is essential to iGluRs clustering at the *Drosophila* NMJ. Moreover, Neto and iGluR clustering are interdependent (Y.-J. Kim et al., 2012). Filamin, an actin cross-linking protein, is required for the clustering of GluRIIA and growth of SSR, but not GluRIIB (G. Lee & Schwarz, 2016). The actin/Spectrin network itself is also found to be crucial at the NMJ since the loss of the actin/Spectrin network disrupts the AZ-GluR alignments (Blunk et al., 2014).

1.4.7 Plasticity and Activity-Dependent Regulation of Gene Expression at the NMJ Synapse

The proper function of the locomotive system requires continuous adaptation of synaptic architecture and neurotransmission at the NMJ. This feature has made the larval NMJ a great candidate to study synaptic plasticity. From embryogenesis to pupation, the growth of a larva takes roughly five days. In this period, the area of a muscle increases by a factor of over 100-fold, while the number of boutons innervating a muscle increases 10-fold (Gorczyca et al., 1993; Keshishian et al., 1993). In order to enable control of the musculature, the glutamatergic motoneurons have to rapidly and continuously modify their structures and connections to muscles by forming new branches, new boutons, and retracting from old connections. Therefore, the larval NMJ has a significant number of signal transduction pathways that allow synaptic size and strength

adjustments. These pathways play key roles in both short-term and long-term plasticity.

The manifestations of the NMJ synapse can be categorized as functional plasticity and structural plasticity. Functional plasticity refers to the changes in the magnitude of synaptic transmission without structural changes at the synapse. These events are usually short-term. Paradigms inspired by facilitation and post-tetanic potentiation are often used to study short-term potentiating plasticity. On the other hand, structural plasticity is thought to require new protein synthesis and therefore takes place over a longer timescale. These structural changes can occur on many levels, including changes in the number of axon branches, the number and size of boutons, and changes in the amount and localization of postsynaptic scaffolding proteins. A milestone in NMJ studies was the discovery that growth of the synapse responds to varying local and external stimuli with changes in neuron morphology, the number of synaptic boutons, and synaptic strength (Budnik et al., 1990; Broadie & Bate, 1993; Jarecki & Keshishian, 1995). These studies provided a quantitative point of view of synaptic plasticity in response to activity. Alterations of pre- and postsynaptic components as well as signalling proteins, by mutation or misexpression, allowed genetic dissection of the key components involved in plasticity. For instance, mutations in channel proteins have been used to alter activities at the synapse to investigate the role of activity in synaptic plasticity. Potassium channel regulatory proteins, Shaker and Ether-a-gogo, repolarize membrane potential after synaptic transmission. Mutations in these genes lead to hyperexcitability, which in turn led to increased branching and increase in bouton numbers at the NMJ (Budnik et al., 1990). In contrast, sodium channels depolarize the neuron during an action potential, and, as expected, duplications in sodium channel gene Paralytic also lead to hyperexcitability at the synapse. Axons in these larvae show decreased number

of branches and complexity (Lnenicka et al., 2002). Likewise, mutations in calcium channel protein, Cacophony, or manipulations of activity of Ca^{2+}/calmodulin protein kinase II (CaMKII) show that Ca^{2+} is a crucial secondary messenger in both pre- and postsynaptic sites (Kawasaki et al., 2000; Rieckhof et al., 2003; Koh et al., 2000).

New proteins synthesized during long-term synaptic changes come from a variety of sources. Some come from the translation of pre-existing mRNAs, while others are expressed via activation of certain transcription factors. At the *Drosophila* NMJ synapse, the regulation of transcription and protein synthesis during plasticity has been studied in remarkable detail. It offers valuable insights into the conserved mechanisms of learning and memory across species. On the transcription level, transcription factors are involved. There are two subtypes of transcription factors: constitutive and inducible (Herdegen & Leah, 1998). Inducible transcription factors are typically found in cells at low levels but can be rapidly expressed in response to activity. Some transcription factors are immediate-early (IE) genes, which are genes that do not require de novo protein synthesis. One example of an IE gene is the activator protein-1, which is a transcription factor that acts upstream of CREB and regulates synaptic plasticity (Sanyal et al., 2002). CREB itself is also a transcription factor required for plasticity, but it is constitutively present. These two types of transcription factors reveal two major ways of regulating transcription: one is to increase the amount of the transcription factor itself, and the other is to alter protein activities via posttranslational modifications such as phosphorylation.

Another source of new protein synthesis in response to stimuli is the translation of pre-existing mRNAs. Abundant experimental evidence indicates localized translation in the dendrite, which is critical in synaptic plasticity (Sutton & Schuman, 2006; Cajigas et al., 2012). Although not many studies have been

undertaken in *Drosophila*, posttranscriptional mechanisms in synaptic plasticity have been studied extensively in mammals (See section 1.3).

1.5 Syncrip

1.5.1 Syncrip in the nervous system

Synaptotagmin binding cytoplasmic RNA interacting protein (SYNCRIP) is also known as heterogeneous nuclear ribonucleoprotein Q (hnRNPQ). As the name implies, SYNCRIP/hnRNPQ has functions in binding Synaptotagmins, a family of membrane-trafficking proteins that are essential for neurotransmitter release in neurons and in association with cytoplasmic RNA. Mammalian SYNCRIP/hnRNPQ has functions in many biological processes, including exosomal miRNA sorting (Hobor et al., 2018), controlling myeloid leukemia stem cell program (Vu et al., 2017), and is found to be a candidate gene for intellectual disability in a meta-analysis (Lelieveld et al., 2016). In the mammalian nervous system, SYNCRIP/hnRNPQ is ubiquitously expressed in the brain and partially colocalizes with Purkinje neuron somata (Tratnjek et al., 2017). It is also part of various neuronal RNA-transporting granules. For instance, it has been identified in kinesin-associated granules containing CamKIIα and Arc (Kanai et al., 2004). It also colocalizes with granules containing Cdc42 mRNA and FMRP, which are crucial for neuronal morphogenesis (H.-H. Chen et al., 2012). Moreover, SYNCRIP/hnRNPQ inhibits translation in vitro by competing with poly(A) binding protein (Svitkin et al., 2013) and affects neurite arborization by interacting with mRNAs encoding components of the actin nucleation-promoting Cdc42/N-WASP/Arp2/3 complex (H.-H. Chen et al., 2012).

In *Drosophila*, CG17838, an hnRNP protein, was identified as the *Drosophila* homolog of SYNCRIP/hnRNPQ and hnRNPR, sharing 47% sequence identity and 60% similarity to mammalian SYNCRIP/hnRNPQ and hnRNPR. CG17838 encodes one of the proteins that bind to *gurken* mRNA, which encodes a localization signal in the oocyte. *Drosophila* Syncrip (Syp) was also shown to specifically bind to *gurken* and *oskar* mRNAs. The localization of these mRNAs is crucial for the determination of axes in the oocyte. Loss-of-function of Syp alleles show *gurken* and *oskar* mRNA mislocalization and misexpression, indicating Syp is necessary for *gurken* and *oskar* mRNA localization and translation regulation (S. McDermott et al., 2012). In *Drosophila* third instar larvae, Syp is present in muscle nuclei as well as postsynaptically at the NMJ. Syp is required for proper NMJ morphology. Syp loss-function-mutants show synaptic overgrowth, whereas overexpression of Syp results in reduced bouton number and branching at the NMJ synapse (S. M. McDermott et al., 2014). Syp is also shown to be linked to a number of mRNAs encoding proteins associated with synaptic function and development, such as *msp-300*, *syd-1*, *neurexin-1 (nrx-1)*, *futsch*, *dlg1*, and *α-spectrin* (S. M. McDermott et al., 2014). In addition, Syp is found to modulate presynaptic vesicle release, but, interestingly, its protein product is only found in the muscle and not in the motoneuron. This finding can be partially explained by Syp modulation of retrograde Bone Morphogenic Protein (BMP) signals from the muscle back to the motoneuron. The study showed that upregulating BMP signalling led to abnormal synapse structure and function (Halstead et al., 2014). A recent study revealed that Syp is indispensable for activity-dependent synaptic plasticity in the NMJ. Syp is shown to posttranscriptionally regulate activity-induced expression of Msp300, an ABP and important structural protein, expression in the muscle. Syp and *msp300* mRNA are shown to interact near the synapse in eukaryotic translation initiation

factor 4E (eIF4E). The eIF4E granules lose their mobility in response to activity and the percentage of eIF4E granules containing *msp300* mRNA also increases in response to stimuli (S. Titlow et al., 2020).

A number of studies demonstrated Syp functions during neuronal development in *Drosophila* neural stem cells. Neural stem cells can generate sets of specialized neurons. These generated daughter neurons can change over time during development. *Drosophila* larval brain stem cells, known as neuroblasts, undergo asymmetric cell division and can generate two neurons of different subtypes (Knoblich, 2008). The sequential differentiation of NBs has been mapped in different parts of the brain throughout larval and pupal development (T. Lee et al., 1999; Yu et al., 2010). An important study identified two sets of genes with age-dependent alterations of expression levels in the mushroom body and antennal lobe. These genes are believed to play key roles in the determination of the temporal fates of neuronal daughter cells generated from neuroblasts at different time points. By intersecting these two sets of genes, a list of 16 common genes was found. Among these genes, IGF-II mRNA-binding protein (Imp) and Syp, two conserved RBPs, exhibit opposite gradients of expression levels: Imp is abundantly expressed at an early stage then gradually declines, whereas Syp increases and becomes one of the most abundant genes at the late larval stage. Imp/Syp gradients are involved in temporal neuronal fate specification in the mushroom body by regulating the temporal transcriptional factor Chinmo translation (Z. Liu et al., 2015). Further study showed that activation of Syp, as well as two additional late NB factors, Broad and E93, require the steroid hormone ecdysone in mid-larval NBs. Ecdysone also simultaneously downregulates Chinmo/Imp (Syed et al., 2017). A recent study revealed that Syp plays a role in the stabilization of *prospero (pros)* mRNA. Pros is an important regulator of the transition between NB proliferation and differentiation.

Upregulation of Pros in larval neurons is required to maintain neuronal identity (Carney et al., 2013). This study found that upregulation of Pros protein is driven by alternative polyadenylation followed by differential posttranscriptional regulation through Syp-mediated mRNA stability (Samuels et al., 2020).

1.5.2 Syncrip in other biological processes

Apart from the nervous system, Syp and its mammalian ortholog are involved in many other biological processes as an RBP. A study found SYNCRIP to regulate a set of mature miRNAs positively and negatively. Specifically, SYNCRIP is part of a microprocessor complex that promotes the processing of pri-let-7a and contributes to the increase of let-7a miRNA (Y. Chen et al., 2020). SYNCRIP was found to act in exosomal sorting of specific miRNAs in hepatocytes (Santangelo et al., 2016). SYNCRIP is shown to promote the amplification of several viruses. SYNCRIP is involved in viral RNA replication of Hepatitis C virus and mouse hepatitis virus as a positive regulator (H. Liu et al., 2009; Choi et al., 2004). Moreover, SYNCRIP facilitates Porcine Parvovirus (PPV) viral DNA replication by mediating the alternative splicing process of PPV mRNA. SYNCRIP binds to the 3'-terminal to promote splicing of NS1 mRNA to produce NS2 mRNA (S. Chen et al., 2021). Interestingly, Syp is also involved in the behavioural response to starvation in *Drosophila*. Starvation-Induced Hyperactivity (SIH) was a prominent behavioural trait when *Drosophila* experienced increased locomotor activity upon starvation. A study performed genome-wide association studies and described a number of genes involved in SIH. Genes encoding ion channels and RBPs, including Syp, are found to be most enriched in SIH genes (Chi et al., 2020). Syp is also discovered to be part of a mechanism that regulates circadian rhythm. Fubp1, a cell-cycle regulator, upregulates the mRNA and protein level of Syp to maintain Period 1 protein

oscillation (T. Kim et al., 2019).

1.6 Basigin and Cofilin

1.6.1 Basigin

Basigin/EMMPRIN is an IgG family glycoprotein found on the surface of human metastasis tumours. Basigin was first discovered in 1982 in cocultures of rabbit fibroblasts and mouse tumour cells of epithelial origin (Biswas et al., 1995). The researchers found that these cells secrete a protein that explains the increased collagenase production in the cocultures and has the exact function in human fibroblasts (Biswas, 1984; Biswas & Nugent, 1987). A later study purified the protein from human lung carcinoma cells and named it tumour cell collagenase-stimulatory factor (Ellis et al., 1989). It was later renamed extracellular matrix metalloproteinase inducer (EMMPRIN) to better represent its role in extracellular matrix metalloproteinase induction in normal and pathological cellular interactions. It was identified to be a member of the immunoglobulin superfamily (Biswas et al., 1995). Basigin has been identified in many species and adopts different names, including CD147 and M6 in humans, HT7 and Neurothelin in chicken, gp42 in mice, and OX-47 in rats (Seulberger et al., 1992).

Basigin has many functions and various binding partners. In humans, BSG has several isoforms: Basigin-1 has three Ig binding domains and is discovered to be retina-specific, while Basigin-2 contains two Ig binding domains and is the most common form. The transmembrane region of both isoforms consists of 23 amino acids and is highly conserved among species (Figure 1.4). However, human and *Drosophila* Basigins have only moderate homology (Muramatsu, 2016). Basigin

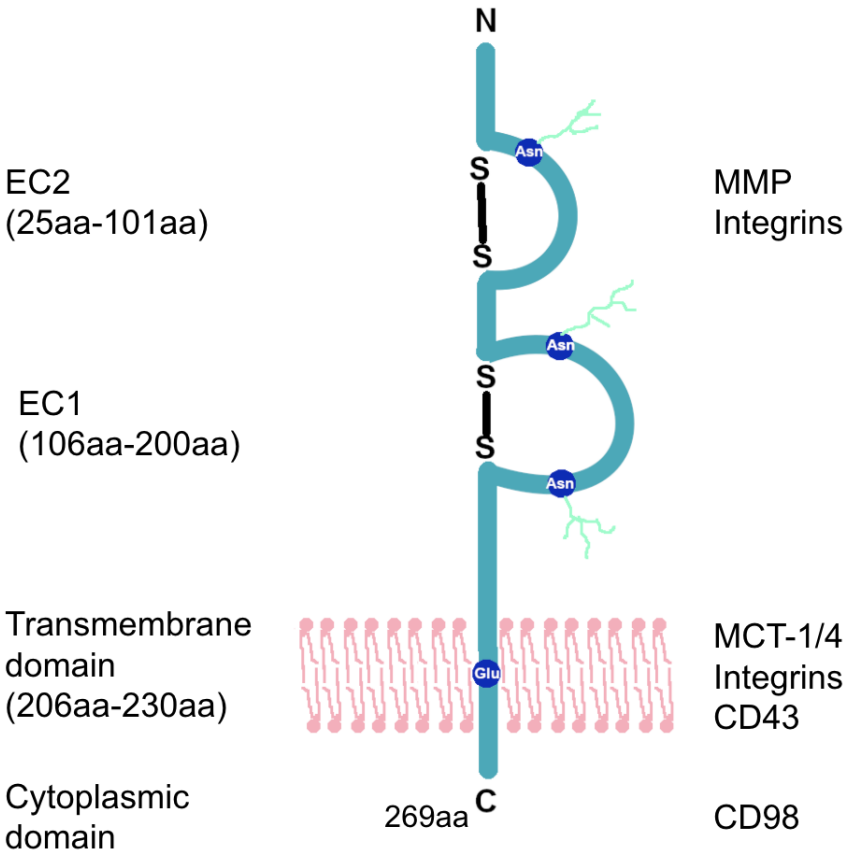


Figure 1.4: **A schematic representation of Bsg domains and binding partners** Adapted from (Li & Nowak, 2020) Figure 1. Bsg consists of two Ig-like extracellular domains, a transmembrane domain, and a cytoplasmic domain. Some of the interactors of Bsg are MMP, Integrins, MCTs, CD43, and CD98, as noted in this chapter.

was first discovered for its involvement in cancer and is best known for its role in tissue remodelling via regulating MMPs. It is found to be overexpressed in a wide range of human malignant tumours and promotes invasiveness, proliferation, and survival of tumour cells (Grass et al., 2014). Bsg plays an important role in reproduction and development. Bsg deficiency can lead to sterility in both male and female mice via different mechanisms (Li & Nowak, 2020). Bsg is also important for eye and tooth development. The loss of Bsg leads to meibomian gland dysfunction, which in turn could lead to ocular surface disease (Mauris et al., 2015). It is also found to delay enamel formation caused by delayed cell differentiation and disturbed epithelial-mesenchymal interactions during tooth development (Khaddam et al., 2014). Furthermore, Bsg is involved in the regulation of immune responses. Studies found that Bsg knockout mice show defective lymphocyte responsiveness and are characterized by the increased mitogenic response of T lymphocytes in mixed lymphocytes reactions (Igakura et al., 1996). Bsg also plays a role in the regulation of inflammatory response by interacting with cyclophilins, a family of proteins that bind to immunosuppressant cyclosporin A (Yurchenko et al., 2010). Later studies revealed that Bsg is involved in the development and activation of T-cells. Bsg has functional roles in T-cell development in the thymus. Furthermore, it suppresses T-cell activation via indirectly mediating T-cell receptor activation (Yao et al., 2014) and inhibiting the nuclear factor of activated T-cells induced by Vav1 and Rac1 (Ruiz et al., 2008). It is also found that human Bsg is a direct marker of a subset of activated and highly suppressive human regulatory T-cells (Solstad et al., 2011).

Bsg is crucial in the sensory and nervous systems. In chicken, Bsg is crucial for chick brain development. It has been described that Bsg is highly expressed in chicken embryo NBs, but not detected in neurons after cytodifferentiation. The

disappearance of Bsg in neurons is hypothesized to be due to glia-neuron interactions, supported by an in vitro study of cocultured chick retina neurons and purified rat astrocytes (Schlosshauer, 1991). Chicken Bsg is also found to be crucial for the development of chick blood-brain barrier endothelium (Seulberger et al., 1992). Bsg has important functions in the retina. Studies in mice showed that Bsg ensures normal morphology and visual functions of rod and cone photoreceptors by interacting with monocarboxylate transporters (MCTs) and binding to a glucose transporter GLUT1 to enhance its activity (Hori et al., 2000; Philp et al., 2003; Ait-Ali et al., 2015). Muller glial cells in the retina depend on glycolysis and lactate exchange between glia and neurons, which is mediated by MCTs (Philp et al., 2003). *Drosophila* Bsg was also shown to be essential to the normal morphology of photoreceptors and normal neuron-glia interactions in the *Drosophila* visual system. Expression of transgenic mouse Bsg in mutant *Drosophila* can rescue defects in the visual system, showing that the mouse Bsg shares the same functions as *Drosophila* Bsg (Curtin et al., 2005, 2007). This was explained in a mutational analysis that introduced mutations in conserved residues in each putative domain and found that the extracellular and transmembrane domains are responsible for Bsg functions while the intracellular domains may not play a role (Munro et al., 2010). Moreover, mutations of Bsg in mice and *Drosophila* lead to loss of sensitivity to noxious odours (Igakura et al., 1996; Anholt et al., 2003), while null mutants in mice also exhibit decreased sensitivity to light and increased sensitivity to electronic foot-shock (Naruhashi et al., 1997). Deficiency of Bsg in mice is also found to cause worsened performance in the Y-maze task, used to assess short-term memory, without leading to any motor dysfunction (Naruhashi et al., 1997). Thus, Bsg is believed to have an important role in sensory functions as well as learning and memory. Further biochemical analysis examined expression patterns of Bsg in mice

central nervous system and found strong expression of Bsg in the limbic system, including the olfactory system, hippocampal formation, amygdala, as well as in retinal neuronal layers and Purkinje cells of the cerebellum (Fan et al., 1998). This evidence confirmed the findings in the previous behavioural study. In *Drosophila*, a study revealed that Bsg is present in both pre- and postsynaptic compartments in the *Drosophila* NMJ, accumulates at periactive zones, and plays a role in restricting synaptic bouton size. It also found that Bsg is required for the normal vesicle distribution and vesicle release, possibly due to the role of Bsg in regulating actin cytoskeleton in the presynaptic compartment. A conserved cytoplasmic juxtamembrane domain is required for Bsg function (Besse et al., 2007).

One important interactor of Bsg is integrin, a transmembrane receptor that connects the extracellular matrix (ECM) with the actin cytoskeleton inside the cell. In mammals, an early study found that Bsg interacts with $\alpha^3\beta_1$ and $\alpha^6\beta_1$ integrins, but it is unclear whether Bsg binds to these integrins directly or indirectly by interacting with other molecules (Berditchevski et al., 1997). There has been evidence of a binding site between Bsg and β1 integrin, as well as a number of molecules that mediate Bsg-integrin interactions, including CD98, CD43, MCT4 and galectin-3 (Muramatsu, 2016). In *Drosophila*, the functional interactions between Bsg and integrin are evident in their ability to affect cellular architecture. Bsg and integrin are found to colocalize both in cultured cells and in the *Drosophila* visual system. Abnormal intracellular structures, such as mislocalized nuclei and cell morphology, are detected in integrin mutants as well as in cells where Bsg-integrin interaction is disrupted (Curtin et al., 2005). The glial cells and ECM have crucial roles in supporting and protecting the *Drosophila* nervous system. A recent study revealed that Bsg negatively regulates integrin in the perineurial glia to maintain the proper ensheathment of

neurons and normal morphology of the nervous system (A. Hunter et al., 2020). Another interactor of Bsg that is worth mentioning is the γ-secretase complex. γ-Secretase complex is a membrane-embedded protease complex that is most known for its role in cleaving β-amyloid precursor protein within the transmembrane region, leading to the production of amyloid β-peptides (Aβ-peptides). Increased production of Aβ-peptides has been associated with the accumulation of amyloid plaques in AD patients. Bsg is suggested to be a subunit within the γ-secretase complex. It is found that the depletion of Bsg, while the expression of other components in the γ-secretase complex remains unchanged, leads to an increase in Aβ-peptide production. This suggests that the Bsg down-regulates Aβ-peptide levels (S. Zhou et al., 2005). However, a later study provided evidence of indirect regulation of Bsg on Aβ-peptide production, postulating that Bsg stimulates extracellular degradation of Aβ-peptides rather than suppressing γ-secretase activity directly (Vetrivel et al., 2008).

1.6.2 ADF/Cofilin

As mentioned above, ADF/cofilin proteins are key actin regulators of actin dynamics in response to a variety of intracellular and extracellular signals. They sever actin to produce barbed ends for polymerization and depolymerize old actin filaments. In mammals, ADF/cofilin proteins have three forms: ADF, cofilin-1 and cofilin-2. ADF and cofilin-1 have similar roles in F-actin depolymerization, though their activities differ, with ADF being more potent in monomer sequestering while cofilin being more efficient in nucleation (Bernstein & Bamburg, 2010). Cofilin-1 is more ubiquitous and extensively studied than cofilin-2, so the following section is based on cofilin-1. Cofilin activity is regulated by LIM kinase (LIMK), where LIMK phosphorylates and inactivates cofilin (Arber et al., 1998). LIMKs are, in turn, activated by downstream targets

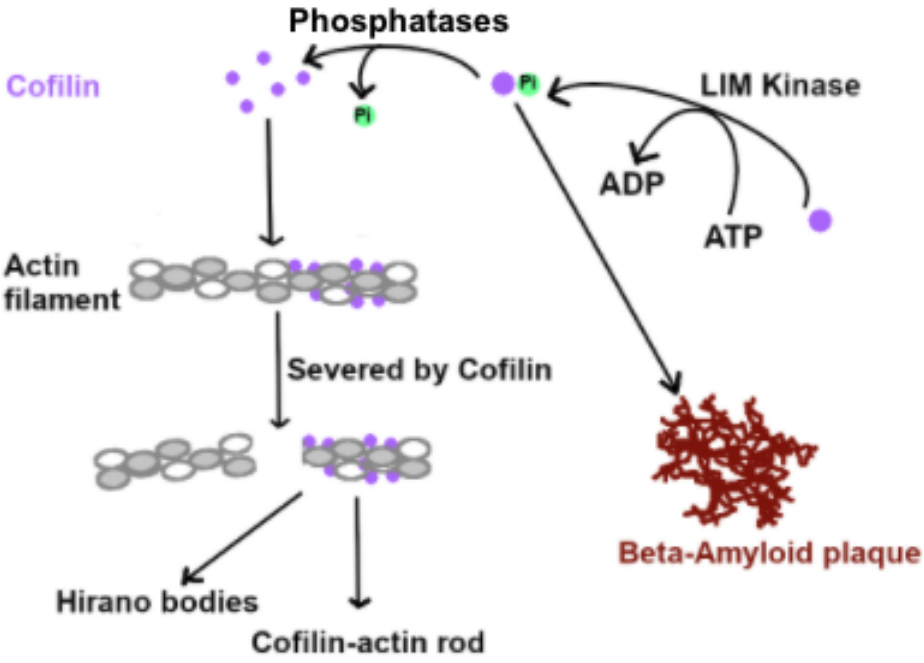


Figure 1.5: **Biochemical activities of Cofilin and its role in diseases** LIM Kinase phosphorylates cofilin and deactivates it. Phosphatases, such as Slingshot, dephosphorylates cofilin. Active cofilin binds to and severs actin filaments. Cofilin-actin is closely related to cellular biological markers of Alzheimer's Disease, which include cofilin-actin rods, Hirano bodies, and β-amyloid plaques.

of small GTPases of the Rho/Rac/Cdc42 family. LIMK1 is activated by p21-activated kinase (Pak1), while LIMK2 is activated by ROCK, a Rho-dependent protein kinase (Edwards et al., 1999; Sumi et al., 2001). LIMK regulation of Twinstar (Tsr), *Drosophila* homolog of ADF/cofilin, was discovered to be evolutionarily conserved between *Drosophila* and mammals (K. Ohashi et al., 2000).

Cofilin dynamically regulates actin assembly and disassembly via severing, promoting elongation, or nucleation (Figure 1.5). The mechanism employed depends on the relative cofilin/actin concentration. At a relatively low concentration, cofilin persistently severs F-actin, while at a high concentration, it

can nucleate filaments (Andrianantoandro & Pollard, 2006). Cofilin also has an important function in modulating actin filament branching by interacting with the Arp2/3 complex. Arp2/3 is a seven-unit actin nucleator that binds to existing actin filaments and serves as a nucleation site for new actin filaments from a branch as a distinctive 70° angle from the existing actin filaments, leading to a dendritic structure (Robinson et al., 2001). A study revealed that cofilin enhances the removal of actin branches by directly competing for binding sites on the actin filament and dissociates Arp2/3 (Chan et al., 2009). Another interesting cellular function of cofilin is its ability to chaperone actin to the nucleus. Actin itself does not possess a nuclear-localization sequence. However, it is crucial for many cellular processes that take place in the nucleus, including chromatin remodelling, the formation of hnRNPs, and the recruitment of histone acetyltransferase complexes to activate gene expression (Zheng et al., 2009). Cofilins and ADFs, on the other hand, have nuclear-localization sequences and can be found participating in the actin nuclear import (Bamburg, 1999; Ruzicka et al., 2007). Arabidopsis ADF9 is found to be a repressor of flowering. It is essential to maintain normal chromatin structure, suggesting some members of the ADF family may be involved in chromatin-level control of gene expression (Burgos-Rivera et al., 2008). It is noteworthy that cofilin also plays a significant role in cell motility and cell migration. Cell migration consists of three distinct steps: a cell initiates protrusion at the front in response to a stimulus, followed by the formation of focal adhesions, and ends with the disassembly of adhesions at both its leading edge and rear end. Cofilin is involved in generating new barbed ends at the leading edge for polymerization as well as depolymerizing old actin filaments. Although it remains undetermined how the activities of cofilin in actin polymerization and depolymerization are coordinated, there has been evidence showing the activation of cofilin is required, and cofilin defines the direction of

cell motility (Carlier et al., 1999; Ghosh et al., 2004). It is shown that the spatiotemporal regulation of cofilin activity by LIMK1 and Slingshot can define migration direction (Nishita et al., 2005).

Cofilin has a variety of functions in the nervous system, including growth cone motility and navigation, synaptic functions, and neurodegeneration. The sensory capacity and motility of neuronal growth cones rely on lamellipodia and filopodia located at the leading edge of extending growth cones. Actin treadmilling regulated by ADF/cofilins determines the morphology and dynamics of the lamellipodia and filopodia. A study found that ADF localized in the growth cones is required for neurite outgrowth, and increased ADF activity promotes neurite extension. Two Rho family GTPases, Rac1 and Cdc42, were identified to regulate ADF/cofilin activation and growth cone guidance in response to external repulsive and attractive stimuli (Kuhn et al., 2000). Cofilin is revealed to be a crucial regulator of dendritic spine morphology and synaptic plasticity. The changes in spine morphology in response to synaptic plasticity are controlled by the reorganization of the actin cytoskeleton (Matsuzaki et al., 2004). Inhibition of ADF/cofilin activities by phosphorylation elicit spine enlargement during LTP (L. Chen et al., 2007), whereas increase of cofilin peptide in neurons induces spine shrinkage and LTD (Q. Zhou et al., 2004). In the mammalian hippocampal synapse, ADF and cofilin share overlapping functions in regulating presynaptic physiology and vesicle trafficking, while cofilin mutant alone can cause defects in postsynaptic plasticity (Wolf et al., 2015).

In *Drosophila* NMJ, cofilin regulates activity-dependent synaptic growth. Increased cofilin activity leads to rapid bouton formation, whereas overexpression of LIMK inhibits cofilin, which in turn inhibits bouton budding. This study also found that the LIMK-cofilin pathway is modulated by the muscle

release of retrograde BMP signals, which regulates presynaptic cytoskeletal rearrangements (Piccioli & Littleton, 2014). Studies also found cofilin to be an important regulator of axon and dendrite branching (Sudarsanam et al., 2020; Nithianandam & Chien, 2018). Apart from the nervous system, *Drosophila* ADF/cofilin homolog is proven to play crucial roles in many biological processes, including cytokinesis, cell motility, cell polarity and photoreceptor morphogenesis (Gunsalus et al., 1995; J. Chen et al., 2001; Blair et al., 2006; Pham et al., 2008). Tissue-specific knockdown of cofilin revealed that cofilin maintains epithelial integrity and Yorkie-dependent tissue growth via inhibition of cell extrusion from the epithelium and c-Jun N-terminal kinase signalling (Ko et al., 2016). Loss of cofilin in muscle leads to impaired formation of the sarcomere, a basic contractile unit for muscle fibre, and therefore causes muscle weakness in *Drosophila* larvae (Balakrishnan et al., 2020).

1.7 Relevance to diseases

1.7.1 Actin-related diseases

The ABPs mentioned above, along with many others, work together to ensure the proper development and functions of the nervous system. Abnormal regulation of the actin cytoskeletons results in diseases prevalent in, but not limited to, the nervous system. Mutations or environmental stress that affect ABPs can lead to serious developmental, neurological, and psychiatric issues as well as addiction. One example is filamin A, an ABP that promotes orthogonal F-actin network. The orthogonal F-actin network is essential to neuronal migration and cortical formation (Fox et al., 1998). Mutations in filamin A can lead to numerous structural abnormalities, including schizencephaly, lissencephaly ("smooth brain"), periventricular heterotopia, and neurological problems, such as poor

motor function, seizures, and even epilepsy (Sheen et al., 2005; Masruha et al., 2006). Some other examples are: Cyclase-associated Protein 1, facilitates cofilin-induced actin turnover, is found to be linked to depression (Nakatani et al., 2007); Epidermal growth factor receptor kinase substrate 8, a barbed-end actin capping protein, is associated with deafness and ethanol addiction (Behlouli et al., 2014; Offenhäuser et al., 2006). Cofilin-actin enriched inclusions, in the forms of irregular aggregates, Hirano bodies or rod-like bundles, are commonly found in diseased brains of a wide range of neurodegenerative disorders. Most prominently, in AD, Aβ induces cofilin-actin rods formation, which further aggravates the generation and secretion of Aβ in toxic forms (Maloney et al., 2005).

Abnormalities in actin and ABPs can also lead to a wide range of diseases outside of the nervous system. Congenital myopathies are linked to five actin and ABP genes (Clarkson et al., 2004). Mutations in ABPs, such as Arp2/3 and Rac2, and missense in actin protein in leukocytes are known to cause neutrophil actin dysfunction and cause diseases, including in Wiskott-Aldrich Syndrome and neutrophil immunodeficiency Syndrome (Nunoi, 2008). It is also confirmed that actin and ABPs play complex roles in cancer progression, including metastasis, DNA damage response and epithelial-mesenchymal transition (Yue et al., 2013; Izdebska et al., 2018). These studies highlight potential therapeutic protein targets associated with the diseases.

1.7.2 mRNA localization and diseases

Dysregulation of mRNA localization could lead to many genetic diseases. Recent studies revealed that both hyperassembly and hypoassembly of RNPs could result in human neurodegenerative diseases, such as spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS) (Shukla & Parker,

2016). Survival motor neuron (SMN) protein is closely associated with SMA, and a severe reduction of SMN can be fatal (Chaytow et al., 2018). SMN deficiency leads to an altered stoichiometry of small nuclear RNAs, which could cause pre-mRNA splicing defects (Zhang et al., 2008). SMN is also found to interact with a number of RBPs. Its localization is involved in the recruitment and transport of RNAs into axons and axon terminals (Dombert et al., 2014). Although the pathogenesis of SMA has not been fully elucidated, these results suggest that axonal mRNA localization and metabolism, regulated by RNPs and SMN, could be the cause of SMA (E. Wang et al., 2016).

Myotonic dystrophy (DM) is a form of muscular dystrophy characterized by progressing muscle wasting and weakness. DM type 1 is caused by an expanded CTG repeat in the Dystrophia Myotonic Protein Kinase (DMPK) gene, while DM type 2 is caused by an expanded CCTG repeat in the zinc finger protein 9 (ZNF9) gene (Brook et al., 1992; Margolis et al., 2006). The Muscleblind-like (MBNL) family of RBPs plays an important role in DM since it regulates splicing and mRNA localization. In DM, CUG and CCUG repeats accumulate in muscle nuclei and sequester MBNLs to prevent them from interacting with their normal RNA targets (E. Wang et al., 2012). Mislocalization of mRNAs in MBNL-depleted tissues is thought to lead to significant physiological consequences. Similar mechanisms may be applied to mRNA localization regulated by other RBPs.

RNA Binding Fox-1 Homolog-1 (RBFOX1), also called ataxin-2 binding protein-1 (A2BP1), is an RBP that is crucial for regulating neuron development and neuronal excitability. It has also been associated with intellectual disability, epilepsy and autism (C. L. Martin et al., 2007). The nuclear isoform of RBFOX1 functions as a splicing regulator, whereas the cytoplasmic isoform of the gene regulates mRNA levels of several genes important to cortical development and

autism (J.-A. Lee et al., 2016). Studies revealed that RBFOX1 binds to 3' UTR of its target mRNAs, including many autism-related genes, in the cytoplasm and promotes their stability in neurons (Ray et al., 2013; Voineagu et al., 2011). These data suggest that understanding RNA regulation would lead to novel therapies for brain disorders.

1.7.3 Basigin in diseases

Bsg has profound implications in a wide range of diseases because of its functions in intracellular communication, immunological responses, and the nervous system. It is most known for its role in malignant tumours. Bsg overexpression is seen in a broad range of human malignant tumours, including melanoma. Bsg regulates hyaluronan synthesis, a significant contributor to cell proliferation and migration, and interacts with hyaluronan-receptors as well as CD44, a cell surface receptor. It also facilitates tumour cell glycolysis by promoting lactate transport in combination with MCTs (Grass et al., 2014). Therefore, Bsg signals an unfavourable prognosis in cancer, suggesting invasiveness and chemoresistance. Bsg is involved in certain infectious diseases, such as malaria. *Plasmodium falciparum* erythrocyte invasion is considered to be an important step in malaria pathogenesis. PfRH5 (RH5), a member of the reticulocyte-binding family homolog, is a parasite ligand crucial for blood-stage growth. RH5 is different from the rest of the members of the family in that it is much smaller and found to be essential to the invasion of all strains of the parasite. Bsg is a receptor of RH5 and is required for erythrocyte invasion (Crosnier et al., 2011). This study also found that a low concentration of anti-Bsg antibodies could block invasion, implying anti-Bsg antibodies may become a treatment for drug-resistant malaria. Notably, Bsg was initially in the spotlight of COVID-19 research. Early research speculated that Bsg might be a receptor for

spike protein of SARS-CoV-2 and potentially facilitate host cell invasion, but this was later disproven. Bsg is not a direct binding receptor of SARS-CoV-2 spike, nor does it affect the susceptibility of human lung epithelial cells (Shilts et al., 2021).

1.7.4 Cofilin in diseases

Due to the important functions of cofilin in neurotransmitter release and synaptic plasticity, deficiency in cofilin can lead to abnormalities in learning, memory, and complex behaviours. Mutant mice, lacking cofilin-1 in the forebrain, show impairments in associative learning (Rust et al., 2010). Experimentally inactivating cofilin-1 in the forebrain leads to decreased anxiety in mice (Goodson et al., 2012). Elevated cofilin activities lead to an increase in accumulation of the AMPA receptor subunits GluR1 and GluR2 in the synapse (Gu et al., 2010). This phenomenon is observed to be independent of morphological changes of synaptic spines and accelerates the extinction of conditioned taste aversive memory (Y. Wang et al., 2013). Recent evidence suggests that cofilin plays an important role in neuronal apoptosis and neurodegeneration. Cofilin-actin rod aggregation can block intracellular organelle trafficking and lead to synaptic losses in both pre- and postsynaptic compartments as well as loss of dendritic spines. Cofilin-actin rods are also found to impair GluR response. This mechanism potentially explains the association between cofilin aggregation and neurodegenerative disorders (Cichon et al., 2012). Mitochondrial translocation of cofilin in early cell apoptosis has been observed, although it is considered not to be required for cell death signalling (Rehklau et al., 2011). A later study proposed another cofilin-mediated apoptosis pathway. It suggests that activated cofilin promotes neuronal apoptosis by forming a complex with p53 and translocating it to mitochondria and nucleus.

Phospholipase D1 negatively regulates this pathway through inactivation of cofilin and inhibition of cofilin/p53 complex formation (T. Liu et al., 2017). Furthermore, cofilin is involved in neuroinflammation. Glial cells, especially microglia and astrocytes, are first activated in response to brain lesions. These glial cells undergo morphological and functional changes that lead to increased cell proliferation, migration, and production of inflammatory cytokines (Alsegiani & Shah, 2020). Studies found that cofilin mediates hemin-induced microglial response and is important for oxidative stress (Sayeed et al., 2017). Cofilin knockdown inhibits lipopolysaccharide-induced microglial activation and reduces the release of pro-inflammatory mediators as well as microglial proliferation and migration (Alhadidi & Shah, 2017). These results suggest inhibition of cofilin could be a potential treatment for brain injuries.

Nemaline myopathy (NM) is a congenital myopathy characterized by proximal weakness, hypotonia, respiratory difficulties and facial weakness. NM can be caused by mutations of a number of genes, including cofilin-2, which is a muscle-specific isoform (Vartiainen et al., 2002). Cofilin-2 in mice is found to be not essential to muscle development but required for muscle maintenance (Agrawal et al., 2012). These results, along with the study conducted by Balakrishnan et al. in *Drosophila*, shed light on the disease progression of NM patients.

1.8 Aims of the project

Activity-dependent synaptic plasticity and new protein synthesis required in this process have been well-studied. The involvement of cytoskeleton and actin, in particular, is well-characterized. Our understanding of RBP regulation of mRNA localization and local translation during synaptic plasticity is increasing rapidly. Actin regulation at a protein level, by a network of ABPs, has been extensively

studied. However, the study of mRNA-based regulation of ABPs is still at its very beginning. An interesting study revealed Musashi (msi-1), an RBP, binds to mRNAs of three components of the Arp2/3 complex and is necessary for regulating memory loss in *C. elegans* (Hadziselimovic et al., 2014). This study underscores the potential mRNA-dependent regulatory network of actin in synaptic plasticity. As previously described, RBP regulation of mRNA localization plays a role in numerous neurological disorders. Furthermore, the mechanism of mRNA-based actin regulation can be generalized to other biological contexts, such as cell migration of fibroblast cells and cancer cells. In this thesis, I focused on the mRNA-level regulation of actin during activity-dependent synaptic plasticity.

The key question I am trying to answer in this thesis is: Whether actin is regulated locally at the **mRNA** level at the **synapse** during **activity**-dependent synaptic formation in *Drosophila* neuromuscular junction (NMJ)?

To answer this question, I set out the following aims:

1. Identify downstream targets of Syncrip that are expressed at the synapse and highly conserved to select my genes of interest (Bsg and Cofilin/Tsr).
2. Study the roles of Bsg and Cofilin/Tsr in synaptic plasticity pre- and postsynaptically.
3. Determine whether they are posttranscriptionally regulated during activity-dependent synaptic plasticity.

2 | Materials and Methods

2.1 Reagents and Fly Stocks

2.1.1 Solutions and Reagents

All solutions and reagents were prepared and autoclaved by the Department of Biochemistry media kitchen, unless otherwise stated. The composition of these solutions is given below:

Table 2.1: Solutions and Reagents

Solution	**Composition**
1M $MgCl_2$	For 1L: 203.3g $MgCl_2$.6H2O in 1L MilliQ water
1M $CaCl_2$	For 1L: 147.2g $CaCl_2$.2H2O in 1L MilliQ water
1M KCl	For 1L: 223.68g KCl in 1L MilliQ water
5M NaCl	For 1L: 292.2g NaCl in 1L MilliQ water
0.5M HEPES, pH 7.5	For 1L: 119.2g HEPES in MilliQ water, pH balanced to 7.5 with NaOH
10x PBS, pH 7.4	For 1L: 80g NaCl, 2g KCl, 14.4g NA_2HPO_4, 2.4g KH_2PO_4 in 900mL distilled water, pH balanced to 7.4, total volume adjusted to 1L with distilled water
20x SSC	For 1L: 175.3g NaCl, Tri-sodium citrate ($2H_2O$) in 800mL distilled water, pH balanced to 7.0 with HCl, total volume adjusted to 1L with distilled water
TE Buffer	10 mM Tris HCl, 1mM EDTA in diethylpyrocarbonate (DEPC) treated water

2.1.2 Fly Food Preparation

Batches of fly food were made by media kitchen following the recipe provided by Antony Knight. In Bucket 1, mix the malt and the molasses, and some of the water to make a easily pourable mixture. In bucket 2, make a suspension of Maize flour, Soya flour and Yeast powder in some of the water. Turn the Joni "120"

heating vessel on, add the remaining water and wait for the temperature to rise to 25-30°C. Add the agar. Continue to heat. Once the temperature reaches 94-96 °C the agar will have dissolved, pour the flour mixture gently into the kettle stirring continuously. Add molasses/malt mixture, stir a bit then turn off stirring paddle. Simmer the mixture for 2-4 minutes. Allow to cool, stirring continuously. When the temperature reaches 75 °C add the "acid mix" and the Nipagin solution. Mix well. Use the proprietary vial filling manifold ("Droso-filler"-Genesee USA) to dispense into bottles and vials. Tubes: The tubes are nominally 25 x 95 mm, fill 10 mL of food in each. Bottles are 55mm square at the bottom and 100mm high, fill 50 mL of food in each.

Table 2.2: Recipe

Ingredient	**Quantity**	**Source**
Organic Maize Fluor	1500g	Brian Drewitt Ingredients
Organic Soya Fluor	1500g	Brian Drewitt Ingredients
Yeast Powder	600g	Brian Drewitt Ingredients
Organic Malt Extract	900g	Brian Drewitt Ingredients
Organic Molasses (Rayners)	900g	Brian Drewitt Ingredients
Agar (80-100 mesh) BPE2641-1	162g	Fisher Scientific
Propionic Acid Free Acid Insect Cell Culture	95mL	Sigma Aldrich
Phosphoric Acid 85% Insect Cell Culture	5mL	Sigma Aldrich
METHYL P-HYDROBENZOATE, 99+%, FCC (Nipagin)	33.6g	Sigma Aldrich
Ethanol	168mL	
Water	24L	

2.1.3 Fly Stocks

Drosophila melanogaster stocks were kept at 18 °C, but transferred to 25 °C for crossed and experimental use. Temperature-sensitive activation was carried out at 30 °C. OrR was the wild type strain. Flies were raised on standard cornmeal-agar medium.

Table 2.3: Fly Stocks

	Genotype	**Description**	**Origin**
wild type	OregonR	wild type	
Msp300::YFP	w^{1118}; PBac{768.FSVS-0}Msp-300^{CPTI003472}	Endogenously YFP-tagged Msp300	University of Cambridge, Department of Genetics CPTI003472
Bsg::YFP	w^{1118}; PBac{544.SVS-1}Bsg^{CPTI100062}	Endogenously YFP-tagged Bsg	University of Cambridge, Department of Genetics CPTI100062
Tsr::YFP	w^{1118}; PBac{754.SVS-1}tsr^{CPTI002237}	Endogenously YFP-tagged tsr, balanced over CyO	University of Cambridge, Department of Genetics CPTI002237
bsg RNAi (1)	y[1]v[1];P{y[+t7.7] v[+t1.8]= TRiP.HMC03195} attP40	UAS-bsg RNAi inserted on the 2nd chromosome	Bloomington stock 52110
bsg RNAi (2)	w^{1118}; P{GD989}v2789	UAS-bsg RNAi inserted on the 3rd chromosome	VDRC ID 2789
tsr RNAi (1)	y[1]sc[*]v[1]sev[21]; P{y[+t7.7] v[+t1.8]= TRiP.HMS00534}attP2	UAS-tsr RNAi inserted on the 3rd chromosome	Bloomington stock 65055
tsr RNAi (2)	P{KK108706}VIE-260B	UAS-tsr RNAi inserted on the 2nd chromosome	VDRC ID 110599
mCherry RNAi	y[1]sc[*]v[1]sev[21]; P{y[+t7.7] v[+t1.8]=VALIUM20-mCherry}attP2	UAS-mCherry RNAi inserted on the 3rd chromosome	Bloomington stock 35785
MHC-Gal4	MHC-Gal4;;	Drives expression in larval and adult muscle	
OK371-Gal4	;OK371-Gal4;	Drives expression in glutamatergic neurons	

D42-Gal4	CyOGFO/Gla;D42-Gal4/Tm6B Tb	Drives expression in motor neurons	
TubGal80ts	w*;P{tubP-GAL80ts}20; TM2/TM6B, Tb1	Encodes GAL80ts expressed under the control of the αTub84B promoter	Bloomington stock 7019
Lifeact-GFP	y1 w*; PUAS-Lifeact-GFPVIE-260B	UAS regulatory sequences drive GFP expression that is tagged with Lifeact, an actin-binding peptide	Bloomington stock 35544
Lifeact-mRuby	y1 w*; PUAS-Lifeact-RubyVIE-19A	UAS regulatory sequences drive mRFPruby expression that is tagged with Lifeact, an actin-binding peptide	Bloomington stock 35545
balancer	CyOGFO/Gla; Tm6Btb/PriA	Doubly balanced line carrying CyOGFP over Gla on the 2nd chromosome and tm6 over Prickle on the 3rd chromosome	

2.2 Fluorescent Labeling

2.2.1 Immunofluorescence (IF) on fixed larval fillet

Wandering third-instar larvae were dissected in 0.3mM Ca^{2+} HL3 on sylgard plates to produce larval fillets with exposed NMJs (Brent et al., 2009). Samples were fixed in 4% paraformaldehyde (PFA) (16% PFA in 0.3% PBSTX) for 30 mins at room temperature (RT). Samples were then rinsed 3x with 0.3% PBSTX (0.3% Triton-X in 1x PBS). Blocking was not necessary because of the high

specificity of the primary antibodies, and therefore omitted. Primary antibodies were added to 0.3% PBSTX at the correct dilution (listed below), and samples were incubated overnight at 4°C with gentle rocking. The next day, samples were rinsed 3x in 0.3% PBSTX. AlexaFluor conjugated secondary antibodies (1:500), 4', 6-diamidino-2-phenylindole (DAPI), and Silicon rhodamine-Actin were added to 0.3% PBSTX. Samples were incubated in secondary antibody solution for 1.5hr at RT in the dark. Samples were briefly rinsed 3x in 0.3% PBSTX and then washed in 0.3% PBSTX for 45 minutes at RT. Finally, samples were incubated in Vectashield for 30 minutes at RT and then mounted on the slide in the mounting medium. Slides were either imaged immediately or stored at -20°C.

Table 2.4: Antibodies and Dyes used for immunofluorescence on fixed larval fillet

Antibody/Dye	**Concentration for IF**	**Description**	**Origin**
α-Dlg1	1:200	Labels Discs large 1, a postsynaptic scaffolding protein	Developmental Studies Hybridoma Bank, https://dshb.biology.uiowa.edu/
α-HRP conjugated to AlexaFluor 405	1:200	Labels neurons	Jackson ImmunoResearch Europe Ltd
α-HRP conjugated to AlexaFluor 488	1:500	Labels neurons	Jackson ImmunoResearch Europe Ltd
DAPI (5mg/mL)	1:500	Labels nuclei	
SiR-Actin (1mM)	1:2000	Labels actin	Spirochrome

2.2.2 Immunofluorescence (IF) on live larval fillet

Wandering third-instar larvae were dissected in 0.3mM Ca^{2+} HL3 on sylgard plates to produce larval fillets with exposed NMJs. AlexaFluor conjugated α-HRP and SiR-Actin were added to 0.3mM Ca^{2+} HL3 at correct dilutions as indicated below. Samples were incubated in the solution for 30 minutes at RT

with gentle rocking in the dark. Samples were then rinsed 3x with 0.3mM Ca^{2+} HL3 before being imaged immediately.

Table 2.5: Antibodies and Dyes used for immunofluorescence on live larval fillet

Antibody/Dye	**Concentration for IF**	**Description**	**Origin**
α-HRP conjugated to AlexaFluor 405	1:50	Labels neurons	Jackson ImmunoResearch Europe Ltd
α-HRP conjugated to AlexaFluor 488	1:125	Labels neurons	Jackson ImmunoResearch Europe Ltd
SiR-Actin (1mM)	1:1000	Labels actin	Spirochrome

2.2.3 Enzymatic Single molecule RNA fluorescent in situ hybridization (smFISH) probe synthesis

smFISH probes were designed using the Stellaris®Probe Designer version 4.2. The sequences of the designed probes are shown in Appendix A. Oligonucleotides were labelled with fluorescent dyes using a protocol adapted from the paper (Gaspar et al., 2017). The oligonucleotides were pooled and elongated overnight at 37 °C in terminal deoxynucleotidyl transferase (TdT) reaction mix with a ddUTP conjugated to a desired dye (see Table 2.5). The fluorescently labelled oligonucleotides were then purified with Zymo Research®Ethanol DNA Purification kit and eluted in TE buffer. OD260 and dye absorption maxima were measured using a NanoDrop spectrophotometer to calculate the degree of labelling and probe concentration. The probes were then diluted to 25 µM with TE buffer and stored at -20 °C.

Table 2.6: TdT Reaction Mix (15 µL)

	Sulfo-Cy3	**AlexaFluor 647**	**ATTO 633**
Oligos (200 µM)	5 µL	5 µL	5 µL
Dye-ddUTP (1 mM)	2.5 µL	3 µL	3 µL
TdT buffer (5x)	3 µL	3 µL	3 µL
TdT Enzyme	0.6 µL	0.6 µL	0.6 µL
Nuclease-free H2O	3.9 µL	3.4 µL	3.4 µL

2.2.4 Single molecule RNA fluorescent in situ hybridization (smFISH) for larval fillet

smFISH was carried out in a protocol adapted from the article (J. S. Titlow et al., 2018). Wandering third-instar larvae were dissected in 0.3mM Ca^{2+} HL3 on sylgard plates to produce larval fillets with exposed NMJs. Samples were fixed in 4% PFA (16% PFA in 0.3% DEPC PBSTX) for 30 minutes at RT. Samples were rinsed 3x in 0.3% DEPC PBSTX, and then permeabilized for 2x 20 minutes in 0.3% DEPC PBSTX. Samples were incubated in Wash Buffer (10% deionized formamide (stored at -80 °C) and 2x SSC in DEPC water) for 30 minutes at 38 °C. Samples were then incubated with 250 nM smFISH probes Hybridisation Buffer (10% deionized formamide, 2x SSC, 10% dextran sulphate, and primary antibodies in DEPC water) overnight at 38 °C. The next day, samples were washed for 2x 20 minutes in Wash Buffer at RT and then washed for 2x 10 minutes in 2x SSC. In the meantime, to prepare RNA-free Block Buffer, 1x RNAsecure and 0.3% Triton-X were added to equal parts of Odyssey and DEPC 1x PBS, and the buffer was then incubated at 60 °C for 1hr. Ribonucleoside vanadyl complex (RVC) was added (at 1:100 dilution) to Block Buffer after it was cooled to RT. Samples were next incubated for 1hr in RNA-free Block Buffer. Samples were then incubated in Counterstain Buffer (DAPI,

GFP-Booster, and secondary antibodies added to Wash Buffer at correct concentration) for 1hr. Samples were rinsed 3x in 0.3% DEPC PBSTX and then washed 3x 15 minutes in 0.3% DEPC PBSTX. Finally, samples were incubated in Vectashield for 30 minutes at RT and then mounted on the slide in the mounting medium. Slides were either imaged immediately or stored at -20 °C.

Table 2.7: Antibodies and Dyes used for immunofluorescence on fixed larval fillet

Antibody/Dye	**Concentration for IF**	**Description**	**Origin**
Probes	1:100	DNA oligos	Integrated DNA Technologies
α-Dlg1	1:160-200	Labels Discs large 1, a postsynaptic scaffolding protein	Developmental Studies Hybridoma Bank, https://dshb.biology.uiowa.edu/
α-HRP conjugated to AlexaFluor 405	1:80-100	Labels neurons	Jackson ImmunoResearch Europe Ltd
GFP-Booster Alexa Fluor 488	1:1000	anti-GFP VHH/Nanobody conjugated to a fluorescent dye	Chrometek
DAPI (5mg/mL)	1:500	Labels nuclei	

2.3 Activity-Dependent Synaptic Plasticity Assay

2.3.1 HL3 Solution Recipes

The Haemolymph-Like Salines (HL3) were prepared as described previously (Roche et al., 2002; Ataman et al., 2008) from solutions listed in Table 2.1.

Table 2.8: HL3 Solution Recipes

Solution	Composition
HL3 0.3mM Ca^{2+}	NaCl 70 mM, KCl 5 mM, $MgCl_2$ 20 mM, $NaHCO_3$ 10 mM, Trehalose 5 mM, HEPES 5 mM, Sucrose 115 mM, Ca^{2+} 0.3 mM, pH 7.2
HL3 1mM Ca^{2+}	NaCl 70 mM, KCl 5 mM, $MgCl_2$ 20 mM, $NaHCO_3$ 10 mM, Trehalose 5 mM, HEPES 5 mM, Sucrose 115 mM, Ca^{2+} 1 mM, pH 7.2
HL3 High K^+	NaCl 40 mM, KCl 90 mM, Ca^{2+} 1.5 mM, $MgCl_2$ 20 mM, $NaHCO_3$ 10 mM, Trehalose 5 mM, HEPES 5 mM, Sucrose 5 mM, pH 7.2

2.3.2 Spaced High K^+ Depolarization Paradigm

The spaced high K^+ depolarization paradigm was used in Ataman et al., 2008. The protocol was described in detail (Ramachandran & Budnik, 2010). Wandering third-instar larvae were dissected in 0.3mM Ca^{2+} HL3 on sylgard plates. The head and tail positions were pinned to shorten the larvae along the vertical axis. The larvae were then transferred to 1mM Ca^{2+} HL3. During the assay, the stimulated samples were washed and submerged in high K^+ HL3 for periods of 2, 2, 2, 4 and 6 minutes, with 15 minutes intervals of being submerged in 1mM Ca^{2+} HL3 in between. The control samples were treated with the same intervals of solution change, except 1mM Ca^{2+} HL3 was used in place of high K^+ HL3. After the last stimulation, the larvae were left to rest in 1mM Ca^{2+} HL3 for 45 minutes. The entire protocol takes approximately 2.5 hrs. The larvae were then stretched to their natural length, pinned on both sides, fixed, and labelled as described in IF or smFISH protocol.

2.4 Image Acquisition and Image Analysis

2.4.1 Acquisition of IF on fixed larval fillet

Mounted samples were imaged using PerkinElmer spinning-disk Ultraview confocal microscopes or Olympus CSU-W1 SoRa laser spinning-disk confocal microscope. Images were acquired using 100x 1.4NA Oil UPlanApo objective (PE) or 100x 1.45NA Oil UPlanXApo (SoRa). Laser units used were solid-state 405, 488, 568 and 640 lasers for both microscopes. NMJs at muscles 6 and 7 in segments 3 to 5 were imaged.

2.4.2 Acquisition of IF on live larval fillet

Dissected samples in the petri dish were imaged using Zeiss LSM 980 with AiryScan2, with 20x 1NA Water Dipping Objective in 0.3 mM Ca^{2+} HL3. The laser units used were solid-state 405, 488, 561 and 634 lasers. Images were acquired with AiryScan 2 detector and Multiplex SR-4Y imaging module. NMJs at muscles 6 and 7 in segments 3 to 5 were imaged.

2.4.3 Acquisition of smFISH on larval fillet

Mounted samples were imaged using Olympus CSU-W1 SoRa laser spinning-disk confocal microscope, with 100x 1.45NA Oil UPlanXApo and 95B camera. The laser units used were solid-state 405, 488, 568 and 640 lasers. NMJs at muscles 6 and 7 in segments 3 to 5 were imaged. Samples from the same experiment were imaged using the same settings.

2.4.4 Image Analysis on smFISH

BigFish pipeline developed by Ph.D. candidate Danail Stoychev in Davis lab was used to quantify nascent mRNA transcripts. (Link to GitHub repository: https://github.com/dstoychev/smFISH_Pypeline). Briefly, the program is written in Python 3. It takes the acquisition parameters and detects spots. Then, a threshold was used to decompose clusters and calculate the number of nascent transcripts by the integrated intensity. Settings for detection and nascent transcript calculation were then applied to all images in a batch setting.

2.4.5 Image analysis to quantify synaptic plasticity

Synaptic plasticity was quantified by counting the number of immature boutons, also known as ghost boutons (GBs), in images of samples that underwent spaced high K^+ depolarization paradigm. Ghost boutons were labelled with α-HRP antibody but lacked the postsynaptic terminal labelled with α-Dlg. The guideline (Gala et al., unpublished) followed to identify ghost boutons in this project was written by Dalia Gala, a DPhil student in Davis lab.

2.5 Bioinformatics and Statistical Methods

2.5.1 Bioinformatics Analysis to Select Genes of Interest

The script and datasets used to select genes of interest can be found in my GitHub repository: https://github.com/gracia-gu/GOISelection. The program was written in Python 3. The packages: g:Profiler was used to find orthologs (Reimand et al., 2016), pandas was used to intersect the datasets (McKinney, 2010), and Matplotlib was used for data visualization (J. Hunter, 2007).

2.5.2 Statistical Analysis

The script used for statistical analysis was written by Dr. Joshua Titlow, a Post-Doc researcher who worked in Davis lab (Link to GitHub repository: https://github.com/jstitlow/Rscripts). The program was written in R and can be run using JupyterLab. Shapiro-Wilk test for normality was used to determine if the data is normally distributed. When two sets of data were being compared if the data was normally distributed, Welch Two Sample t-test was used. Otherwise, Wilcoxon rank-sum test was used. When more than two sets of data were being compared, one-way ANOVA with Tukey Post-hoc was used if the data was normally distributed. Otherwise, Dunn's test with Bonferroni correction was used. For data visualization, ggplot2 was used (Wickham, 2016).

3 | Results

3.1 Summary of Previous Work

Davis lab has conducted a large-scale survey on mRNA and protein localization in the *Drosophila* larval nervous system using smFISH and discovered a high level of discordance between the distribution of mRNA and protein from individual genes (Titlow et al., unpublished). Syp and Imp, two RBPs that are known to be required for brain development, were found to bind to transcripts of hundreds of other genes directly. In a recent study, Syp was found to posttranscriptionally regulate Msp300, an ABP required for activity-dependent synaptic plasticity at the larval NMJ (S. Titlow et al., 2020). In response to activity, Msp300 protein levels increase while msp300 mRNA levels remain unchanged. Davis lab proposed that posttranscriptional regulation of ABPs could be a conserved method and was interested in finding other genes that behave like Msp300. Individual nucleotide resolution crosslink and immunoprecipitation (iCLIP) experiment was performed by Ph.D. candidate Jeff Lee from Davis lab to determine the direct RNA binding targets of Syp in the *Drosophila* nervous system. The data analysis was done following a pipeline described previously (Busch et al., 2020). 197 genes were identified in the *Drosophila* larval brain and neuromuscular synapse. Gene ontology analysis on these genes revealed that many of them have functions in actin-binding, actin filament binding and other cytoskeletal-related processes (Lee et al., unpublished).

3.2 Candidate Gene Selection

To select genes that are known to be expressed at the synapse and are highly conserved, three datasets: neurite transcriptome (Kugelgen & Chekulaeva, 2020), Syp iCLIP in rats (Khudayberdiev et al., 2020), and Syp iCLIP in *Drosophila* (Lee et al., unpublished), were intersected using the method described in section 2.5.1. A list of 13 genes was found (Table 3.1; Appendix B), and all of them have actin-binding or actin-related functions. Notably, Arp3 and arpc5 are subunits of the Arp2/3 complex, which is involved in F-actin polymerization and branching. Cpa encodes a capping protein that binds to the barbed end of F-actin, blocking the exchange of actin monomers at this end. Tsr (Cofilin) severs F-actin and facilitates actin depolymerization. To better understand how Syp might regulate the mRNA transcripts of these genes, the binding domains were identified in *Drosophila* Syp iCLIP. Syp bound to the 5' UTR, the 3' UTR, the coding sequence (CDS) or a combination out of the three, suggesting that there is no universal mechanism of Syp regulation of the list of 13 genes. Syp could be involved in a variety of processes in posttranscriptional regulation, including mRNA stability, localization, and translation. Syp binding domains of bru1, CG10915, and Dg are different in the *Drosophila* brain versus the whole larva, implying different regulation mechanisms might be used in the brain and the rest of the larva.

Table 3.1: List of 13 *Drosophila* Syncrip binding targets

Gene Names	**Description**	**Syncrip Binding Domains in *Drosophila* Brain**	**Syncrip Binding Domains in *Drosophila* whole Larva**
Actin-Binding			
Arp3	An ATP-binding component of the Arp2/3 complex, involved in actin polymerization and branching	5' UTR	5' UTR
Arpc5	Part of the Arp2/3 complex, which is involved in F-actin polymerization	3' UTR, 5' UTR	3' UTR, 5' UTR
cpa	Encodes an ABP binds to the fast growing end of actin to restrict the addition/loss of monomers	3' UTR	3' UTR
SCAR	Encodes a primary regulator of the Arp2/3 complex	3' UTR	3' UTR
tsr	Cofilin: exhibits F-actin depolymerizing activity and regulates actin cytoskeleton dynamics	3' UTR, CDS, 5' UTR	3' UTR, CDS, 5' UTR
Actin-Related			
bru1	Encodes a RBP whose product is required for gametogenesis, developmental patterning, and muscle organization	5' UTR	5' UTR, 3' UTR
Bsg	Encodes an IgG family glycoprotein that promotes cytoskeleton rearrangement	3' UTR, CDS, 5' UTR	3' UTR, CDS, 5' UTR
CanB2	Calcineurin B2: a calcium-binding and calmodulin-binding protein	3' UTR	3' UTR
CG10915	Functions by slowing the dissociation of Cortactin from Arp2/3 nucleated branches		3' UTR

Dg	Dystroglycan: encodes a ECM receptor that connects ECM to the actin cytoskeleton	5' UTR	5' UTR, CDS
Ggamma1	encodes a protein involved in the G-protein coupled receptor signaling pathway	3' UTR, 5' UTR	3' UTR, 5' UTR
flw	serine/threonine protein phosphatase type 1β(PP1β): regulation of nonmuscle myosin	3' UTR	3' UTR
Rho1	Encodes a GTPase signaling protein that acts as a molecular switch and functions as key regulator of the actin cytoskeleton	3' UTR, CDS, 5' UTR	3' UTR, CDS, 5' UTR

To further narrow down candidate genes, a visualization of Syp iCLIP *Drosophila* and neurite transcriptome datasets was created to select genes that are highly expressed and conserved across the animal kingdom. The reads per million (RPM) data from Syp iCLIP in *Drosophila* larval brain, Syp iCLIP in the whole larva, and the mean transcripts per million (TPM) from the neurite transcriptome meta-analysis were plotted against the number of datasets in which the gene has a TPM > 10 in the neurite transcriptome study (Figure 3.1). Bsg (Nptn in mice), present in all 15 datasets, is the most highly expressed in the whole larva (Figure 3.1A). Tsr (Cfl1 in mice), present in 14 out of 15 datasets, is the most highly expressed in neurite transcriptome data and has a local peak in the larval brain (Figure 3.1B, C). Therefore, Tsr and Bsg were selected as genes of interest in this project.

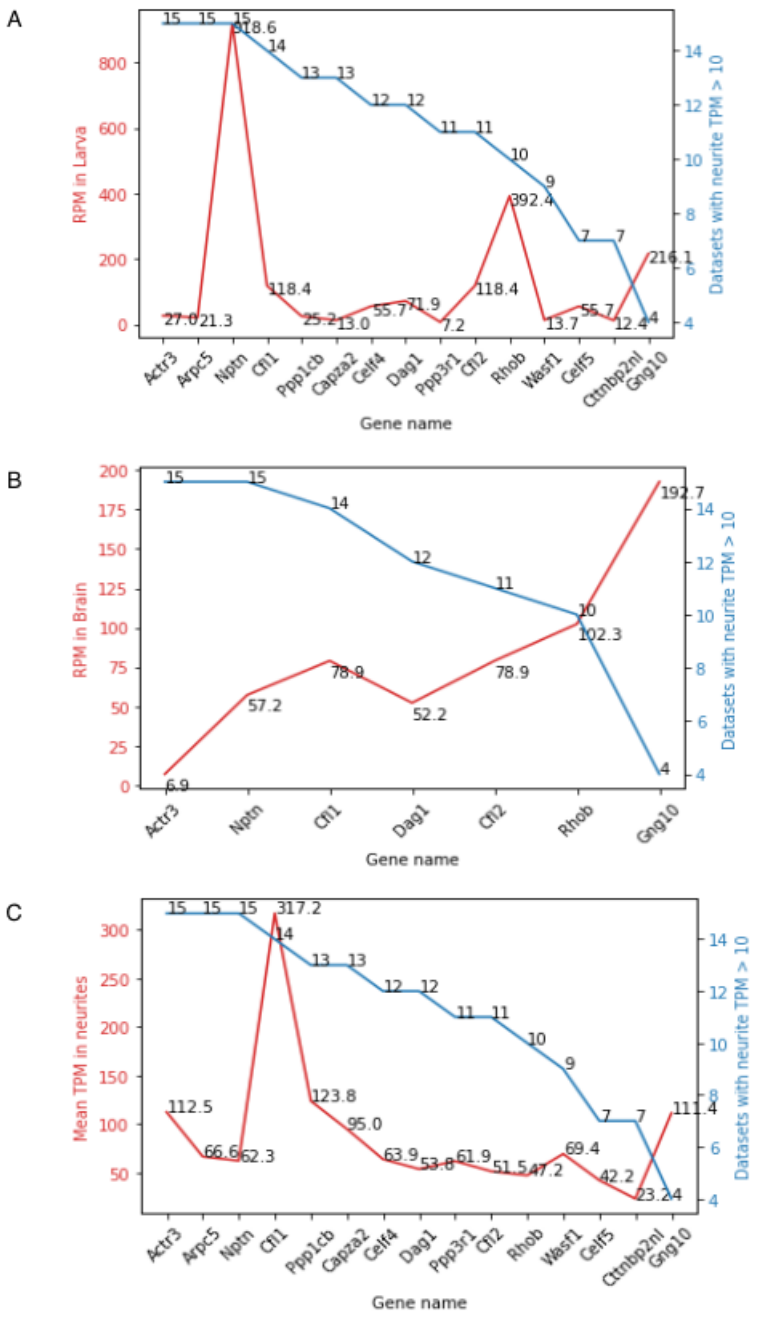


Figure 3.1: **Candidate Gene Selection.**
Blue lines in all three subfigures represent the number of neurite datasets in which each gene has TPM > 10. Red lines in subfigures A, B, and C represent RPM in *Drosophila* whole larva Syp iCLIP, RPM in *Drosophila* brain Syp iCLIP, and mean TPM in neurite dataset respectively. Nptn and Cfl1 are mouse orthologs of Basigin and Cofilin in *Drosophila*.

3.3 The role of Bsg in synaptic plasticity

3.3.1 Loss of *bsg* in the postsynaptic compartment promotes synaptic plasticity

Bsg is a transmembrane protein that promotes cytoskeletal rearrangements. Bsg is found to localize on both pre- and postsynaptic membranes and is important to regulate bouton size during development (Besse et al., 2007). To examine the function of Bsg in the postsynaptic compartment, *bsg* RNAi was expressed using *bsg* RNAi (1) line under the MHC-Gal4 driver, a muscle-specific driver. UAS-*mCherry* RNAi expressed under the same MHC-Gal4 driver was used as a control. The effectiveness of the RNAi was quantified using smFISH (Supplementary B.1). The number of boutons was quantified at each synapse to identify whether *bsg* knockdown driven by a muscle-specific driver leads to a developmental phenotype. The diameters of boutons at each NMJ were measured manually in ImageJ, and the average diameter was calculated. Although *bsg* knockdown larvae had slightly higher mean bouton numbers and sizes, no statistically significant difference between *bsg* knockdown and control was observed (Figure 3.2D). Therefore, the developmental effect caused by the loss of *bsg* in the postsynaptic compartment was non-significant.

The role of Bsg in the postsynaptic compartment during plasticity was then studied by applying spaced high K^+ stimulation on both third-instar *bsg* knockdown and control larvae. Larvae expressing *bsg* RNAi under the muscle-specific driver produced, on average, around twice as many ghost boutons (GBs) as the control (Figure 3.2E). Lack of *bsg* in the postsynaptic compartments facilitates GB formation and synaptic plasticity. This result implies that Bsg is potentially involved in the formation of postsynaptic

terminals in response to stimulation.

3.3.2 Loss of *bsg* in the presynaptic compartment leads to an increase in bouton size

To study the role of Bsg in the presynaptic compartment, *bsg* RNAi was expressed using *bsg* RNAi (1) line under the OK371-Gal4 driver, a motoneuron-specific driver. UAS-*mCherry* RNAi expressed under the same OK371-Gal4 driver was used as a control. As mentioned above, Bsg plays a role in restricting bouton size during development. The numbers and average diameters of boutons at each NMJ in both *bsg* knockdown and control were quantified in ImageJ to identify the effect of presynaptic Bsg during development. Although *bsg* RNAi NMJs had a lower average number of boutons compared to control, the difference was not statistically significant. However, the average bouton size of *bsg* RNAi was over 60% larger than that of the control (Figure 3.3). The increase in bouton size in presynaptically *bsg* knockdown larvae is consistent with the known function of Bsg. Due to the developmental effect of *bsg* deficiency in the presynaptic compartment, a conditional knockdown experiment, for instance, using the Gal4/Gal80 temperature-sensitive system, is needed to isolate the function of presynaptic Bsg during synaptic plasticity from development.

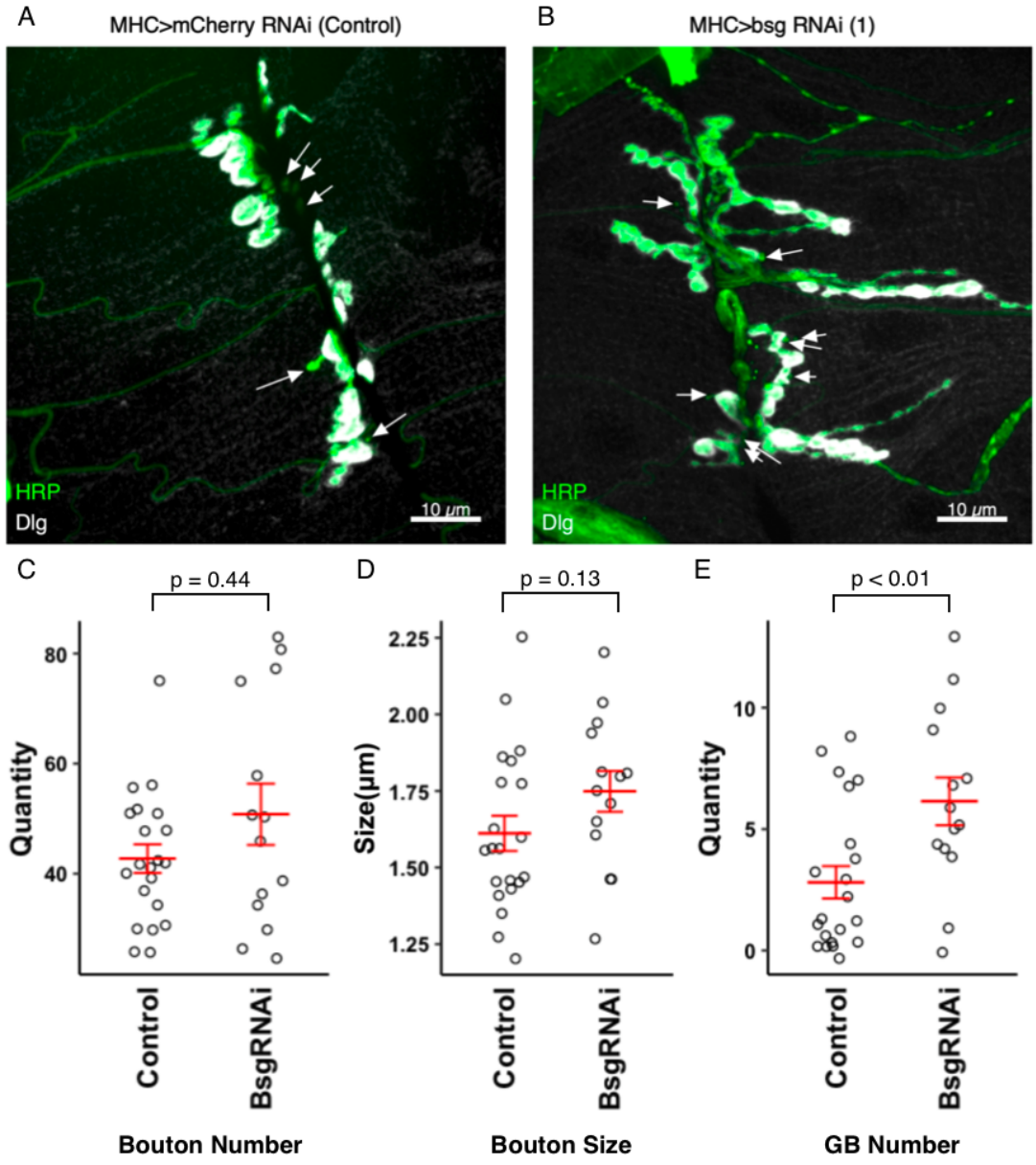


Figure 3.2: **Postsynaptic *bsg* knockdown promotes GB formation during spaced K^+ stimulation.**
A, B: NMJs were labelled with α-HRP (axon) and α-Dlg (postsynaptic density). Arrows indicate GBs. C-D: Quantifications of bouton number and bouton size, measured by diameter, show no statistically significant difference in *bsg* knockdown compared to control. Bouton size E: Quantification of GB number shows that *bsg* knockdown leads to an increase in GB number compared to control.

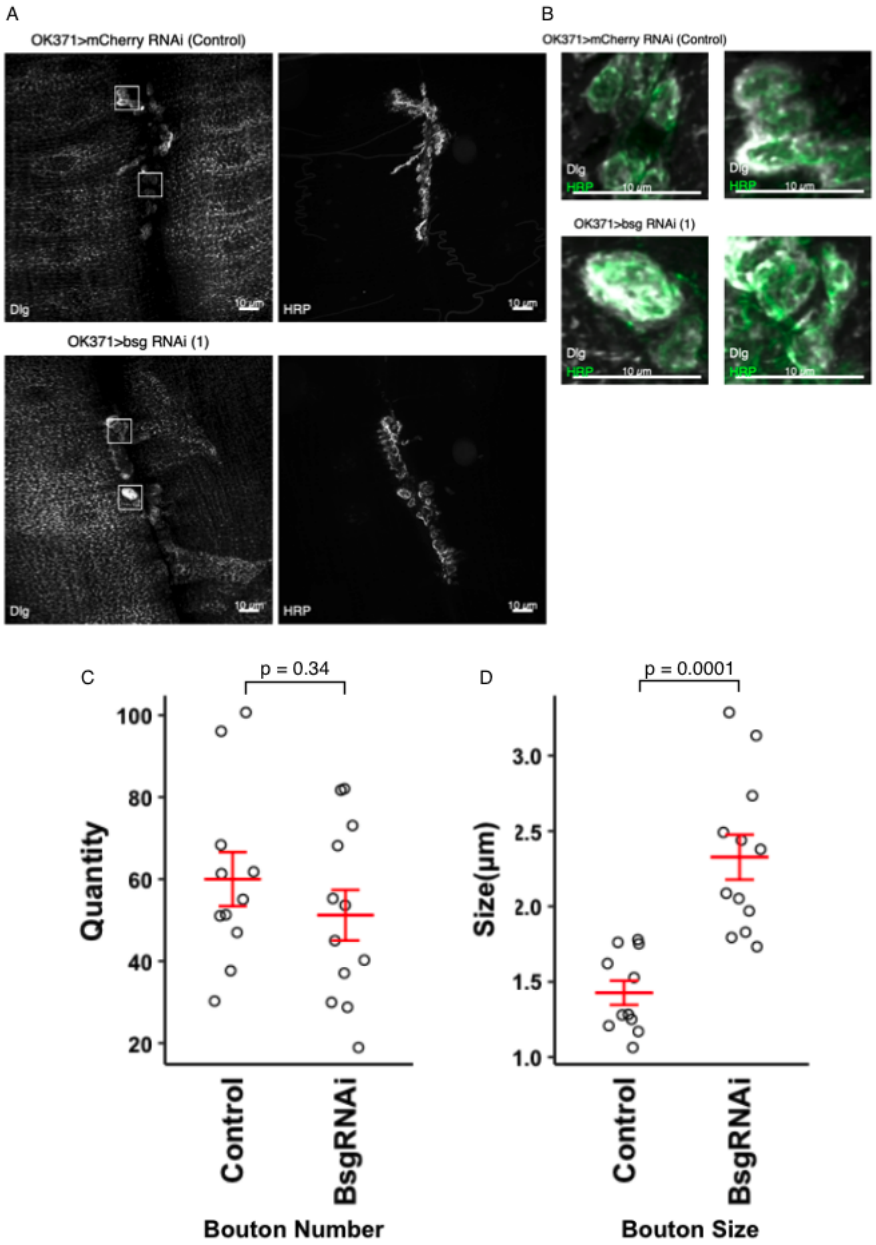


Figure 3.3: **Loss of *bsg* presynaptically leads to an increase in bouton size during development.**
A: NMJs were labelled with α-HRP (axon) and α-Dlg (postsynaptic density). Squares highlight clusters of boutons shown in panel B. B: Close-ups of selected regions in panel A. Larger bouton size is observed in *bsg* RNAi driven by OK371-Gal4 driver compared to control. C: Quantifications of bouton number shows a lower average in *bsg* knockdown relative to control, although the difference is not significant. D: Quantification of bouton size shows a notable increase in bouton size in *bsg* knockdown relative to control.

3.4 Function of Tsr (Cofilin) in synaptic plasticity

3.4.1 Loss of *tsr* in the postsynaptic compartment affects muscle structure but does not have a significant effect on synaptic plasticity

While it is clear that Tsr has a significant function in regulating actin, whether Tsr is involved in synaptic plasticity in *Drosophila* is still unclear. To investigate the function of Tsr in the postsynaptic compartment, *tsr* RNAi was expressed using *tsr* RNAi (1) line under the MHC-Gal4 driver, and UAS-*mCherry* RNAi expressed under the same MHC-Gal4 driver was used as a control. *Drosophila* with muscle-specific Tsr knockdown exhibited muscle weakness, slow movement speed in the larval stage and were smaller for those that reached the adult stage. After dissection and IF, third-instar larvae with *tsr* knockdown showed severely disrupted muscle and synaptic structures, especially in the postsynapse, compared to control (Figure 3.4). The phenotype in the muscle has been observed previously (Balakrishnan et al., 2020). The effectiveness of the RNAi was measured using smFISH by quantifying RNA transcripts in the muscle of *tsr* knockdown and control (Supplementary B.1).

In order to separate the function of Tsr during synaptic plasticity from its developmental effect, a conditional knockdown of Tsr with Gal80ts/Gal4/UAS was employed. The desired *Drosophila* line was produced through genetic crossings and was kept at 30 °C for 48 hours prior to experiment to allow proper muscle development up to second-instar larva as well as a deprivation of Tsr RNA and protein during spaced high K^+ stimulation. *Wild-type* OregonR (OrR) flies were used as a control. Conditional *tsr* knockdown larvae showed none to mild levels of disrupted muscle structure but maintained regular synaptic

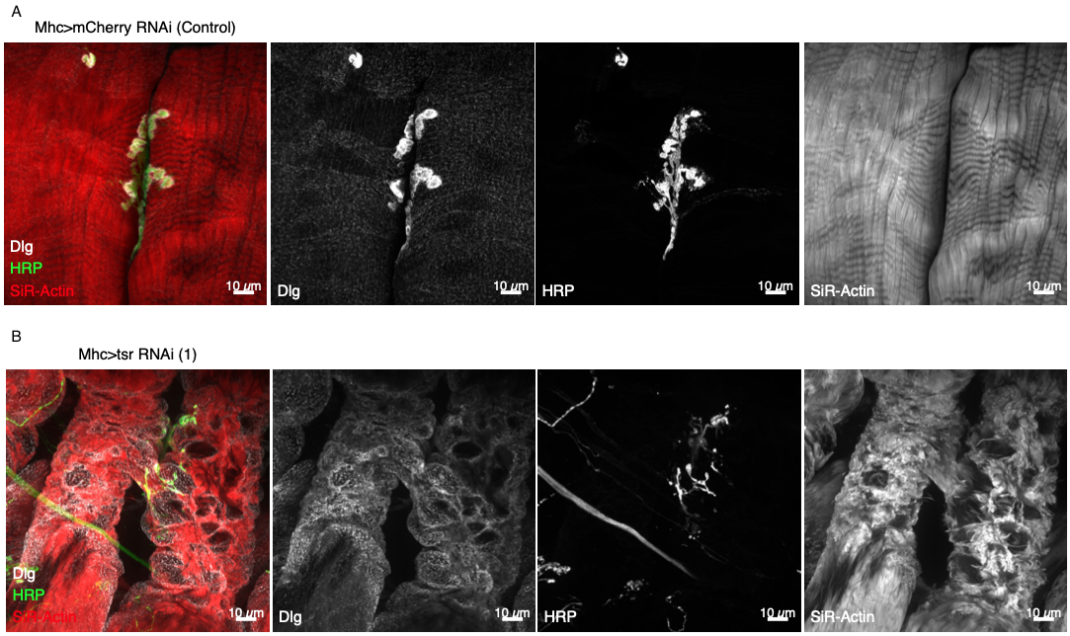


Figure 3.4: **Postsynaptic *tsr* RNAi under MHC-Gal4 driver affects muscle and synaptic structures during development**
A, B: *mCherry* RNAi and *tsr* RNAi driven by MHC-Gal4 driver respectively. NMJs were labelled with SiR-actin (actin), α-HRP (axon) and α-Dlg (postsynaptic density). Abnormal muscle structure, characterized by the formation of actin aggregates, and synaptic morphology, characterized by the loss of defined postsynaptic compartment, in *tsr* RNAi larvae are shown compared to control.

morphology (Figure 3.5A-D). After the stimulation, the numbers of GBs were quantified in both *bsg* knockdown and control. Although the *tsr* knockdown group had a slightly higher mean, no statistically significant difference between the GB numbers of control and conditional *tsr* knockdown was observed (Figure 3.5E). This result suggests that deficiency of Tsr in the postsynaptic compartment might increase but K^+-induced synaptic plasticity but the result is inconclusive.

3.4.2 Loss of *tsr* in the presynaptic compartment has an inhibitory effect on synaptic plasticity

To investigate the function of Tsr in the presynaptic compartment, *tsr* RNAi was expressed using *tsr* RNAi (1) line under the OK371-Gal4 driver, and UAS-*mCherry* RNAi expressed under the same OK371-Gal4 driver was used as a control. Larvae with motoneuron-specific *tsr* knockdown showed normal muscle and synaptic structures compared to control (Figure 3.6A-D). However, flies with *tsr* knockdown were lethal in the pupal stage and did not survive to adulthood. Spaced high K^+ stimulation was performed on both OK371>*tsr*RNAi and control larvae. The numbers of GBs were quantified after the experiment. The result showed that *tsr* knockdown larvae produced over 60% fewer GBs than control larvae. Fewer GBs formation in larvae lacking *tsr* in the presynaptic compartment indicates that the loss of *tsr* in motoneurons inhibited GB formation (Figure 3.6E). GBs formed in *tsr* knockdown larvae were small and close to existing boutons and branches, potentially the product of budding. Large GBs away from the main branch were not observed in *tsr* knockdown larvae. This result suggests that different actin reorganization mechanisms might be involved for different types of GB formation.

Since Tsr is crucial in maintaining actin dynamics, it is possible that the loss of

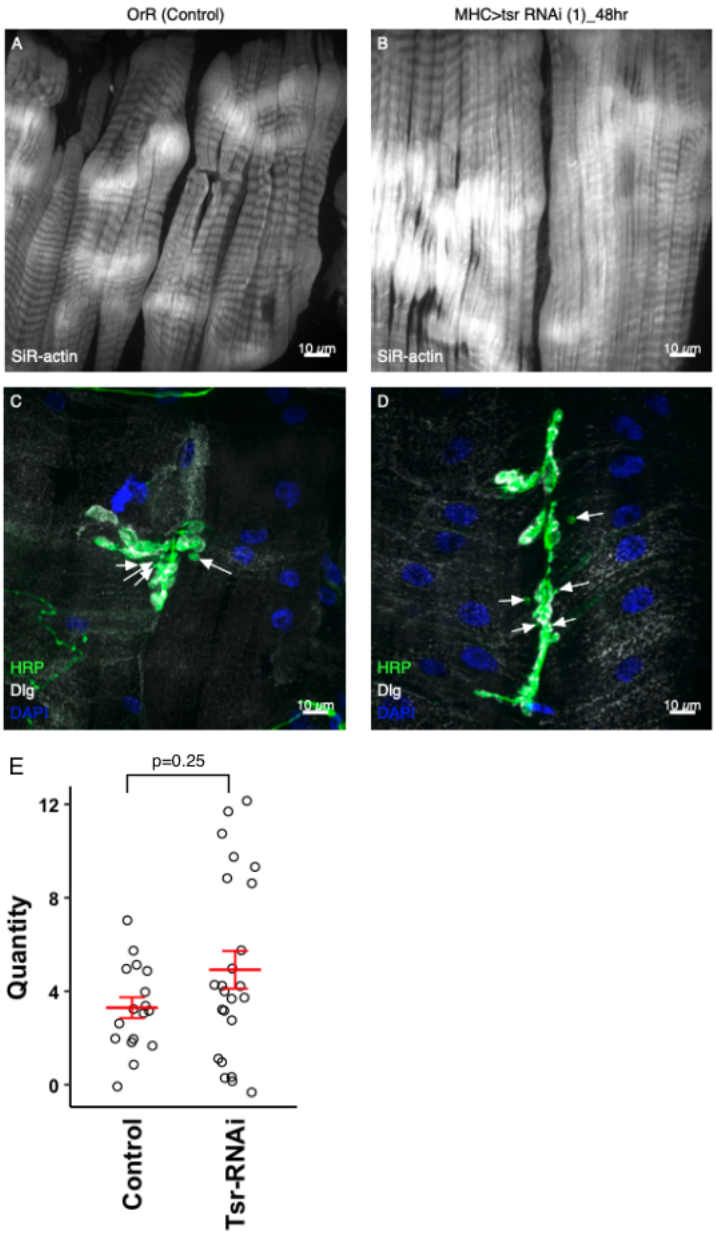


Figure 3.5: **Activity-dependent synaptic plasticity in postsynaptic conditional *tsr* knockdown and *wild-type* control.**
NMJs were labelled with SiR-actin (actin), α-HRP (axon), α-Dlg (postsynaptic density), and DAPI (nuclei). A, B: staining of actin showing muscle structures in control and conditional *tsr* knockdown larvae. C, D: Staining of the synapses in control and conditional *tsr* knockdown larvae. Arrows indicate GBs. No defects in muscles and the synapse are seen here. Quantification of GBs show a slightly higher average bouton number in *tsr* conditional knockdown relative to control, but the difference is not statistically significant.

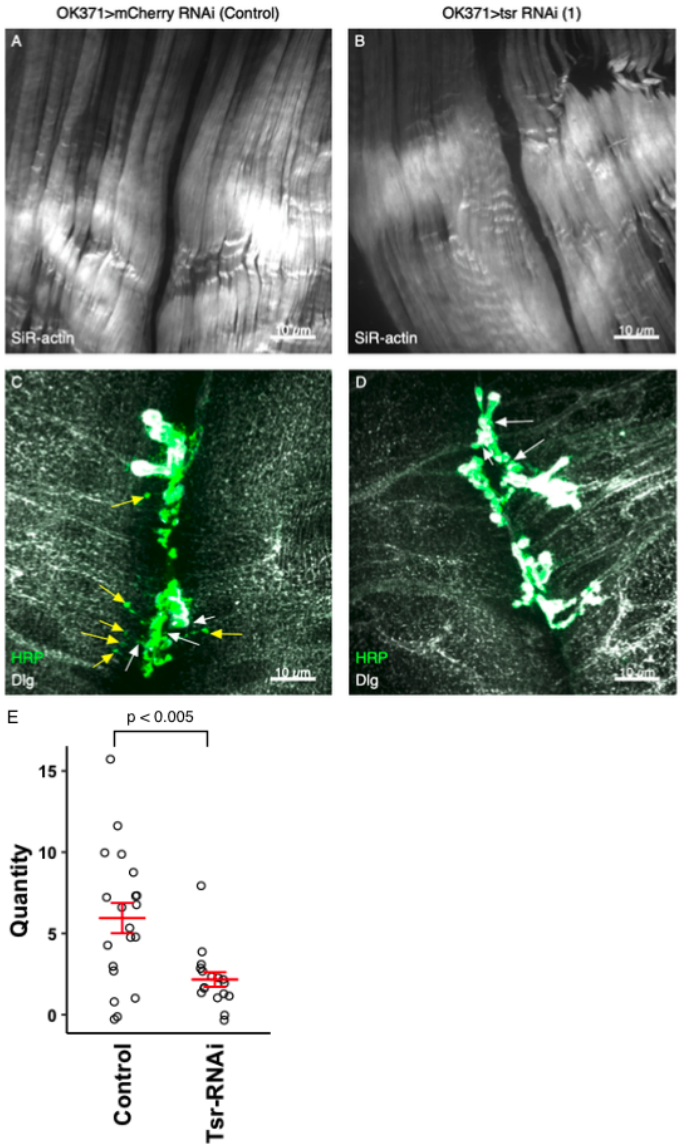


Figure 3.6: **Loss of *tsr* in motoneurons inhibits GB formation during activity-dependent synaptic plasticity.**
NMJs were labelled with SiR-actin (actin), α-HRP (axon), and α-Dlg (postsynaptic density). A, B: Actin staining in the muscle. C, D: Staining of the synapses. E: Quantification of GBs in control and *tsr* knockdown larvae. Arrows indicate GBs and yellow arrows indicate GBs formed away from the main branch. A-D: *tsr* knockdown driven by OK371-Gal4 does not exhibit any abnormal phenotype in muscle and the synapse in the larval stage. C-E: The average number of GBs formed in *tsr* knockdown is less than 40% of that of the control after K^+ stimulation. GBs away from the main branch are only seen in control and not in *tsr* knockdown larvae.

tsr in motoneurons could lead to an abnormal cytoskeleton in the axons. Abnormalities in actin structures could, in turn, prevent important structural proteins involved in synaptic plasticity from reaching the synapse. To observe F-actin structure in motoneurons, a *Drosophila* line that expresses Lifeact-GFP under D42-Gal4 driver, a motoneuron-specific driver, was generated. Here, D42-driver was used instead of OK371-Gal4 for the ease of setting up genetic crosses. Lifeact is a small peptide that binds to F-actin to allow actin visualization in live organisms. D42>Lifeact-GFP line is then crossed to the *tsr* RNAi (2) line to express *tsr* knockdown in motoneurons. Here, *tsr* RNAi (2) line was used instead of *tsr* RNAi (1) line because *tsr* RNAi (1) driven under D42-Gal4 driver was lethal in the larval stage, while *tsr* RNAi (2) generated a weaker knockdown phenotype in muscle (Balakrishnan et al., 2020). *mCherry* RNAi crossed to the same D42>Lifeact-GFP was used as control. Images were acquired in live samples. Actin was observed in motoneurons and presynaptic boutons, labelled with α-HRP. Similar expression patterns were observed in both *tsr* knockdown and control. However, the concentration of actin was not quantified due to the autofluorescence signal in the muscle (Figure 3.7). Nevertheless, this result suggests that larvae with *tsr* knockdown in motoneurons were likely to have functional actin cytoskeletons. Therefore, a deficiency of *tsr* led to reduced GB formation and synaptic plasticity but did not have a noticeable development effect in larvae motoneurons and axon terminals.

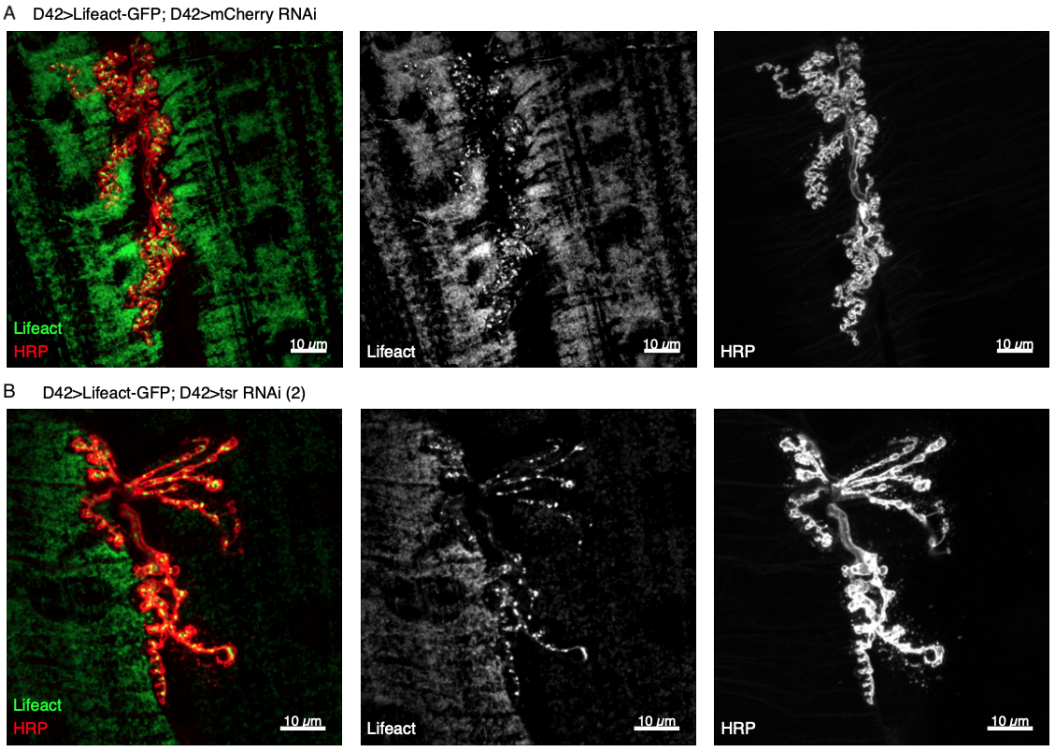


Figure 3.7: **Live-imaging of presynaptic actin in *tsr* knockdown and control under D42-Gal4 driver.**
NMJs were labelled with α-HRP (axon) and Lifeact-GFP (actin). A: Lifeact-GFP and *mCherry* RNAi expressed under D42-Gal4 driver (Control). B: Lifeact-GFP and *tsr* RNAi expressed under D42-Gal4 driver. Actin was detected in axons and presynaptic compartments in *tsr* knockdown as well as control.

3.5 Activity-induced expression of Bsg and Tsr

3.5.1 Localization and expression of *bsg* mRNA and Bsg protein during synaptic plasticity

Third-instar larvae with Bsg::YFP protein trap insertions were used to investigate the localization and levels of *bsg* transcripts and Bsg proteins. *bsg* mRNA transcripts can be seen to localize throughout the muscle, with highly concentrated transcription foci in muscle nuclei. Bsg protein was seen to colocalize with α-HRP and α-Dlg stainings of the pre- and postsynaptic membrane, same as shown in a previous study (Besse et al., 2007). Bsg was found around all mature boutons and only around some GBs. This result suggests that Bsg is involved in GB maturation (Figure 3.8).

The spaced high K^+ stimulation paradigm was used to determine the effect of increased synaptic activity on *bsg* mRNA expression. The larvae were divided into two groups, the stimulated and the mock, and the experiment was carried out as described in section 2.3.2. GB numbers were quantified in both groups to show that the stimulation experiment was successful (Supplementary B.2). In response to stimuli, the numbers of nuclear transcripts, measured by comparing the integrated intensity of transcription foci to that of cytoplasmic mRNA, and cytoplasmic transcripts increased in the muscle. The numbers of nuclear transcripts and cytoplasmic transcripts of the stimulated group more than doubled relative to control. The spatial distribution of *bsg* transcripts in stimulated larvae was indistinguishable from control (Figure 3.9C, D). Bsg protein level was measured by the average intensity of YFP at the NMJs adjusted by the area of the synapse. An average of a 24% increase was observed at the NMJ synapse in response to spaced stimulation (Figure 3.9E). A decrease of the Bsg::YFP protein

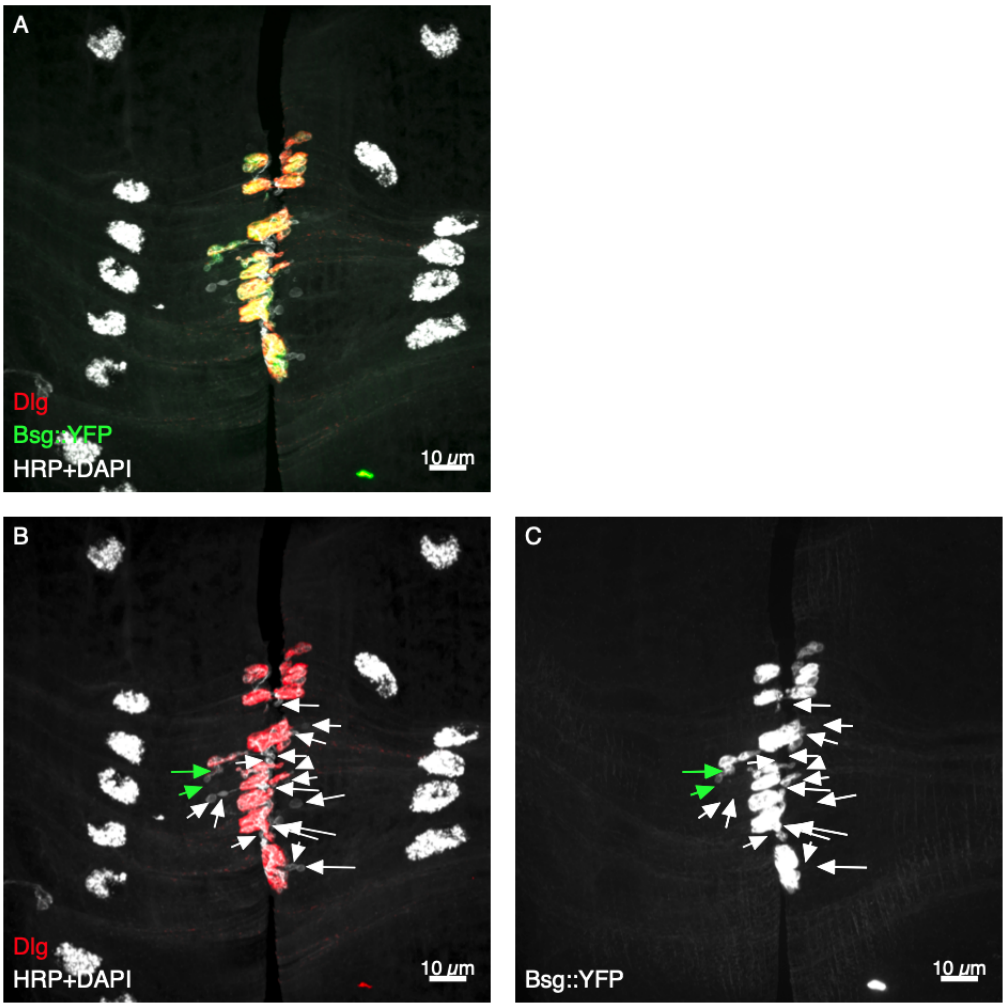


Figure 3.8: **Bsg::YFP protein localization at the synapse in stimulated larva.** NMJs were labelled with α-HRP (axon), α-Dlg (postsynaptic density), GFPBooster (YFP), and DAPI (nuclei). A: Image of an NMJ with all three channels present. Bsg proteins colocalize with pre- and postsynaptic markers at the synapse. B: The same image with Dlg and HRP channels. GBs are indicated with arrows. Green arrows indicate Bsg presence in GBs. C: The same image with only Bsg::YFP channel. Bsg is present in all mature boutons and some GBs.

to *bsg::YFP* RNA ratio in stimulated larvae compared to control was shown (Figure 3.9F). These results suggest that Bsg transcription is activity-dependent and transcriptional regulation of Bsg is potentially involved during synaptic plasticity. The activity-induced enrichment of Bsg protein to the synaptic membranes underscores its relation to activity-dependent synaptic plasticity.

3.5.2 Localization and expression of *tsr (cofilin)* mRNA and Tsr (Cofilin) protein during synaptic plasticity

Third-instar larvae with Tsr::YFP protein trap insertions were used to study the localization and level of *tsr* mRNA transcripts, as well as those of Tsr proteins. These larvae are lethal when homozygous, so the chromosome carrying Tsr::YFP is balanced over a CyO chromosome. The amount of *tsr* RNA and Tsr protein in these larvae might be different from that in *wild-type*. IF images showed that both *tsr* mRNA and Tsr protein are abundant and evenly distributed in the larval muscle (Figure 3.10A, B). Transcription foci were observed in muscle nuclei.

To determine whether activity-dependent synaptic plasticity affects *tsr* mRNA localization and expression, spaced high K^+ stimulation paradigm was performed. GB numbers were quantified in both stimulated and mock-treated control groups (Supplementary B.2). In response to elevated neuronal activity, the number of nuclear transcripts was not significantly altered. However, a notable increase in the number of cytoplasmic transcripts was observed. The stimulated group showed around 74% more transcripts compared to the control group. The spatial distribution of *tsr* transcripts in stimulated larvae was indistinguishable from control. Tsr protein level was measured by the average intensity of YFP in the muscle. A 53% increase on average was also shown in the stimulated group compared to mock (Figure 3.10C-E). Notably, Tsr protein

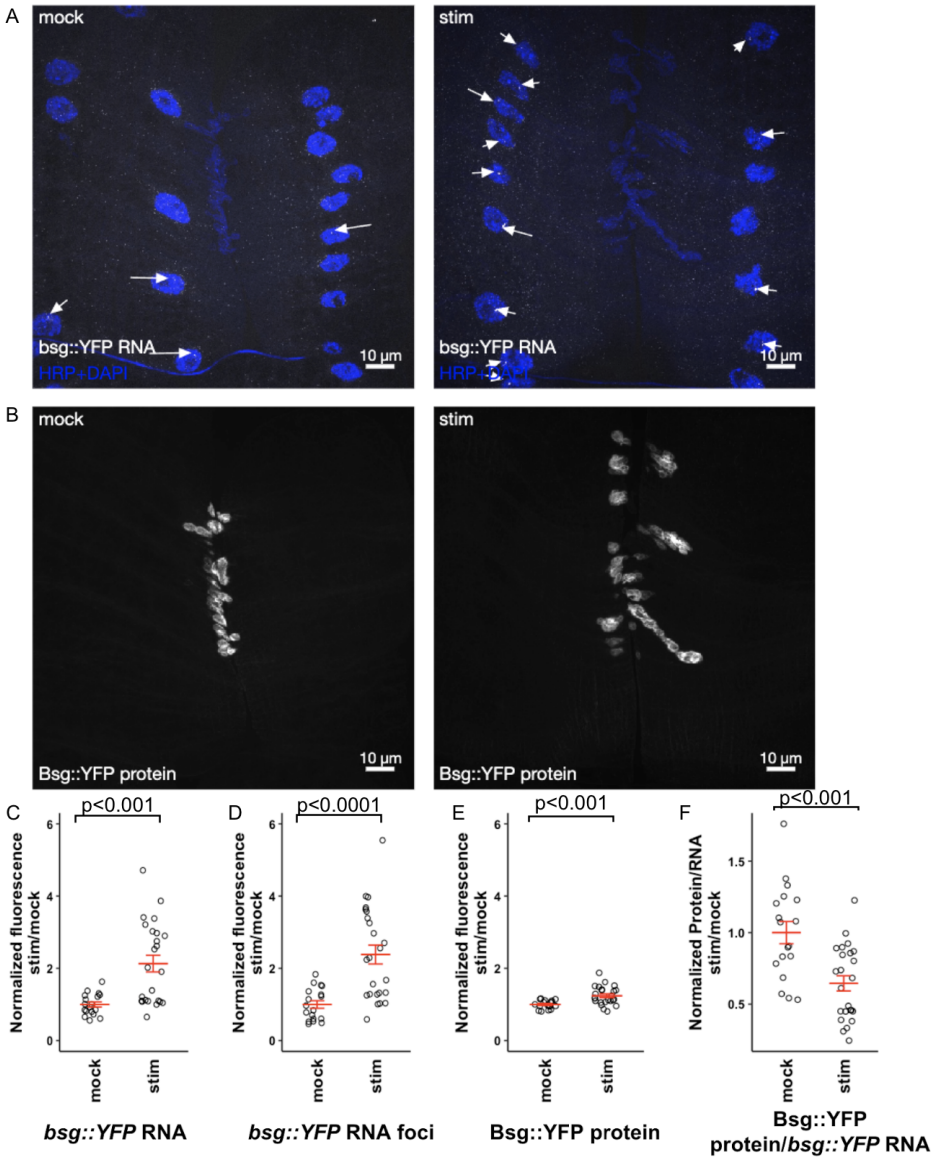


Figure 3.9: **Activity-induced expression of Bsg at the RNA and protein levels.** NMJs were labelled with α-HRP (axon), anti-*yfp* probes (*bsg::YFP* RNA), GFPBooster (Bsg::YFP protein), and DAPI (nuclei). A: *bsg::YFP* RNA in mock and stimulated larvae NMJs. Arrows indicate transcription foci. B: Bsg::YFP protein in mock and stimulated larvae NMJs. C, D: Quantification of smFISH shows an increase in overall *bsg* mRNA transcripts number and an increase transcription foci intensity in stimulated compared to mock. E: Quantification of Bsg::YFP protein level uses the average intensity of YFP signals adjusted over the size of the synapse. An increase of protein level can be seen in stimulated larvae relative to control. F: Bsg::YFP protein to *bsg::YFP* RNA ratios in mock and stimulated larvae. A decrease of the protein to RNA ratio can be observed.

accumulation inside the nuclei of stimulated larvae was increased (Figure 3.10B). An increase of the Tsr::YFP protein to *tsr::YFP* RNA ratio in stimulated larvae compared to control was observed (Figure 3.10F). The enrichments of *tsr* mRNA and Tsr protein at the synapse imply that the transcription and translation of Tsr are activity-dependent. The results also suggest that *tsr* mRNA transcripts were rapidly translated in the cytoplasm while the resulting protein was transported into the nuclei. Therefore, posttranscriptional regulation of Tsr was involved during synaptic plasticity.

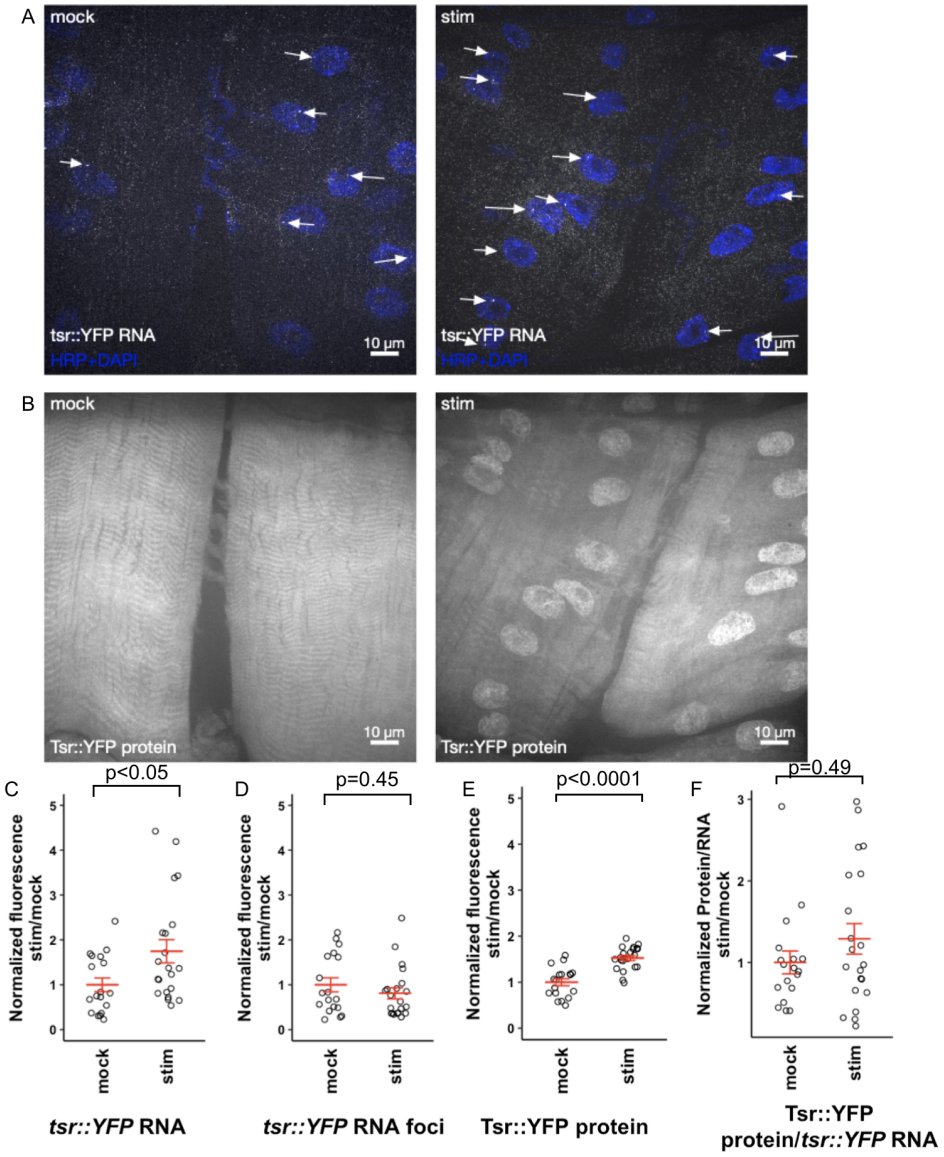


Figure 3.10: **Activity-induced Tsr expression at RNA and protein levels.** NMJs were labelled with α-HRP (axon), anti-*yfp* probes (*tsr::YFP* RNA), GFPBooster (Tsr::YFP protein), and DAPI (nuclei). A: *tsr::YFP* RNA in mock and stimulated larvae NMJs. Arrows indicate transcription foci. B: Tsr::YFP protein in mock and stimulated larvae NMJs. C, D: Quantification of smFISH shows a slight increase in overall *tsr* mRNA transcripts number in stimulated compared to mock, but foci intensity remains unchanged. E: Quantification of Tsr::YFP proteins show a significant increase in protein levels in stim relative to mock and notable accumulation in the nuclei. F: Tsr::YFP protein to *tsr::YFP* RNA ratios in mock and stimulated larvae. An increase of the protein to RNA ratio can be observed.

4 | Discussion

In this project, the roles of two actin-binding or actin-related genes, Tsr (Cofilin) and Bsg, were investigated during activity-dependent synaptic plasticity at the *Drosophila* NMJ. The activity-induced gene expression of these two genes was also studied. Motoneuron-specific knockdown of *tsr* was shown to inhibit GB formation. This result is expected based on previous studies in *Drosophila*. Since the presynaptic overexpression of active *tsr* leads to increased budding (Piccioli & Littleton, 2014), presynaptic *tsr* knockdown has the reverse effect. This effect can be attributed to the role of Tsr in promoting actin turnover and presynaptic cytoskeletal rearrangements, which is required for structural synaptic plasticity. No large boutons connected by neurite extensions or large "jettisoned" boutons away from the main branch were seen in *tsr* knockdown. This result suggests the Tsr might be required to form certain types of GBs or enlargement of GBs (Figure 4.1). Although muscle-specific knockdown did not show a significant effect on activity-dependent synaptic plasticity in this study, evidence in mammalian studies suggests Tsr is crucial in postsynaptic plasticity (Wolf et al., 2015). The conditional knockdown of *tsr* in the postsynaptic compartment could be repeated with larger sample size and a potentially longer knockdown duration without compromising synaptic structure. Increasing knockdown duration will better guarantee the depletion of *tsr* mRNA and Tsr protein during the experiment. A proper control could be set up by applying the same genetic crossings and temperature shift prior to experiment to *mCherry* RNAi flies. Furthermore, one unexpected result was that *tsr* RNAi (1) driven by D42-Gal4 led to larval lethality. OK371-Gal4 and D42-Gal4 are both motoneuron specific drivers, while, interestingly, OK371-Gal4 is known to be expressed earlier than D42-Gal4 in the larval stage (Xia et al., 2012). This result could suggest that the

D42-Gal4 line might be a much stronger driver than the OK371-Gal4 line used in this experiment. This could be confirmed by comparing RNA levels in knockdown driven by OK371-Gal4 and D42-Gal4. The result could also suggest that the D42-Gal4 driver used was leaky. This could be confirmed by expressing a fluorescent marker with D42-Gal4 driver and observe the expression pattern. While larvae expressing *tsr* RNAi (1) under MHC-Gal4 driver showed a more severe muscle phenotype compared to *tsr* RNAi (2), the two RNAi constructs had similar levels of knockdown of *tsr* transcripts (Supplementary C.1). This suggests that *tsr* RNAi (1) line could potentially have off-targets effect. However, a study showed that expressing *wild-type* Tsr together with either of the RNAi constructs could rescue the muscle structures (Balakrishnan et al., 2020). This study attributed the milder effect of *tsr* RNAi (2) to insufficient *tsr* knockdown.

Bsg is an essential gene in development, and it is involved in a variety of biological contexts in many different organisms. In *Drosophila*, Bsg was shown to be required for proper morphologies of the NMJ synapse and of spines of peripheral nerves during development (Besse et al., 2007; A. Hunter et al., 2020). However, the role of Bsg in synaptic plasticity has never been studied. In this project, the larvae with deficiency of postsynaptic Bsg showed elevated GB formation compared to control, potentially due to defects in cytoskeletal rearrangements in the postsynapse. In addition, Bsg protein was discovered to be present in some, but not all, GBs. These results imply that Bsg might have functions in the formation of postsynaptic terminals and facilitating bouton maturation (Figure 4.2). The muscle-specific *bsg* knockdown did not significantly alter the NMJ morphology during development, while the presynaptic *bsg* knockdown larvae had a larger bouton size relative to control. This result can be expected from a previous study in the *Drosophila* NMJ (Besse et al., 2007). The paper found that expressing *wild-type* Bsg solely in neurons in

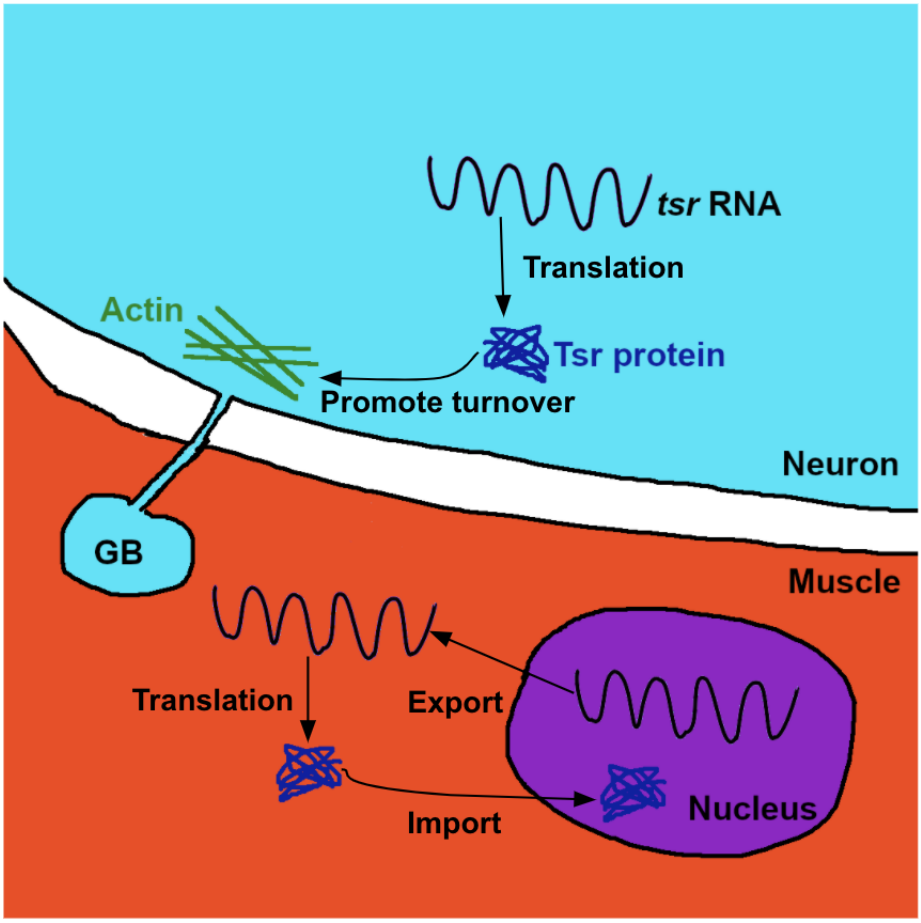


Figure 4.1: **Schematic of the function of Tsr (Cofilin) during activity-dependent synaptic plasticity**
In the neuron, Tsr protein interacts with actin to promote actin turnover and increase GB budding in response to activity. In the muscle, localized *tsr* transcripts are translated in response to activity and the Tsr protein produced is imported into muscle nuclei to aid transcription. Activity-dependent expression of *tsr* is posttranscriptionally regulated in the postsynpase.

Bsg mutants more significantly rescued the mutant phenotype than solely expressing *wild-type* Bsg in the muscle. Therefore, presynaptic Bsg could have a more significant effect during development compared to postsynaptic Bsg. The study also showed that Bsg modulates the organization of presynaptic actin, potentially indirectly via interacting with α- and β-integrin subunits on the cell membrane. From these results, it is suspected that Bsg plays a role in presynaptic plasticity. Future research could investigate whether Bsg is involved presynaptically during activity-dependent plasticity using a conditional knockdown experiment to eliminate the developmental phenotype of presynaptic *bsg* knockdown. Another experiment could be set up to investigate when Bsg arrives in GBs and whether the presence of Bsg affects the maturation of GBs.

A critical study from Davis lab that inspired this project was on Syp regulation for activity-induced Msp300 expression (S. Titlow et al., 2020). In this recent study, Msp300 protein levels were found to increase in wild-type muscles, while msp300 mRNA levels were not significantly changed in response to increased neuronal activity. This result implies that Syp directly regulates Msp300 translation, but not msp300 mRNA transcription or turnover. One objective of this project was to examine whether the same regulatory mechanism also could be applied to other ABPs or actin-related proteins that are binding targets of Syp. Bsg was found to behave differently from Msp300 because the result the Bsg smFISH suggests regulation at the transcriptional level. Further study is needed to determine whether Bsg translation is activity dependent by measuring the ratios of *bsg* RNA and Bsg protein before versus after stimulation. The result from the Tsr (Cofilin) smFISH experiment suggests that postsynaptic Tsr protein translation occurs locally in the muscle and is required for transcription in the nuclei. In fact, mammalian studies showed that cofilin-1 is required for RNA polymerase II transcription elongation (Obrdlik & Percipalle, 2011). Future

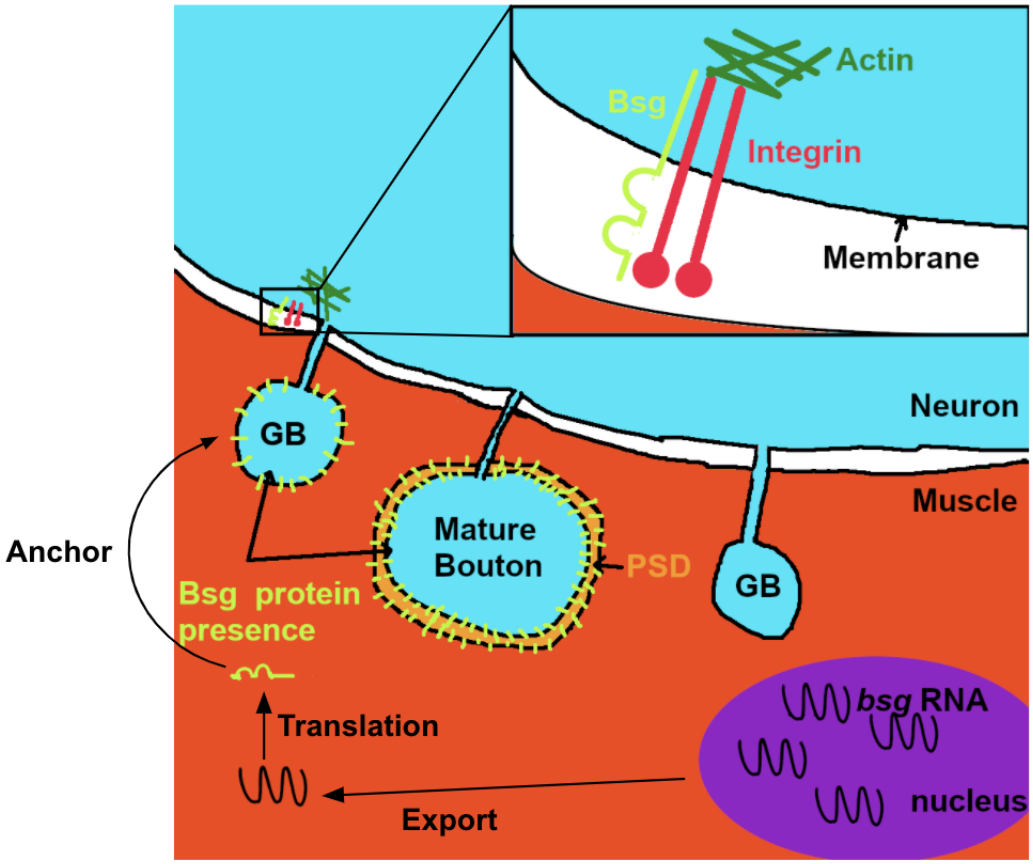


Figure 4.2: **Schematic of the function of Bsg during activity-dependent synaptic plasticity**
Bsg is a transmembrane protein that can be found on both pre- and postsynaptic membranes. Bsg is essential to restrict bouton size. Bsg functions during activity-dependent synaptic plasticity by interacting with integrin and, therefore, indirectly promoting cytoskeletal rearrangements. Bsg could play a role in the formation of postsynaptic density and signal the maturation of GBs. As postsynaptic terminals form around the GBs, Bsg is recruited and anchored on pre- and postsynaptic membranes. Bsg shows increased transcription in muscle nuclei in response to activity.

study is needed to determine if Syp is required in modulating Bsg and Tsr protein levels posttranscriptionally. The study can be done by repeating the spaced high K^+ stimulation experiment in Syp mutant larvae and comparing changes in mRNA as well as protein levels to those in *wild-type* larvae.

Imp, similar to Syp, is a highly conserved RBP required for brain development. Previous studies from Davis lab found that Syp and Imp co-regulate NB differentiation. While Syp primarily functions at the postsynapse, Imp was found to be an important mRNA regulator at the presynaptic sites (Akins, 2009). Tsr and Bsg are also downstream targets of Imp (Hansen et al., 2015). A direction for future research is to investigate whether Imp posttranscriptionally regulates *bsg* and *tsr* in the presynaptic compartment during activity-dependent synaptic plasticity. The same spaced high K^+ stimulation paradigm can be used to induce plasticity. However, smFISH and imaging of the cell body of the motoneuron could also be needed. At the beginning of this project, a list of 13 Syp binding targets was selected, and all of which have functions in regulating the actin cytoskeleton. These genes are potentially involved in synaptic plasticity as well. Another direction for future research is to determine the roles of these genes in pre- and postsynaptic compartments during activity-dependent synaptic plasticity. If they are found to play a role in this process, their activity-induced expression and how the expression is regulated by RBPs, such as Syp and Imp, could also be investigated. The results from this study would help elucidate whether an RBP regulatory mechanism is shared among actin-interacting genes.

In this project, synaptic plasticity was represented solely using structural plasticity, which is the formation of GBs. However, it would be interesting to also investigate the roles of these genes in functional synaptic plasticity. Functional activity-dependent synaptic plasticity can be measured by recording miniature excitatory junction potentials (mEJPs) during and after spaced high K^+

stimulation. Changes in mEJPs measured in postsynaptic muscle could provide a physiological readout of presynaptic modifications (Ataman et al., 2008). Similarly, the same method can be used to compare synaptic changes in RBP mutant compared to *wild-type* to analyze the RBP modulatory effects on mRNAs in functional plasticity.

Another potential direction for future studies is to observe how actin and ABPs interact during activity-dependent synaptic plasticity. In the early stage of this project, live-imaging of actin interactions with ABPs using Zeiss LSM 980 with AiryScan2 was explored. Although SiR-actin and Lifeact made live actin staining possible, many difficulties remained with live-imaging actin at the NMJ synapse. Due to the low refractive index of HL3 solution and slow scanning speed, it was concluded that the microscope did not have sufficient speed or resolution to capture fast interactions between actin and ABPs. Different imaging approaches and post-processing techniques could be explored to circumvent these problems – Deep SIM microscope and super-resolution radial fluctuations (SRRF) analytical approach to name a few. An alternative experiment to infer actin and ABPs interactions from fixed images was attempted. An issue encountered with this experiment was that the high concentration of actin in the muscle made it challenging to identify the presence of actin in GBs. A way to investigate presynaptic actin-ABP interactions is to express Lifeact with a presynaptic driver. Lifeact-mRuby should be used to avoid unwanted autofluorescence in the GFP channel. However, the stability of Lifeact during fixation needs to be analyzed. The issue will remain for identifying interactions between actin and ABPs that function in the muscle, for example, Msp300.

In conclusion, a list of conserved genes regulated by RBP Syp was identified. Two highly expressed genes, Bsg and Tsr, were selected to identify their roles during activity-dependent synaptic plasticity. Their expressions were induced by

increased synaptic activities. This study lays the foundation for investigating the functions of ABPs and actin-related proteins in synaptic plasticity. Future studies are needed to provide a complete picture of RBP regulations of mRNAs encoding ABP or actin-related proteins. Extending the study to other actin-related genes can deepen our understanding of the molecular basis of activity-induced synaptic modifications and how the actin cytoskeleton is involved in this process.

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A | smFISH probe sequences

tsr

Probe No.	Sequence	Probe Position	Percent GC
1	tcacgaacaaccggacga	30	56.00%
2	tgtttaaccttccgcctt	51	44.00%
3	caaattgctgcttcgccg	89	56.00%
4	ttcctgtagtttttcgct	118	39.00%
5	tcagacacagttacacca	147	44.00%
6	cctcgtatgtagtcttgc	170	50.00%
7	agcgatgctttttgtcct	197	44.00%
8	gcttttcatcgcgaatgt	227	44.00%
9	ccacagtctccacatcaa	248	50.00%
10	aactggtcgtactcggcg	276	61.00%
11	ggtccgcatttctggata	303	50.00%
12	actggtgcatgtactcga	351	50.00%
13	aactctcagaggtgcctt	372	50.00%
14	ggaacagcttttgcttct	393	44.00%
15	cttggcagtatcgggaca	422	56.00%
16	aggagctggagtacaaca	454	50.00%
17	gcgacttcttgagagcat	475	50.00%
18	tatacttttggacgccca	496	44.00%
19	ccgggaggcttcggaaag	528	67.00%
20	cggagtttctcctcgacg	551	61.00%
21	gtttattggcggtcggtg	572	56.00%

22	gcaatgcacagtgctcgt	593	56.00%
23	tgcatgccttgtttgtta	614	39.00%
24	cgtgtttccatcgctgtt	635	50.00%
25	attggcgatctcaacagg	656	50.00%

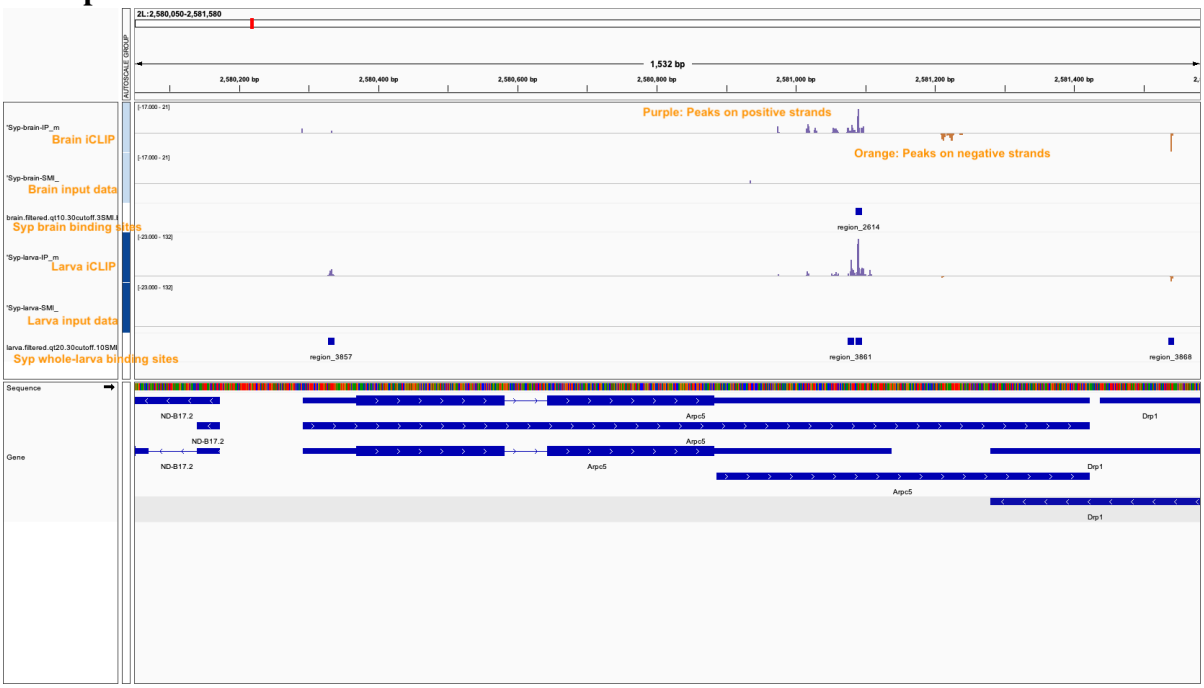
bsg

Probe No.	Sequence	Probe Position	Percent GC
1	tcatagtttggcaccagc	5	50.00%
2	ttcatttgatgctcggca	26	44.00%
3	gagcggcgatctgatgtc	51	61.00%
4	cgtaccgtctttcacgtt	81	50.00%
5	agcggtgccgttcttttt	121	50.00%
6	gcgacctctgagagaagg	151	61.00%
7	acttgttctcgtctgcga	179	50.00%
8	tcgttcgtatccgtctta	207	44.00%
9	ctcgcaactgtatttgcc	229	50.00%
10	cgatttccttggacacgc	254	56.00%
11	ctcgtacaacaacgcggg	282	61.00%
12	tccacaacggctgtattt	307	44.00%
13	gtcaccgacatcttttca	328	44.00%
14	ttggtacccacaacgctg	349	56.00%
15	aaggtccatgtcaactct	370	44.00%
16	gcgatctgtggcatttgt	405	50.00%

17	acaccattatcgtctggt	434	44.00%
18	ccagtgtcagaatggcgt	457	56.00%
19	ctctgtcgtccaatgtca	481	50.00%
20	ttcgtccaatgcatttgt	505	39.00%
21	caccgtagacattggccg	526	61.00%
22	tggcaggagtggtggtgt	547	61.00%
23	cacgcacagttgtgacgt	568	56.00%
24	acaaggcggcaaatttgc	593	50.00%
25	gcacagatgcccaggaaa	615	56.00%
26	atgcacagaatcagcacc	636	50.00%
27	gttgcgtcgcttctcata	667	50.00%
28	agtatcactctcctccag	694	50.00%

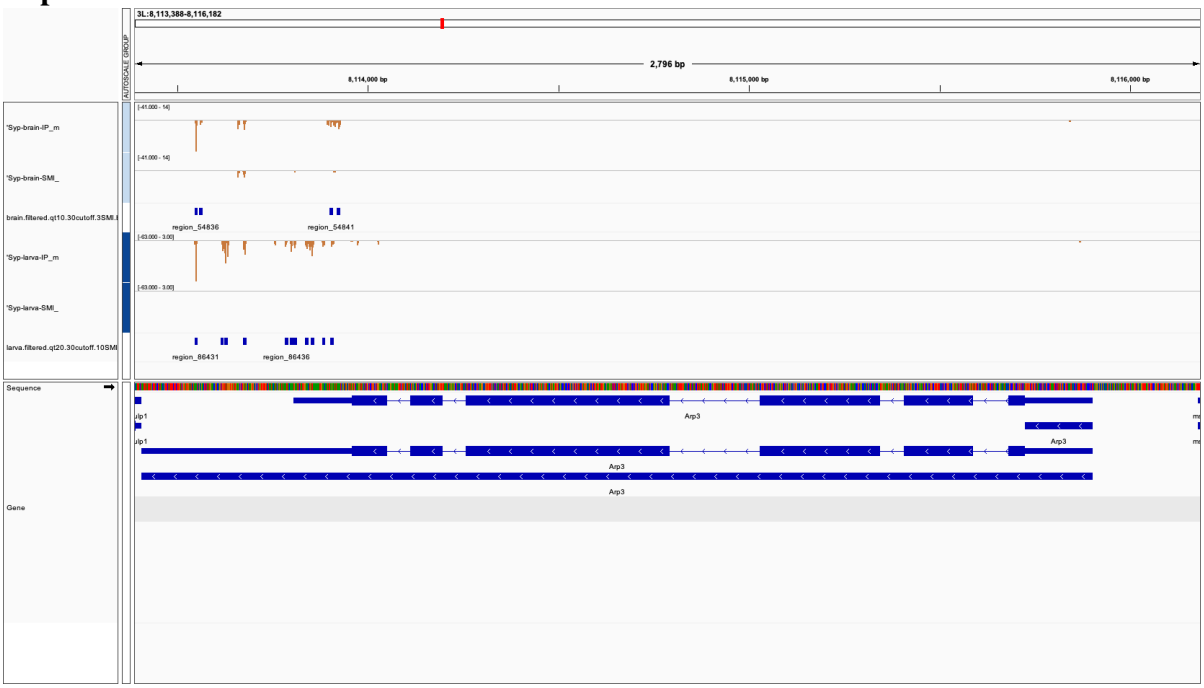
B | Syp iCLIP Binding Profiles of Candidate Genes

Example

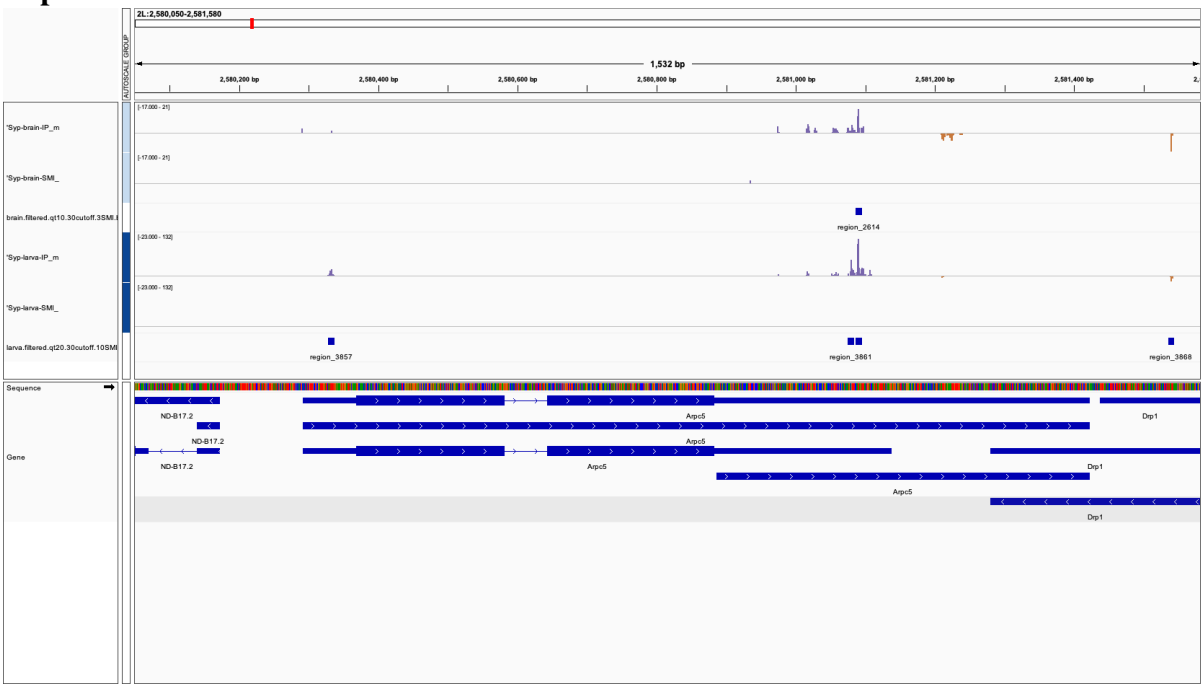


Please see the example figure for legends. The significant binding peaks were filtered so that the immunoprecipitation coverage is at least 3 times more enriched compared to the input coverage.

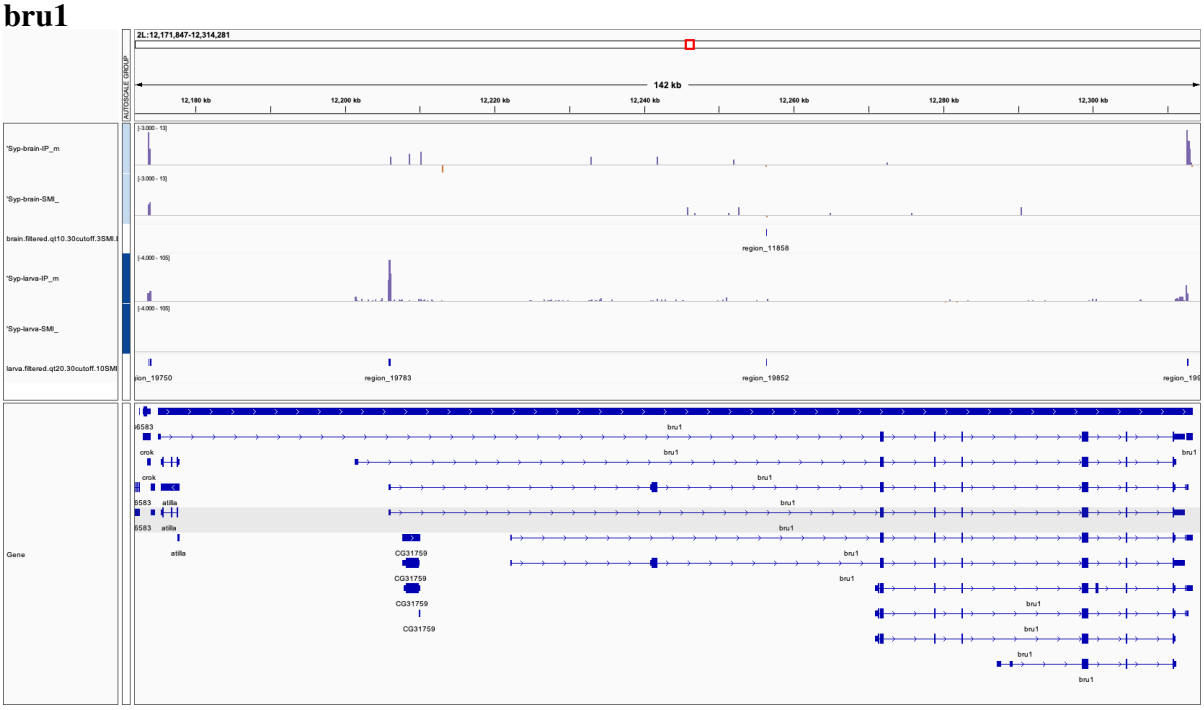
Arp3


3L:8,113,388-8,116,182
2,796 bp
8,114,000 bp
8,115,000 bp
8,116,000 bp
region_54836
region_54841
region_86431
region_86436
Sequence
Gene
Arp3

Arpc5


2L:2,580,050-2,581,580
1,532 bp
2,580,200 bp
2,580,400 bp
2,580,600 bp
2,580,800 bp
2,581,000 bp
2,581,200 bp
2,581,400 bp
region_2614
region_3857
region_3861
region_3868
Sequence
Gene
ND-B17.2
Arpc5
Drp1

bru1


2L:12,171,847-12,314,281
142 kb
12,180 kb
12,200 kb
12,220 kb
12,240 kb
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12,300 kb
region_11858
region_19750
region_19783
region_19852
Gene
bru1
CG31759
atilla
crok

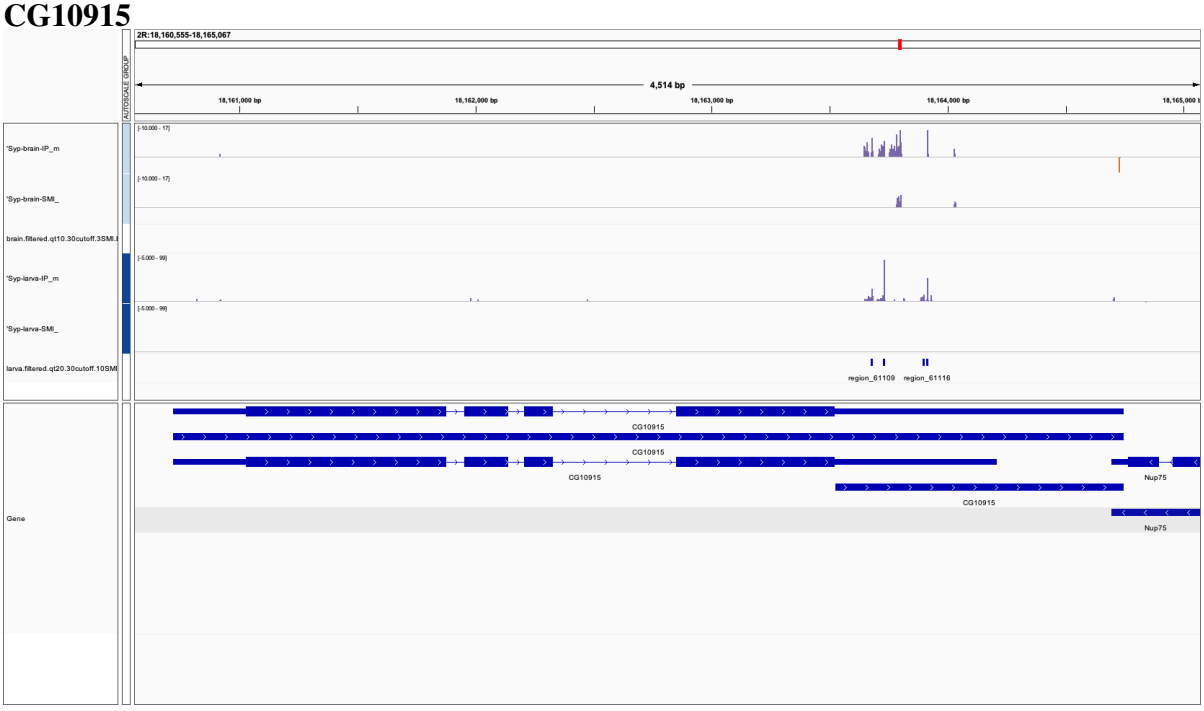
Bsg


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37 kb
8,090 kb
8,100 kb
8,110 kb
region_7699
region_7714
region_7730
region_7864
region_13394
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Mcr
Bsg
mon2
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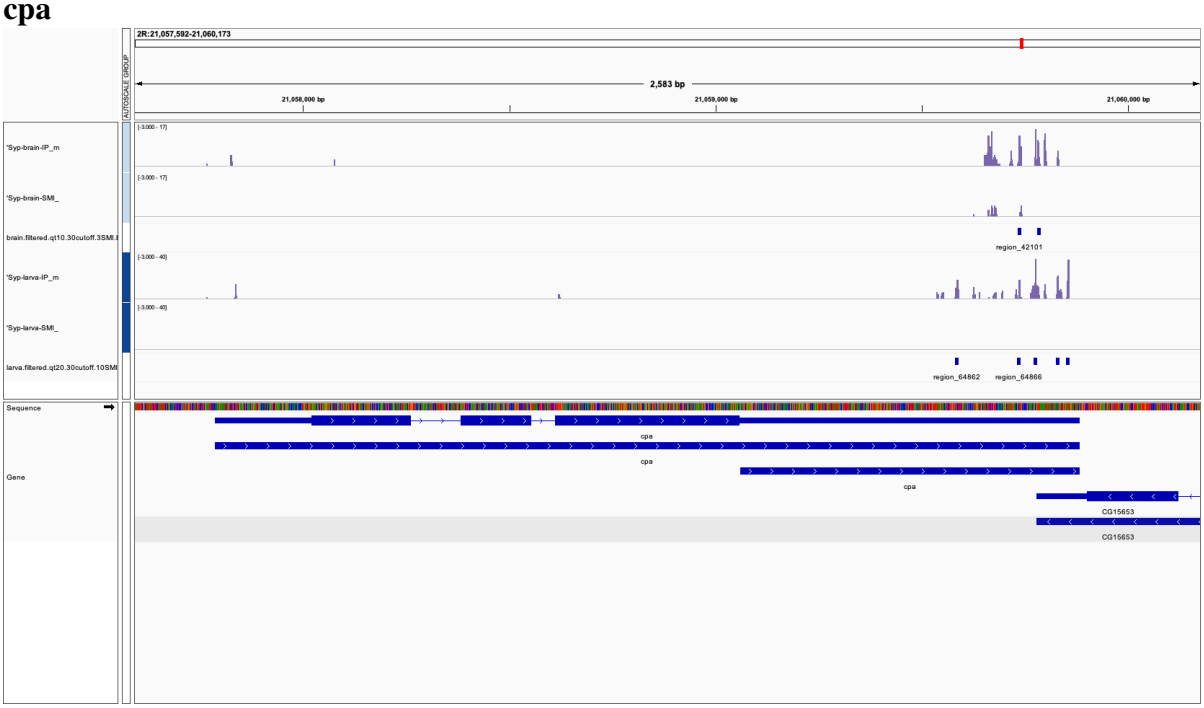
CanB2



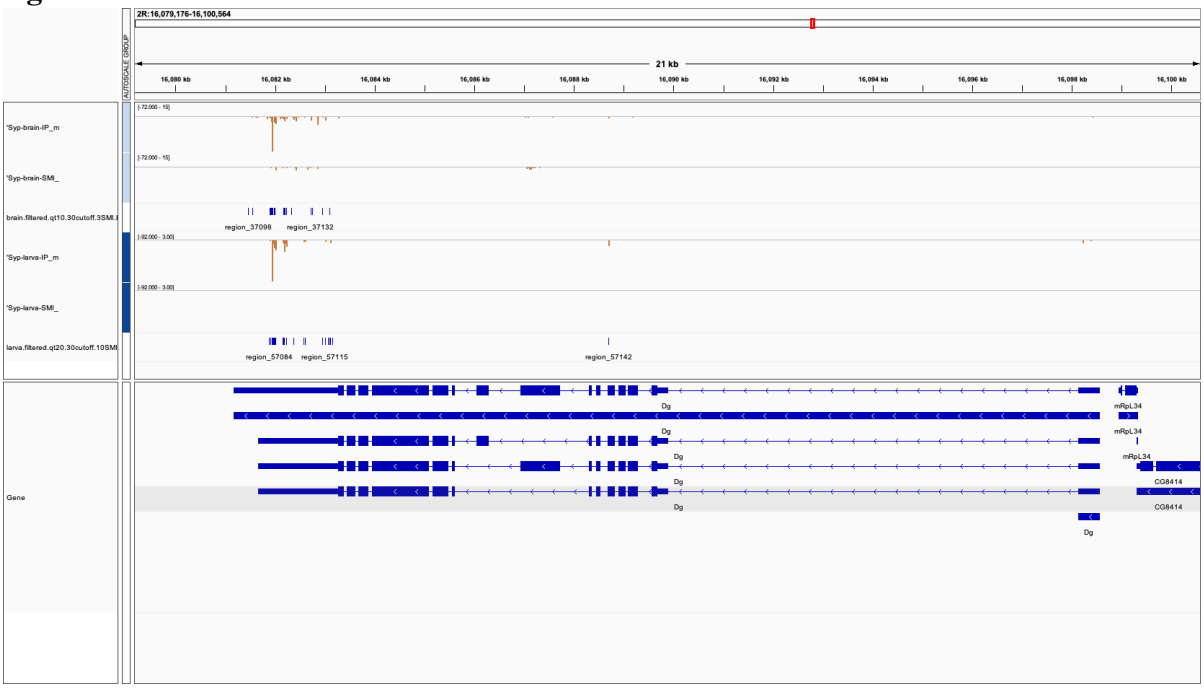
CG10915



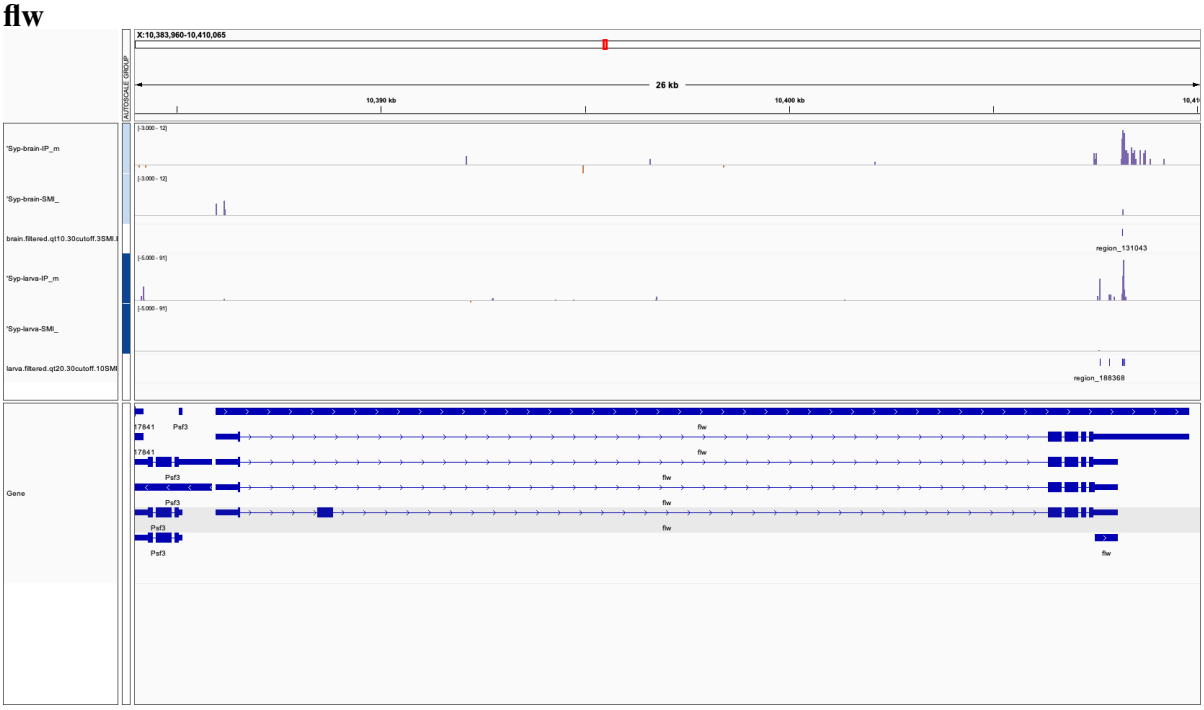
cpa


2R:21,057,592-21,060,173
2,583 bp
21,058,000 bp
21,059,000 bp
21,060,000 bp
region_42101
region_64862
region_64866
Sequence
Gene
cpa
CG15653

Dg


2R:16,079,176-16,100,564
21 kb
region_37098
region_37132
region_57084
region_57115
region_57142
Gene
Dg
mRpL34
CG8414

flw


X:10,383,960-10,410,065
26 kb
10,390 kb
10,400 kb
'Syp-brain-IP_m
'Syp-brain-SMI_
brain.filtered.qt10.30cutoff.3SMI
'Syp-larva-IP_m
'Syp-larva-SMI_
larva.filtered.qt20.30cutoff.10SMI
region_131043
region_188368
Gene
l7841
Psf3
flw

Ggamma1


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8,903,000 bp
8,904,000 bp
8,905,000 bp
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region_29112
region_29118
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region_44449
Gene
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Ggamma1
CCT8

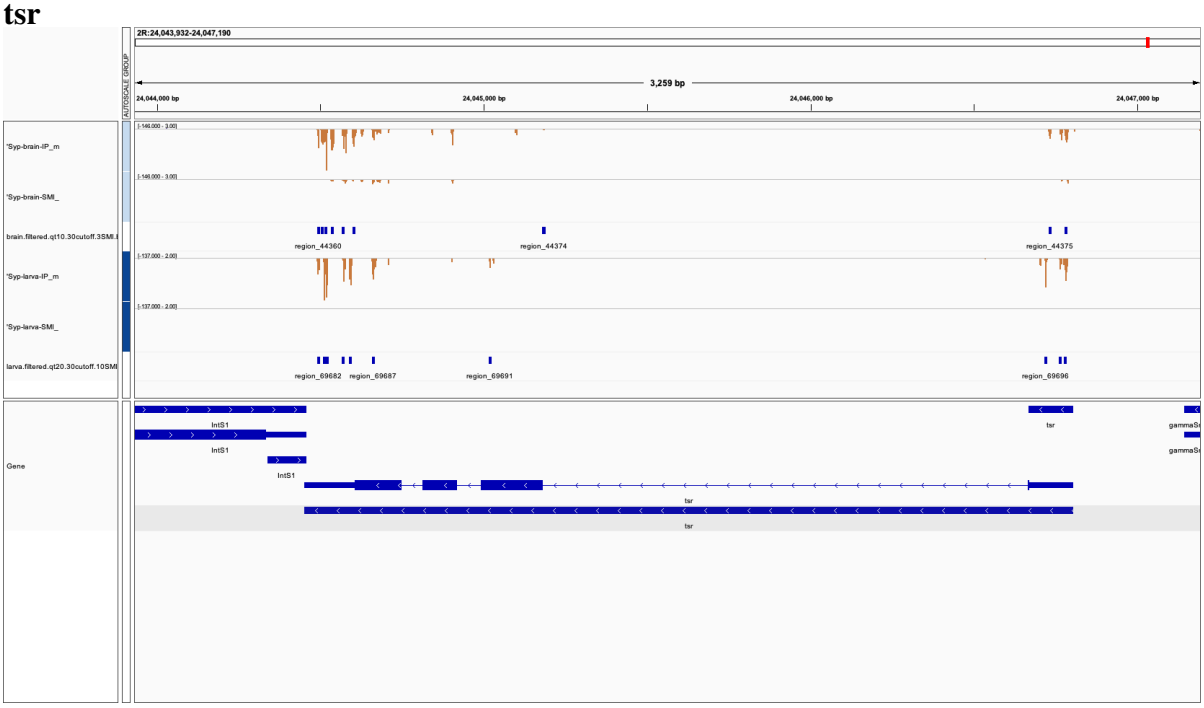
Rho1


2R:16,102,369-16,107,766
5,399 bp
16,103,000 bp
16,104,000 bp
16,105,000 bp
16,106,000 bp
16,107,000 bp
'Syp-brain-IP_m
'Syp-brain-SMI_
brain.filtered.qt10.30cutoff.3SMI
'Syp-larva-IP_m
'Syp-larva-SMI_
larva.filtered.qt20.30cutoff.10SMI
region_37192
region_37209
region_37211
region_37212
region_37218
region_37222
region_37227
region_37241
region_57162
region_57177
region_57182
region_57185
region_57192
region_57193
region_57202
Gene
CG8414
Rho1
Ric

SCAR


2L:10,976,523-10,982,910
6,389 bp
10,977,000 bp
10,978,000 bp
10,979,000 bp
10,980,000 bp
10,981,000 bp
10,982,000 bp
'Syp-brain-IP_m
'Syp-brain-SMI_
brain.filtered.qt10.30cutoff.3SMI
'Syp-larva-IP_m
'Syp-larva-SMI_
larva.filtered.qt20.30cutoff.10SMI
region_11234
region_18534
Gene
SCAR
piwi

tsr


2R:24,043,932-24,047,190
3,259 bp
24,044,000 bp
24,045,000 bp
24,046,000 bp
24,047,000 bp
AUTOSCALE GROUP
'Syp-brain-IP_m
'Syp-brain-SMI_
brain.filtered.qt10.30cutoff.3SMI.l
'Syp-larva-IP_m
'Syp-larva-SMI_
larva.filtered.qt20.30cutoff.10SMI
region_44360
region_44374
region_44375
region_69682
region_69687
region_69691
region_69696
Gene
IntS1
tsr

C | Supplementary Figures

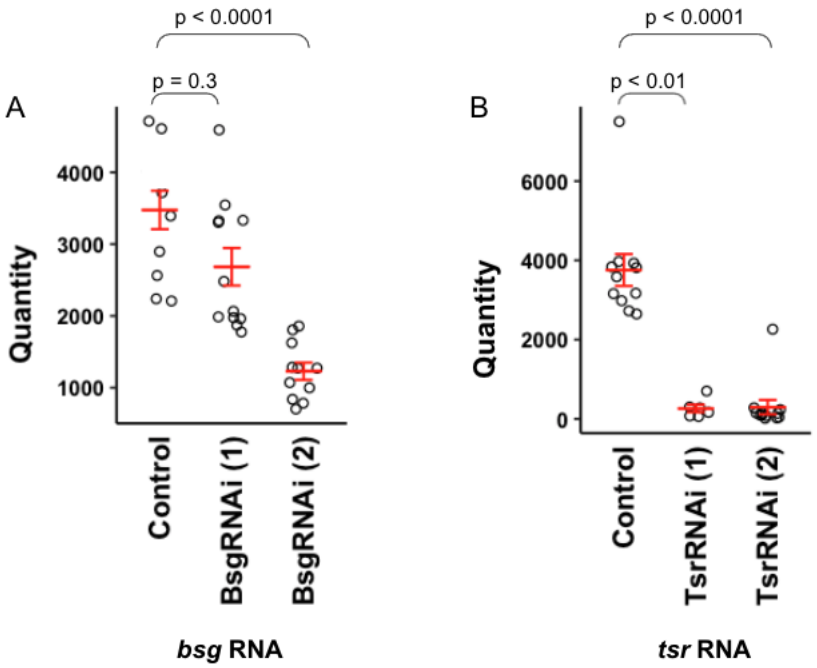


Figure C.1: **Quantification of *bsg* and *tsr* after RNAi under MHC-Gal4 driver.** smFISH probes were used detect RNA transcripts and αHRP was used to label the synapses. A: smFISH quantification of *bsg* RNA transcripts in control, *bsg* RNAi (1), and *bsg* RNAi (2). A decrease in RNA levels in both *bsg* RNAi lines is shown, while *bsg* RNAi (2) is able to greater reduce the number of *bsg* transcripts. B: smFISH quantification of *tsr* RNA transcripts in control, *tsr* RNAi (1), and *tsr* RNAi (2). Both *tsr* RNAi lines are shown to almost completely deplete *tsr* transcripts.

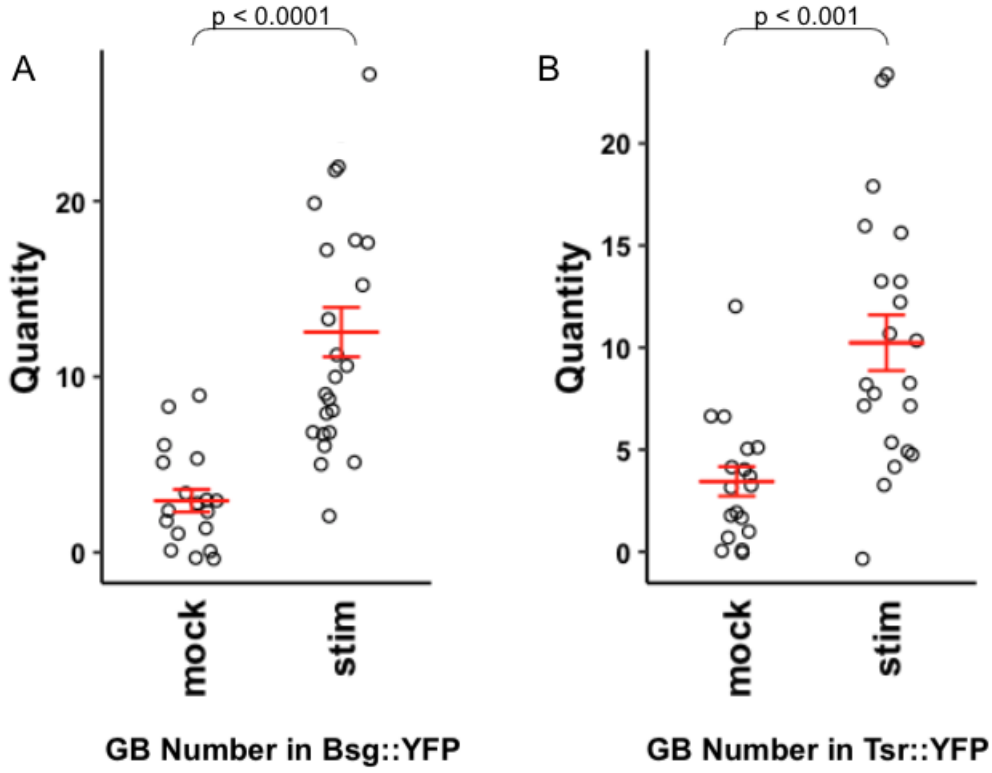


Figure C.2: **Quantification of GBs after spaced K^+ stimulation in Bsg::YFP and Tsr::YFP larvae.**
A: Quantification of GBs in mock and stimulated Bsg::YFP larvae. B: Quantification of GBs in mock and stimulated Tsr::YFP larvae. Larvae in both experiments show activity-dependent synaptic plasticity induced by stimulation, since significantly more GBs were produced in stim relative to mock.