Advanced Review

Development and plasticity of the *Drosophila* larval neuromuscular junction

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The *Drosophila* larval neuromuscular system is relatively simple, containing only 32 motor neurons in each abdominal hemisegment, and its neuromuscular junctions (NMJs) have been studied extensively. NMJ synapses exhibit developmental and functional plasticity while displaying stereotyped connectivity. *Drosophila* Type I NMJ synapses are glutamatergic, while the vertebrate NMJ uses acetylcholine as its primary neurotransmitter. The larval NMJ synapses use ionotropic glutamate receptors (GluRs) that are homologous to AMPA-type GluRs in the mammalian brain, and they have postsynaptic scaffolds that resemble those found in mammalian postsynaptic densities. These features make the *Drosophila* neuromuscular system an excellent genetic model for the study of excitatory synapses in the mammalian central nervous system. The first section of the review presents an overview of NMJ development. The second section describes genes that regulate NMJ development, including: (1) genes that positively and negatively regulate growth of the NMJ, (2) genes required for maintenance of NMJ bouton structure, (3) genes that modulate neuronal activity and alter NMJ growth, (4) genes involved in transsynaptic signaling at the NMJ. The third section describes genes that regulate acute plasticity, focusing on translational regulatory mechanisms. As this review is intended for a developmental biology audience, it does not cover NMJ electrophysiology in detail, and does not review genes for which mutations produce only electrophysiological but no structural phenotypes.

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INTRODUCTION

Chemical synapses are specialized junctions between cells that mediate transmission of information *via* small molecule and/or peptide neurotransmitters. The presynaptic terminals of these synapses contain neurotransmitter-filled vesicles and the machinery necessary for neurotransmitter release. The postsynaptic partners, which can be other neurons or nonneuronal cells, have specialized postsynaptic structures containing receptors that bind to the neurotransmitter(s) released by the presynaptic cell and transduce electrical and/or chemical signals.

Excitatory synapses in the vertebrate nervous system that use glutamate as their primary neurotransmitter are characterized by postsynaptic densities (PSDs), which are very large protein complexes that contain ionotropic glutamate receptors (GluRs) and numerous scaffolding and signaling proteins. These types of synapses exhibit plasticity, which is a process whereby the connections between the neuron and its partner are modified in response to neuronal activity. Synaptic plasticity usually involves both structural and functional changes, and it is thought to be the foundation of learning and memory. Plastic changes are also observed during synaptic development and maturation. Many of the molecules and mechanisms used for synaptic plasticity during development are reused later for plasticity linked to learning and memory in mature neurons.[1] Thus, the

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study of synaptic plasticity during development can provide important insights into learning and memory mechanisms.

Studies performed in invertebrate genetic model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* have provided important insights into the molecular mechanisms involved in synaptic development and function.[2] These organisms have nervous systems with fewer cells than those in vertebrates and are amenable to gene discovery through forward genetic screening. Many genes involved in nervous system development and function that are conserved between invertebrates and vertebrates have been identified in such screens.

In this review, we focus on neuromuscular junction (NMJ) synapses in *Drosophila* larvae. These synapses are glutamatergic and similar to those in the vertebrate central nervous system (CNS). Larval NMJ synapses use ionotropic GluRs that are homologous to AMPA-type GluRs in the mammalian brain, and they have postsynaptic scaffolds that resemble those found in mammalian PSDs. Many of the vertebrate synaptic components also have *Drosophila* orthologs, including Neurexin,[3] Neuroligin,[4,5] PSD-95,[6] and Phosphodiesterase 4 (PDE-4).[7,8] The *Drosophila* larval neuromuscular system is relatively simple, containing only 32 motor neurons in each abdominal hemisegment, and its NMJs are large, individually specified, and easy to visualize and record from. As discussed below, fly NMJ synapses also exhibit developmental and functional plasticity while displaying stereotyped connectivity. Because of these features, the *Drosophila* larval NMJ is an excellent genetic model for glutamatergic synapses in the mammalian brain (CNS).[9–12]

NMJ DEVELOPMENT

A Brief Overview of *Drosophila* NMJ Development

Motor neurons are individually specified and are generated in lineages deriving from at least 10 different neuroblasts.[13,14] Their muscle targets, which are also individually specified, are produced by cell fusion events. During stages 13–15 of embryonic development, motor neurons extend their axons into the musculature. Motor axons leave the CNS in three pathways: the segmental (SN) and intersegmental (ISN) nerve roots and the transverse nerve (TN). In the periphery, the SN and ISN split into five nerve pathways, designated as the SNa (innervates lateral muscles), SNc (innervates ventral muscles), ISN (innervates dorsal muscles), ISNb (innervates ventrolateral muscles (VLMs)), and ISNd (innervates other ventral muscles).[15] Each motor axon follows a genetically determined pathway to a specific muscle fiber or group of fibers.[16] These are shown in both immunohistological composite (ISN root-derived branches only, Figure 1(c)) and as a schematic in Figure 1(d).

After an axonal growth cone makes contact with its target muscle, postsynaptic GluRs and Discs large (Dlg), the *Drosophila* ortholog of the mammalian PSD-95 postsynaptic scaffolding protein, begin to cluster at the contact site.[17,18] The growth cone then differentiates into a presynaptic terminal. By the end of embryonic development, functional NMJs, each containing a few synaptic boutons, have formed on each muscle fiber (Figure 3(c) and (d)). Boutons are oval-shaped structures that house synapses. Boutons contain multiple active zones (neurotransmitter release sites), and each of these is apposed to a GluR cluster. The presynaptic bouton at larval NMJs eventually becomes surrounded by an infolded membranous structure called subsynaptic reticulum (SSR), which contains neurotransmitter receptors, scaffolding proteins, and postsynaptic signaling complexes.

Early neural development is often characterized by an initial overproduction of synaptic connections, followed by a period of selective elimination of improper processes. This phenomenon was first observed in vertebrates[19] and has been studied extensively at the visual system and the NMJ. In the visual system, the refinement of the connections is necessary for the formation of the retinotopic maps in the mammalian brain. Relay of visual information from the retina to the primary visual cortex in the brain occurs through the lateral geniculate nucleus (LGN) located in the thalamus. Initially, axon terminals of retinal ganglion cells (RGCs) from the two eyes form ectopic connections and overlap within the different layers of the LGN. Later on, these connections are refined to form specific eye layers. This segregation of RGC inputs involves retraction from incorrect target layers and synapse formation in the correct layer.[20,21] At the vertebrate NMJ, multiple motor neurons initially innervate the same muscle fiber. As development progresses, all but one of the motor neurons are eliminated.[22] Activity is critical for this refinement: altering the activity of the motor neurons results in the more active neuron being maintained and stabilized.[23]

Synaptic refinement also occurs at the *Drosophila* NMJ. However, this refinement is most similar to the process that happens in the vertebrate visual system as opposed to the vertebrate NMJ. In

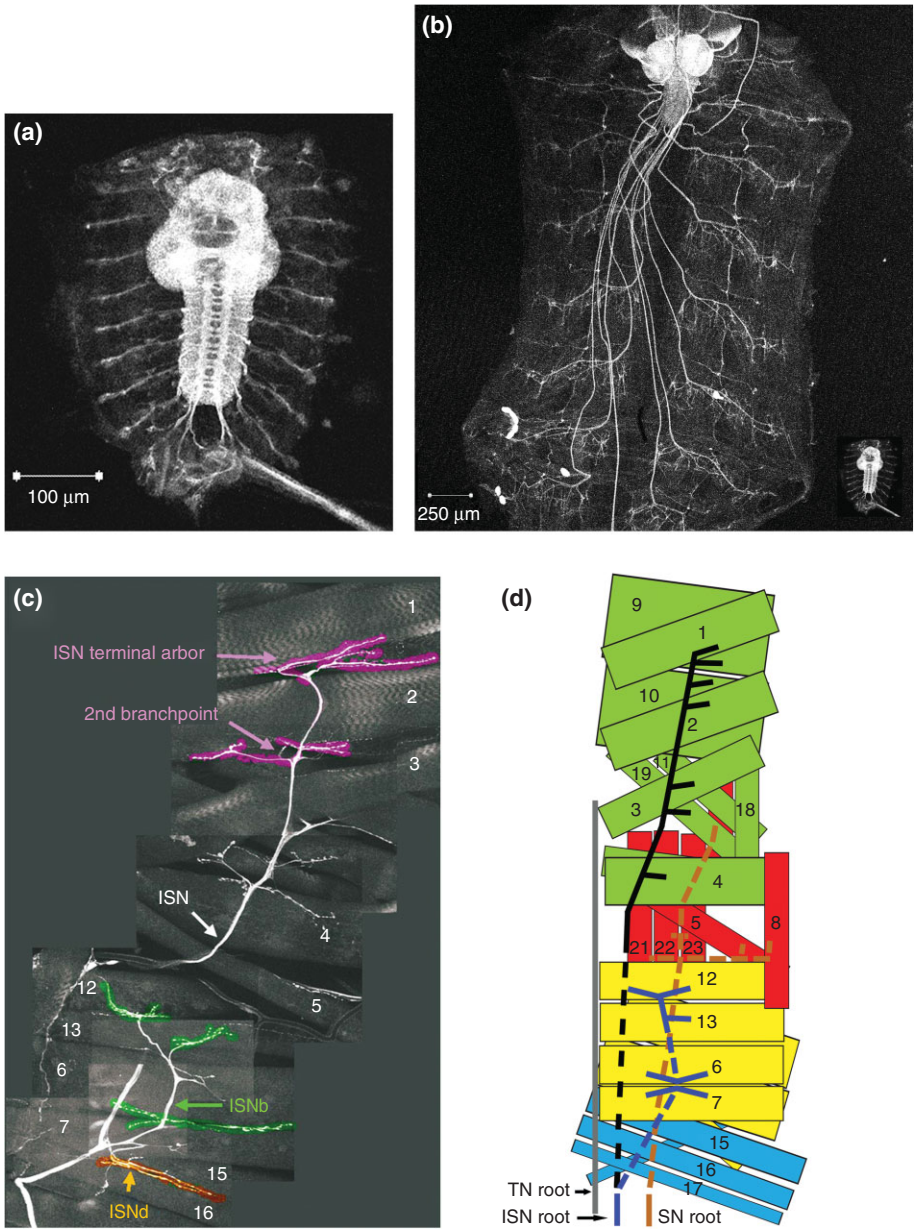

FIGURE 1 | Growth of the larva and its neuromuscular system. (a) A dissected late stage embryo 'fillet' stained with anti-horseradish peroxidase (anti-HRP), which stains neuronal membranes. The bright structure in the center is the ventral nerve cord (VNC), with the brain at the top and the ladder-like axon array extending downward from the brain. Extending outwards from each segment of the VNC are the motor and sensory axon tracts. (b) A third instar larval fillet expressing green fluorescent protein (GFP) in all neurons. The brain and VNC are now located at the anterior end, and the motor/sensory nerves run posteriorly from the VNC to reach each body segment. Note the stereotyped array of nerve endings in each segment. The inset in (b) shows the embryo from (a) at the same scale as the larva, illustrating the dramatic growth of the animal during larval life (the embryo is about the same size as a newly hatched first instar larva). (c) Innervation pattern of the intersegmental nerve (ISN) and its ISNb and ISNd branches in a third instar larva. This is a composite of many confocal images of GFP-labeled neurons in an abdominal hemisegment. Numbers indicate muscles innervated by the different branches of the ISN. (d) A schematic representing the three nerve roots: ISN, the segmental nerve (SN), and the transverse nerve (TN), and their respective innervation patterns. For clarity, not all muscles and nerve branches are shown. For the SN root, we show only the SNa nerve; SNc is not depicted. The ISNd branch of the ISN root, visible in C, has also been omitted from the diagram. The dashed lines are used to indicate the sections of the nerves that lie under (ventral to) the muscle(s). Scale bars in (a) and (b) are 100 and 250 µm, respectively.

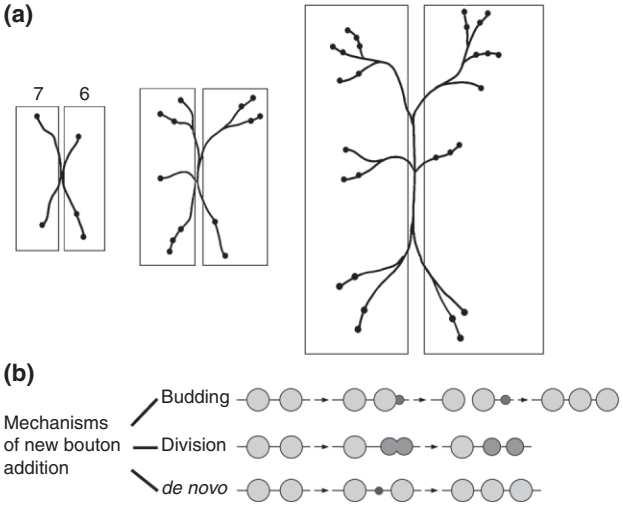

FIGURE 2 | Neuromuscular junction (NMJ) expansion and synaptic growth. (a) A cartoon depicting patterned growth of the Type 1b boutons on muscles 6 and 7 NMJ during larval development from the first instar (left panel) to the third instar (right panel). As the muscles increase in size, the NMJs add more branches and boutons. (b) During NMJ growth, new boutons are added by any of the these mechanisms[31]: (1) asymmetric budding of a preexisting bouton, similar to cell division in yeast, (2) symmetric division of a bouton, and (3) *de novo* formation of a bouton from the axonal membrane.

early embryonic development, motor neurons form ectopic contacts on nontarget muscles. These misplaced synapses are then eliminated in late-stage embryos by an activity-dependent process.[24–27] An additional form of refinement occurs after embryogenesis at the level of synaptic gain control once the motor neurons have reached their appropriate muscle targets. Here, the NMJ arbor must grow in order to maintain the proper synaptic drive that is needed because of the dramatic increase in muscle fiber size. From hatching of the embryo to the late third instar, the surface area of each muscle fiber increases by up to 100-fold (Figures 1(a) and (b) and 2(a)). During this growth period, boutons are continuously being added (and some are eliminated), and these processes result in the number of boutons and the number of active zones per bouton both increasing by up to 10-fold.[28,29] The final increase in the number of active zones by up to 100-fold matches the increase in muscle surface area (Figure 1(b)). Another round of synapse elimination occurs during metamorphosis.[30]

In addition to the structural changes that result because of expansion in muscle size, *Drosophila* NMJs also undergo plastic changes in response to short-term perturbations of neuronal and muscle activity. Some of these involve structural alterations in the NMJ, and will be reviewed here. In others, such as facilitation and homeostatic compensation,

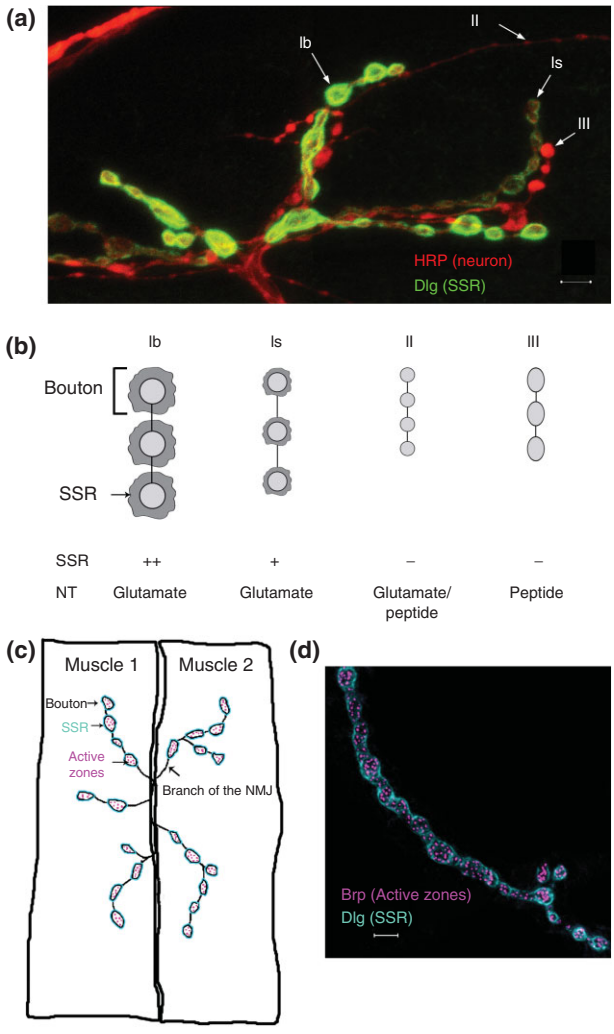

FIGURE 3 | Types and structure of boutons at the *Drosophila* larval neuromuscular junction (NMJ). (a) Type Ib, Is, II, and III boutons on muscle 12 are indicated by arrows. Type Ib and Is boutons differ in size, morphology, physiology, and the amount of subsynaptic reticulum (SSR) that surrounds them. The SSR is stained by Discs large (Dlg) antibody, which labels both Type Ib and Is boutons. Type Ib boutons are surrounded by more SSR membrane as compared to the Type Is boutons, resulting in the differential staining of the two types. Dlg is absent in Types II and III boutons. Anti-HRP labels the presynaptic neuronal membrane and allows visualization of all bouton types. (b) A diagram depicting the differences in bouton types seen in (a). Other than the size and morphological differences, the boutons also differ in the neurotransmitter utilized. (c) A schematic showing a NMJ on arbitrary muscles labeled 1 and 2. One of the branches of the NMJ is indicated. Active zones localized inside each bouton are represented as pink dots. The SSR, which consists of postsynaptic muscle membrane surrounding each bouton, is shown in blue. (d) Immunohistological staining showing Type Ib boutons on muscle 4 stained with a Bruchpilot (Brp) antibody that labels the presynaptic active zones, which are visualized as punctate structures. Also stained with a Dlg antibody to show the SSR. Scale bars are 5 μm.

electrophysiological changes that alter transmitter release and/or postsynaptic responses are observed. In many of these cases, these changes are not associated with major alterations in NMJ structure, and thus are not discussed in this review.

Patterned Growth of the Larval NMJ

The region of contact between the motor neuron and the muscle is the NMJ. The presynaptic terminals of *Drosophila* larval NMJs are organized into branched arbors that are composed of chains of synaptic boutons. There are three types of boutons: Types I, II, and III (Figure 3(a)). These differ in size and shape, the neurotransmitter that is released, the amount of SSR that surrounds them, and the subunit composition of the GluRs with which they are associated (Figure 3(b)). In this review, we consider only Type I boutons, which are glutamatergic and can be divided into two classes: 1b (large) and 1s (small). Type II and III boutons are modulatory and use other neurotransmitters. In a third instar larva, a typical NMJ has approximately 20–50 Type I boutons on each muscle, with each individual bouton housing about 10 active zones (Figure 3(c) and (d)). Most studies examine NMJs on muscles 6/7 (this NMJ has twice as many boutons because it represents two muscles), muscle 4, or muscle 12, in segments A2–A5.[16]

The structure of a larval NMJ is stereotypic and shows a similar arborization pattern for a specific muscle in different abdominal segments. Numerous studies have examined development of this complex structure from a first larval instar to a third instar using fixed larval preparations. However, live imaging of NMJ development has allowed the study of the mechanisms involved in bouton addition and branch formation.[31] Live imaging was first done by using a chimeric transmembrane green fluorescent protein that was targeted to the SSR. This construct allowed visualization of postsynaptic structures that outline Type Ib synaptic boutons through the cuticle of live larvae. The same NMJ could be viewed multiple times during development, from first instar through third instar. These studies showed that location of new bouton formation was either between preexisting boutons or at the end of a branch of the NMJ arbor. The new boutons arose by asymmetric budding of a mature (parent) bouton (similar to cell division in yeast), by symmetric division of a preexisting bouton, or by *de novo* formation of a bouton from the axonal membrane (Figure 2(b)).

During NMJ development, many transient structures are either stabilized or retracted during formation of the complex terminal arbor. As the above study examined synaptic boutons indirectly through visualization of a postsynaptic marker protein that surrounds these boutons, nascent presynaptic structures could not be observed. These include synaptopods, presynaptic debris, and ghost boutons. These transient structures are seen at normal NMJs during development and seem to be remnants of the synaptic refinement process. However, under various conditions, such as acute stimulation of motor neurons, these structures are stabilized and not properly eliminated.[32] Synaptopods are highly dynamic presynaptic filopodial extensions that can only be visualized in live larval preparations.[33–36]

Molecular Mechanisms Involved in NMJ Growth

The mechanisms that regulate the different stages of bouton formation, development and maintenance are not fully understood. Our current knowledge of the molecules involved is based on genetic and biochemical analyses of mutants that have morphological NMJ phenotypes. The purpose of this section is to review some of the genes involved in bouton growth and classify them based on their mutant phenotypes. The ultimate goal of investigators would be to understand the molecular mechanisms involved in the life history of a bouton from birth through maturity, as well as those that are required for branch formation during development of the terminal arbor. The genes discussed below function either cell autonomously on the presynaptic side (in motor neurons) or in transsynaptic pathways involved in signaling from muscles to the neurons. Transsynaptic signaling pathways are also discussed in a separate section below. Table 1 is a partial list of genes that are implicated in NMJ growth, which includes many genes in addition to those explicitly discussed in the text.

For the purposes of organization, we group the genes that play a role in the development of the larval NMJ into four categories. Each of these categories includes genes that encode proteins that function in a variety of different pathways. The first category consists of genes whose products promote NMJ growth. These are defined as those for which loss-of-function (LOF) mutants have smaller terminal arbors. In the second category are genes whose products inhibit NMJ growth, and LOF mutants for these genes have expanded terminal arbors. The third category discusses genes involved in neuronal activity that alter NMJ growth. The fourth category encompasses genes that regulate the formation and maintenance of

TABLE 1 | *Drosophila* NMJ phenotypes

Effects in Mutant → Protein ↓	Bouton Number	Bouton Size	Ghost Boutons	Presynaptic Debris	Presynaptic Retractions	Satellite Boutons	References
Cytoskeletal Proteins and Adaptors							
Nervous wreck (Nwk)	incr	decr				incr	37, 38
Wsp	incr					incr	38
Adducin/Hu-li tai shao (Hts)	incr				yes		39
Spastin	incr	decr					40
PP2A	decr	incr					41
aPKC	decr						42
Ankyrin2	decr	incr			yes		43, 44
Spectrin - pre- and post synaptic RNAi	decr (post)						45, 46
Dynactin complex - centractin and Glued/P150					yes		47
Dliprin-α	decr						48
LIM kinase	incr				yes		49, 50
Stathmin	decr				yes		51
Diaphanous (Formin) Rho GTPase	decr	incr					52
Futsch	decr	incr					53
Cell Adhesion							
Neurexin	decr						54
Neuroligin	decr						54
Fasciclin II	incr						29, 55
Fasciclin II pre- and post synaptic overexpression	incr					incr	55
Fasciclin II pre- or post synaptic overexpression	decr						55
Teneurin-a and Teneurin-m	decr	incr					56
Syndecan	decr						57
Dally-like protein (Dlp)							57
Dlp overexpression	decr						57
Endocytic Proteins							
Cdc42						incr (very slightly)	58
RabII						incr	59
Dynamin (shi^{ts1} at non permissive temp)						incr	60
Dapl60 (dynamin associated protein 160)						incr	61, 62
Endophilin						incr	60
Synaptojanin						incr	60
Spinster	incr						63
VAP 33A	decr	incr					64
VAP-33A overexpression	incr	decr					64
Exocytosis							
Complexin	incr						65
Receptors							
Draper	decr	incr	incr	incr			33
APPL overexpression						incr	66

TABLE 1 | *Continued*

Effects in Mutant → Protein ↓	Bouton Number	Bouton Size	Ghost Boutons	Presynaptic Debris	Presynaptic Retractions	Satellite Boutons	References
Arrow	decr						67
Dlar	decr						57
Arrow overexpression	incr					incr	67
Transcription Factor							
Dad						incr	68
Kinases							
Shaggy						incr	69
Protein Synthesis							
Pumilio	decr	incr					70
Nanos	incr						71
FMRP	incr					incr	72, 73
miR-8 sponge in muscles	decr						74
Protein Degradation							
Hiw	incr	decr					75, 76
Fat facets overexpression	incr						77
Anaphase- promoting complex/Cyclosome (APC/C)	incr						78
Wnd (wallenda MAPKKK) overexpression	incr	decr					79
cAMP							
Dunce (cAMP phosphodiesterase)	incr						80
Rutabaga (adenylate cyclase)	wt						80
Dunce Rut	wt						80
Eag Sh	incr						81
Dnc Eag	more incr						81
Dnc Sh	more incr						81
Channels							
K+ channels sei- and slo-						incr	82
Cac (Calcium channel)	decr						83, 84
K+ channels eag and Sh combined	incr						81
Ligand							
Wg	decr		incr				85
Wg overexpression	incr					incr	67, 85

boutons and do not fall into the other three groups. Disruption of these genes produces boutons that are arrested at various stages of development. For each of these categories, only a few genes that fall into these groups are discussed. In the last part of this section, we describe how many of these genes may work in parallel to affect the same downstream effectors that regulate NMJ growth.

Genes that Promote Synaptic Growth

LOF mutations for genes in this category produce phenotypes that are characterized by decreases in the number of boutons and are sometimes associated with an increase in their sizes (Figure 4(c) and (d)). A large subset of genes in this category alters the *microtubule (MT) cytoskeleton*. The cytoskeleton of NMJ presynaptic terminals can be divided into core and membrane-associated components. MTs and MT-binding proteins are part of the core cytoskeleton. The membrane-associated cytoskeleton is a filamentous network of spectrin molecules linked together by actin and attached to cell adhesion proteins in the plasma membrane.[86] The presynaptic MT cytoskeleton is most easily visualized using antibodies against Futsch,

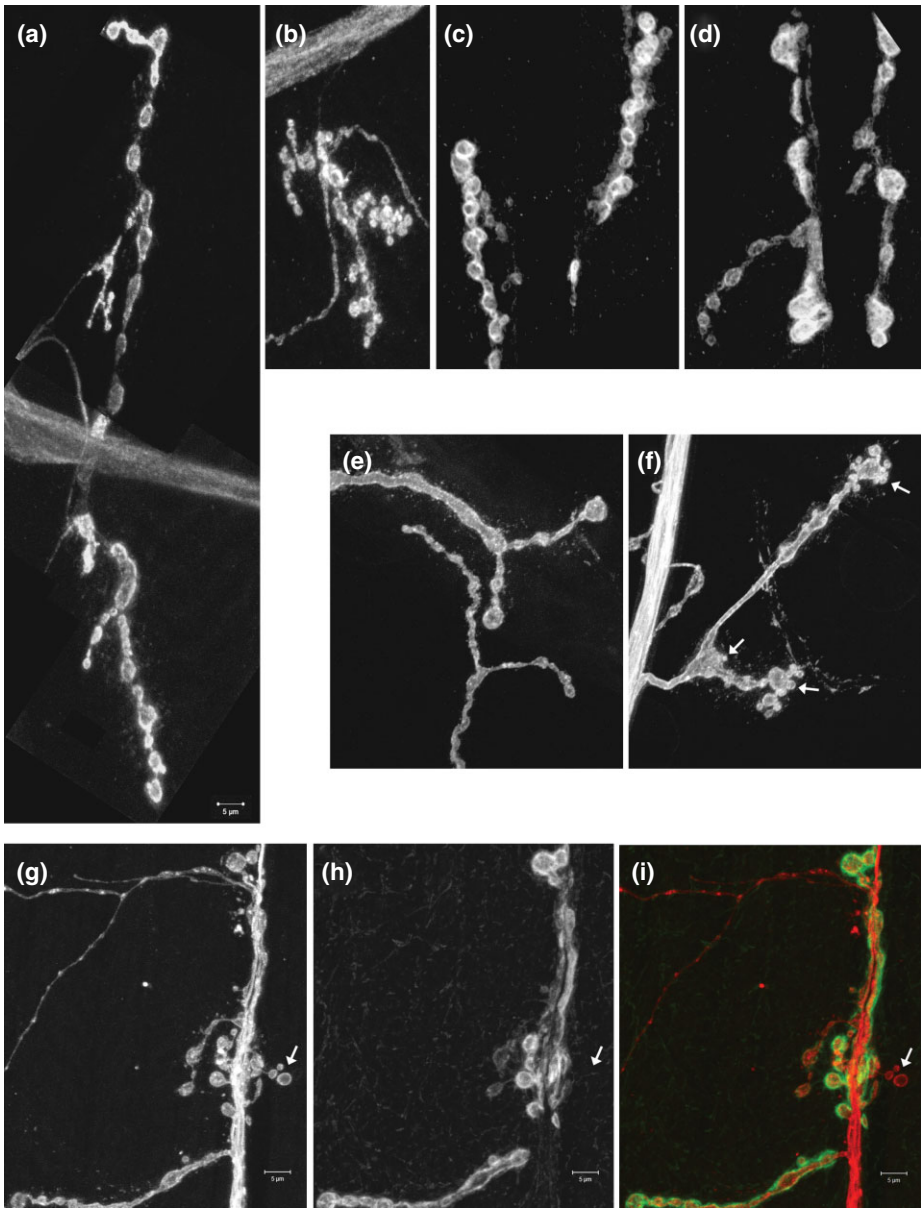

FIGURE 4 | Examples of neuromuscular junction (NMJ) phenotypes. (a, b, e, and f) Muscle 4 NMJs; (c, d, g, h, and i) muscle 6–7 NMJs. (a–f) are labeled with anti-HRP; (g–i) are double-labeled with anti-HRP and anti-Dlg. (a) Wild type. (b) A mutant NMJ with boutons that are greater in number but smaller in size than in wild type. Entire muscle 4 NMJs are shown in (a) and (b). (c) Wild type. (d) A mutant NMJ with boutons that are fewer in number but larger in size than in wild type. Partial muscle 6–7 NMJs are shown in (c) and (d). (e) Wild type. (f) An NMJ with a satellite bouton phenotype. Satellite boutons (arrows) resemble 'budding' structures and are seen here on the terminal parent boutons and on the branching bouton. (g–i) An NMJ with ghost boutons. Ghost boutons [arrow in (i)] appear as boutons that have the presynaptic marker, anti-HRP [(g); red in (i)], but lack postsynaptic markers, such as Dlg [(h); green in (i)]. Scale bars are 5 μm.

which encodes the *Drosophila* MT-associated protein (MAP) 1b ortholog.[53] Fragmentation of the MT network is correlated with decreased bouton numbers in *futsch* mutants.[42,53,87] Similar phenotypes are seen in mutants lacking atypical protein kinase C (aPKC).[42] aPKC activity is thought to stabilize MTs during bouton maturation and Futsch is required for the aPKC-mediated MT stabilization.

Some genes that function within the *Wnt signaling pathway* also promote NMJ growth by altering the MT cytoskeleton. Two of the Wnt pathway genes for which mutations affect the MT cytoskeleton are *arrow* (*arr*) and *dishevelled* (*dsh*). *arr* encodes a co-receptor of Frizzled 2 (Fz2), the receptor for the secreted Wnt protein Wingless (Wg). Dsh is a cytoplasmic phosphoprotein that is downstream

of Fz2. Presynaptic loss of either of these molecules results in a decrease in bouton number, accompanied by abnormal enlargement of some of the boutons. Loss of presynaptic Arr or Dsh also causes disorganization of the MT cytoskeleton.[67]

Genes that Negatively Regulate Synaptic Growth

The second category of genes includes those for which LOF mutants have increased numbers of boutons, sometimes accompanied by a decrease in their sizes (Figure 4(a) and (b)). These genes may normally function as negative regulators of NMJ growth. It is important to note, however, that investigators often only report bouton numbers and do not quantitate bouton size, so some mutations that increase bouton number may not increase the total number of NMJ active zones because there is a corresponding decrease in bouton size and/or active zones per bouton. We also distinguish phenotypes with increases in the number of normal boutons from those that are characterized by the presence of 'satellite' boutons. Satellite bouton phenotypes are described in another section below. Mutant NMJs with larger numbers of normal boutons may have longer synaptic arbors with unaltered branching patterns, or may have increased numbers of secondary and tertiary arbor branches. Genes within this category encode proteins involved in a variety of signaling pathways, and a few of these are highlighted here.

Another cytoskeletal modulator, Spastin, appears to be a negative regulator of bouton growth. *spastin* mutant NMJs show a slight increase in the number of boutons along with a decrease in bouton size. Spastin is an *MT-severing* protein belonging to the family of AAA ATPases. One might have expected that a decrease in Spastin activity would produce less severing of MTs and consequently should result in an increase in Futsch labeling. Instead, Futsch and tubulin staining in NMJs are reduced in *spastin* mutant NMJs, particularly at terminal boutons. The data suggest that severing of MTs into smaller segments may facilitate transport of MTs from the axon into the NMJ.[40,88]

NMJ growth is also regulated by *protein degradation pathways*. The ubiquitin-proteasome system (UPS) has many ubiquitinating and deubiquitinating enzymes that function in almost all developmental decisions.[89] Two E3 ubiquitin ligase complexes known to function in the cell cycle are SCF (Skp/Cullin/F-box) and the anaphase-promoting complex/cyclosome (APC/C). The APC/C complex is composed of core and catalytic subunits. The catalytic subunits are APC2 and APC11. Cdc27 is one of the core subunits and Cdh1 is an activator subunit that regulates activity of the APC/C complex.[77,90,91] APC2, Cdc27, and Cdh1 localize to the *Drosophila* larval NMJ. APC2 is a negative regulator of bouton growth. Lack of APC2 (*morula*) in neurons results in an increase in bouton number, although bouton size does not change. One of the substrates of the APC/C complex is DLiprin-α, a scaffolding protein that promotes bouton growth. In an *apc2* mutant, DLiprin-α is not ubiquitinated and the protein accumulates at the NMJ. This lack of degradation results in an increase in bouton number.[78]

Highwire (Hiw) is a ubiquitin ligase that is part of the SCF complex, and fat facets (Faf) is a deubiquitinating protease. Both are required presynaptically to control bouton growth.[75–77] *hiw* and *faf* mutants have greatly expanded presynaptic NMJ arbors. Bouton number and NMJ span are increased, but bouton size is decreased. Hiw controls NMJ growth by regulating the MAP kinase signaling pathway through Wallenda (Wnd), a dual leucine zipper kinase (DLK) that is orthologous to MAPKKK.[92] When overexpressed, *wnd* displays an overgrowth phenotype similar to that of a *hiw* LOF mutant.[79]

Neuronal Activity and Synaptic Growth

Neuronal activity plays a critical role in synaptic growth at the *Drosophila* NMJ. Double LOF mutants that have reductions in the levels of two *voltage-gated K^+ channels,* Ether-a-go-go (Eag) and Shaker (Sh), or *eag* mutants expressing a Shaker dominant-negative subunit, have hyperexcitable neurons. These mutants have increased numbers of boutons, suggesting that neuronal activity can positively regulate NMJ growth.[81,93] However, the increase in bouton number seen in such mutants might also be due to satellite bouton formation (see below). cAMP plays a role in the activity-dependent effect of Eag and Sh on synaptic growth, as shown by analysis of the phenotypes of double mutant combinations involving genes that regulate cAMP levels (*dunce*, *rutabaga*, and others) and those that alter electrical activity. It has been suggested that neuronal activity increases the amount of intracellular calcium, which subsequently affects signaling through the cAMP pathway.[80,81]

The importance of *calcium regulation* in bouton growth is further evident when investigating calcium channels. The voltage-gated N-type Ca^+ channel Cacophony (Cac) is expressed presynaptically and functions in neurotransmitter release. An independent role of Cac is in regulation of bouton formation, because *cac* mutants have reduced numbers of boutons and terminal arbor branches. The data suggest that calcium entry through Cac channels has dual roles:

it triggers synaptic vesicle fusion and also promotes bouton formation.[83,84]

Genes that Affect Maintenance of Bouton Structure

On the basis of LOF phenotypes, we suggest that another category of genes includes those involved in maintaining the integrity of bouton structure during development of the NMJ. Mutants for genes within this group have NMJs that display increased numbers of boutons that are arrested (or captured by fixation) at various stages of development. These include 'ghost boutons', presynaptic retractions (also known as 'footprints'), and satellite boutons. Synaptopods are not included in this list, although they might also be increased in number in mutant NMJs, because they can only be observed in live preparations, and most studies of mutant phenotypes are of fixed samples.[32]

Ghost boutons are newly formed, 'immature' boutons that contain synaptic vesicles, but no active zones, and have not recruited postsynaptic elements, such as Discs large (Dlg)[94] (Figure 4(g)–(i)). They express the neuronal membrane marker recognized by anti-HRP antibody, the cell surface protein Fasciclin 2 (Fas2), and the synaptic vesicle markers cysteine string protein (CSP) and Synapsin. However, they lack the postsynaptic Dlg protein and GluRs. In addition, they rarely contain any Bruchpilot (Brp), which is an active zone component. Ghost boutons are transitional structures that are in the process of being stabilized into mature boutons and are not a result of degeneration of mature boutons. This was shown by live imaging of wild-type NMJs where ghost boutons, although rare, do exist.[32] Stimulation of motor neurons results in an increase in ghost bouton numbers. Ghost boutons are also prominent in *drape*r mutants. *draper* encodes an engulfment receptor.[33] A more detailed description of Draper function is provided in the transsynaptic section below. The process of ghost bouton formation at the Drosophila NMJ is comparable to synapse elimination in vertebrates.[95]

Presynaptic retractions, in which previously formed boutons disappear, are marked by footprints, which are postsynaptic relics that mark the spots that had been occupied by the boutons. Presynaptic retractions have been observed during normal growth for both Type Ib and Is boutons, with a moderate frequency (18% of NMJs) during early larval development and a much lower frequency in the third instar (6% of NMJs).[47] These structures are characterized by a simultaneous lack of Synapsin and Bruchpilot staining (presynaptic) and positive Dlg and glutamate receptor immunoreactivity (postsynaptic). Presynaptic retractions differ from ghost boutons not only by the pre- and postsynaptic molecules that are retained (see above), but also by the morphological changes that each encompasses: the former involves an entire arbor (with many boutons), whereas the latter involves a single bouton. In addition, the presynaptic retraction seems to occur later in the time course of bouton growth as indicated by the markers retained.

The genes implicated in presynaptic retraction are those that regulate the cytoskeletal architecture. Increased retraction of synaptic boutons occurs when components of the core or the membrane cytoskeleton are disrupted. The presynaptic Dynactin complex, which includes the Arp-2 (centractin) subunit and P150/Glued, binds to the microtubule component of the core cytoskeleton. Mutants for *Arp-2* or *P150/Glued* show disorganization of the MT network. These mutants also display a high frequency of presynaptic retractions.[47]

Proteins within the membrane cytoskeleton that affect synaptic retraction include Spectrin and Hu-li tai shao (Hts), the Adducin ortholog, which binds spectrin and caps actin filaments to stabilize synapses. *hts* mutants show an elevated number of retractions. In addition, Hts is a negative regulator of NMJ growth. When Hts is knocked down presynaptically *via* RNAi, the number of boutons increases and small-diameter membrane protrusions are seen at the ends of Type Ib synaptic terminals. Spectrin also affects presynaptic stability. In its absence, the cell adhesion molecules (CAMs) Fas2 and Neuroglian (Nrg) disappear, and this is followed by synaptic retraction.[45] A final player is Ankyrin2-L (the long isoform of Ankyrin2), which is thought to link the core MT cytoskeleton to the spectrin–actin membrane cytoskeleton. In its absence, the MT skeleton becomes disorganized and this results in an increase in synaptic retractions.[43,44]

Satellite boutons are small boutons that bud from a parent bouton that is present in a branch of the terminal arbor. As satellite boutons contain Synapsin and Brp and are apposed to postsynaptic Dlg and GluRs, they presumably contain functional synapses. Satellite boutons are more prevalent in mutants that display NMJ overgrowth. In wild-type larval NMJs, a branching parent bouton normally has no more than two new branches.[37] Mutants that exhibit the satellite bouton phenotype have parent boutons with 3–5 small boutons budding from the parent bouton[37,60,96] (Figure 4(e) and (f)). Satellite boutons are also seen budding off from the axonal segment that connects two adjacent boutons.[66] The satellite bouton phenotype is distinct from that of mutant NMJs that have many small-sized boutons (e.g., *hiw*, see above), because it is characterized

by parent boutons of normal size with many small boutons attached to them.

Genes for which LOF mutants have satellite bouton phenotypes encode molecules that are implicated in endocytosis, Wnt signaling, and control of neuronal activity. Loss of endocytic molecules, including Dynamin, Dap-160, Endophilin, Synaptotagmin, and Synaptojanin, produces NMJs with large numbers of satellite boutons.[60] Nervous wreck (Nwk) is an adaptor protein that localizes to the periactive zone.[37,58] It has been shown to interact with components of the endocytic machinery and negatively regulates Bone morphogenetic protein (BMP) signaling by direct interaction with the BMP receptor Thickveins (Tkv).[38,68] Mutations in *nwk* also produce large increases in the numbers of satellite boutons. Altering the levels of some of the components of the transsynaptic Wnt signaling pathway, including Wg, Arr, and Dsh, produces satellite bouton phenotypes. Neuronal activity changes resulting from K^+ channel mutants *seizure (sei)* and *slowpoke (slo)* also result in satellite bouton formation. Lee and Wu carried out a detailed study on molecules important for the formation of satellite boutons in these mutants.[82] They found that these satellites were suppressed by pre- or postsynaptic cAMP signaling and that Dlg was required. Their data suggested that there is postsynaptic involvement for the early steps in satellite formation, but that the later steps are regulated presynaptically.

Other proteins that regulate satellite bouton formation include the fly homolog of the amyloid precursor protein (APPL) and Fas2, the *Drosophila* NCAM ortholog.[66] APPL is a transmembrane glycoprotein that might function as a G_o-coupled receptor. Satellite boutons form when APPL is overexpressed and is not internalized, resulting in excess APPL protein on the plasma membrane. When Fas2 is selectively overexpressed on either side of the synapse, bouton number is decreased. However, overexpressing Fas2 simultaneously on both sides of the NMJ results in the formation of satellite boutons.[55]

Mechanisms Involved in Development and Maintenance of NMJ Arbors

The genes described in the above four sections ultimately converge to control the growth of boutons and the arborization pattern of the larval NMJ. Positive and negative regulators of bouton growth (first and second categories, respectively), modulators of neuronal activity (third category), and genes with structural bouton phenotypes (fourth category) affect NMJ development through a variety of molecular pathways. Signaling through each of these pathways is likely to be continuously modulated by antagonistic and cooperative pathways whose input is dependent on the physiological states of the muscles and neurons. Here, we describe some of the systems within which the proteins described above function in order to control the pattern of NMJ development.

Numerous studies indicate that the cytoskeleton is the primary driver in forming the presynaptic structures during development of the *Drosophila* neuromuscular system.[97] In both vertebrate and invertebrate systems, the presynaptic terminal can be compartmentalized into the core and membrane-associated cortical cytoskeletons, as described above. Downstream cytoplasmic molecules that affect polymerization of actin or tubulin structures play roles in bouton formation and growth. Many of the MT severing proteins, extracellular matrix molecules and CAMs (detailed in the next few paragraphs), converge through indirect pathways to these downstream effector molecules to alter the presynaptic cytoskeleton. Some of the known downstream proteins are ADF/Cofilin, LIM kinase, and Futsch. Cofilin depolymerizes actin,[98] whereas LIM kinase promotes actin polymerization by inactivating Cofilin.[99] LIM kinase is activated by p21-activated kinase (PAK), which is in turn stimulated by Cdc 42 and Rac.[100,101] These small Rho-like GTPases control the formation of polymerized actin structures. Some of the genes listed in Table 1 seem to affect the MT-associated neuronal protein Futsch directly or indirectly. Futsch colocalizes with microtubules in boutons and may increase their stability.[53] Some of the actions of the Wnt signaling pathway target Futsch presynaptically (*via* receptors present on the neuronal membrane and cytoplasmic proteins located intracellularly).[67,69] This is a form of autocrine signaling because the Wnt ligand, Wg, is released by the motor neuron.

NMJ development is affected by a variety of proteins that alter MT dynamics. Katanin, Spastin, and Fidgetin are enzymes that sever MTs *in vitro*.[102,103] Spastin is important for NMJ development, but it remains to be seen if the other proteins have roles in the neuromuscular system[88,104,105] (see category 2). Atlastin, an integral membrane protein GTPase that affects microtubule stability in muscles, has been shown to bind to Spastin *in vitro*.[106] It is not known if this interaction is relevant to bouton growth. Spastin function, like LIM kinase function, seems to be regulated by PAK.[40] In mammalian cells, PAK induces phosphorylation of Stathmin, a MT binding protein, resulting in changes in actin polymerization. At *Drosophila* NMJs, Stathmin acts presynaptically in neurons to affect NMJ development.[51]

The extracellular matrix molecules Syndecan and Dally-like (Dlp) are cell surface heparan sulfate proteoglycans (HSPGs). These affect NMJ growth by interacting with leukocyte-antigen-related-like (Lar), a transmembrane receptor protein expressed in neurons.[48,57] Lar is a receptor tyrosine phosphatase (RPTP) whose cytoplasmic domain interacts with several downstream signaling proteins. For the growth of boutons, Lar signals via Trio, a Rho-GEF (GEF, guanine nucleotide exchange factor), and Diaphanous, a Rho effector, to control the actin and microtubule cytoskeleton.[52] Lar also interacts with Dliprin-α (Syd-2 ortholog), which is involved in the organization of active zones.[48,107] Syd-1, a Rho-GTPase activating protein (Rho-GAP) is required for the correct localization of Dliprin-α to active zones.[108] To assemble both pre- and postsynaptic proteins across the synaptic cleft, Owald et al.[109] showed that Syd-1 recruits the CAM Neurexin and its postsynaptic partner, Neuroligin. These proteins assemble earlier than the localization of Bruchpilot (active zone protein) and the postsynaptic GluRs.

Many synaptically localized CAMs affect morphology and growth of boutons at the NMJ.[110,111] Fas2 can be a positive or a negative regulator of bouton formation, depending on whether it is expressed on both sides of the synaptic cleft or on one side, respectively. Fas2 stimulates growth by signaling through APPL and a cytosolic APPL-binding protein protein, Mint.[55] Fas2 homophilic interactions across the cleft may trigger the phosphorylation of APPL. The latter molecule could relay a signal to microtubules by binding to the heterotrimeric GTP binding protein G_o[55] and thus stimulate bouton growth by affecting MT dynamics.

Ubiquitylation and sumoylation are two processes that affect diverse cellular processes. SUMO (*S*mall *U*biquitin like *Mo*difier) proteins are small protein tags that are covalently attached to other proteins to modify their function. Sumoylation is similar to ubiquitylation, but has different functions. The latter is used to tag proteins for degradation, whereas the former is used mainly for modification of proteins. Although there is extensive evidence (see Section *Genes That Negatively Regulate Synaptic Growth* above) for roles of ubiquitylating proteins in NMJ development in *Drosophila*, sumoylation has not been shown to play a role in bouton growth.[89] Recently, Berdnik et al. have shown the involvement of a SUMO protease participating in the *Drosophila* olfactory system.[112] It will be of interest to examine the roles of the sumoylation machinery in the growth and development of boutons in the *Drosophila* NMJ.

Transsynaptic Signaling Pathways

Two of the most extensively studied transsynaptic pathways that regulate development of synaptic arbors at *Drosophila* NMJs are the Wnt pathway and the BMP pathway. Excellent recent reviews exist for these pathways, so we do not discuss them here.[35,113–117] The Neurexin–Neuroligin transsynaptic pathway has also recently been reviewed.[54] Below we discuss two less well-known transsynaptic pathways.

The Draper/Ced-6 Signaling Pathway

Draper is an engulfment receptor molecule that is involved in removal of neuronal cell fragments during programmed cell death in the *Drosophila* brain.[118] At the larval NMJ, the Draper/Ced-6 pathway functions to clear presynaptic neuronal debris and ghost boutons that have not stabilized. This pathway operates in the muscles and in the glial cells that surround the synaptic boutons. In *draper* mutants, the number of boutons decreases and the number of ghost boutons increases. The latter is due to inability of the muscle cells to phagocytose immature ghost boutons. The fact that synaptic growth is decreased in *draper* mutants suggests that accumulated presynaptic debris not cleared by Draper inhibits growth at the NMJ.[33] We classify this pathway as transsynaptic in this review because the results suggest that presynaptic neuronal debris contains signaling molecules that might activate the Draper/Ced-6 pathway in muscles and glia to clear the debris and thus allow synaptic growth.

Synaptotagmin-4 Retrograde Signaling Pathway

Synaptotagmin-4 (Syt 4) localizes to vesicles in the postsynaptic muscles. Syt 4 mRNA and protein expression are modulated by neuronal activity. In wild-type NMJs, increasing neuronal activity (by increasing temperature or in hyperexcitability mutants) results in increased numbers of boutons.[81,119] In *syt 4* mutants, there is no synaptic overgrowth when neuronal activity is increased.[120] Thus, Syt 4 seems to control the postsynaptic signal that promotes bouton growth when induced by activity.

TRANSLATIONAL REGULATORY MECHANISMS AND ACUTE PLASTICITY AT THE NMJ

Translational regulation is used to modulate protein expression and localization in a variety of biological contexts, including early embryonic development,

cell differentiation, and neuronal plasticity. In both *Drosophila* and vertebrates, translation of many specific mRNAs is regulated during early embryonic patterning. Translationally regulated *Drosophila* maternal mRNAs that are essential for development include *hunchback*, *oskar*, *gurken*, and *nanos (nos)*.[121–123] In many cases, translational regulation involves protein–RNA and/or RNA–RNA interactions within the 3′ untranslated region (UTR) sequences of the regulated transcripts.

Long-lasting changes in the structure and function of synapses are required for the storage and processing of information. Regulated 'local' postsynaptic protein synthesis is an attractive long-term plasticity mechanism because it provides a way to maintain synaptic states beyond the lifetime of any individual protein in the synapse. Newly synthesized proteins could in principle be selectively directed only to those synapses that have undergone modification. It is known that components necessary for translation are present in mammalian dendrites, including polyribosomes,[124,125] mRNAs, and miRNA machinery.[126,127] Dendritic protein synthesis is required for long-term maintenance of changes in synaptic efficacy. However, it has not been demonstrated that newly synthesized proteins are actually selectively routed to dendritic spines containing potentiated synapses.

Although Ib and Is synapses, which derive from different neurons, can be separately regulated,[128] there is no evidence that single boutons of the same type within a single *Drosophila* NMJ are independently controlled. However, the *Drosophila* NMJ is still a useful system in which to study translational regulation, because it exhibits both developmental and short-term plasticity, and control of postsynaptic mRNA translation is essential for these events. Some translational control mechanisms may operate throughout the entire postsynaptic muscle fiber, while others may be specific to the postsynaptic SSR, which has been shown to contain polyribosomes.[129,130] In this section of the review, we will consider some translational regulatory mechanisms that function at the *Drosophila* NMJ to regulate synaptic growth and plasticity, focusing on translational repression by microRNAs (miRNAs) and the RNA-binding proteins fragile X mental retardation protein (FMRP), Pumilio (Pum), and Nanos (Nos) (Figure 5).

miRNAs

miRNAs are 21-25 nucleotide noncoding RNAs that regulate gene expression by binding to target mRNAs and recruiting a repressor complex known as the RNA-induced silencing complex (RISC), which includes Dicer, Argonaute proteins, and other components.[131,132] RISC not only functions in the biogenesis of miRNAs and siRNAs (silencing RNAs), but also is required for their activity. The degree of complementarity between the miRNA and its target mRNA determines the mode of regulation: near-perfect complementarity results in cleavage of the duplex, whereas partial complementarity leads to translational repression.

miRNAs are known to be important regulators of neural development and function in a variety of systems.[133–135] However, the only miRNA whose individual function at the NMJ has been characterized thus far is miR-8. This miRNA is also implicated in neurodegeneration,[136] Wnt signaling,[137] and innate immune homeostasis.[138] miR-8 was found to be a regulator of NMJ growth in a forward genetic screen. miR-8 activity at the NMJ was examined using a deletion of miR-8 and a modified 'microRNA sponge' system, which can inactivate specific miRNAs.[139] Knocking out miR-8 function presynaptically had no effect. However, postsynaptic knockout resulted in a decrease in the number of synaptic boutons and branches. The 3′ UTR of the *enabled* (*ena*) mRNA has one predicted miR-8 binding site, and if miR-8 indeed binds to and represses *ena* mRNA translation, Ena protein levels should increase when miR-8 activity is inhibited. This was in fact observed: muscle expression of the miR-8 sponge resulted in elevated levels of postsynaptic Ena. It was also shown that postsynaptic overexpression of Ena can phenocopy the loss of miR-8.[74] These results provide evidence for a role of miRNA-mediated translational repression in regulating synaptic growth at the NMJ. Some important questions remain, however. First, are *ena* mRNA and/or miR-8 localized to the SSR? Second, the genetic interaction between miR-8 and *ena* does not necessarily indicate that miR-8 directly controls Ena expression. It will be important to determine whether miR-8 binds to the *ena* 3′ UTR.

Although miR-8 is the only miRNA that has been shown to regulate NMJ growth thus far, Dicer and miR-284a control GluRIIA and GluRIIB protein levels at the NMJ[34] (Figure 5). *GluR* mutations can affect bouton numbers,[140–142] so miRNA-mediated effects on translation of *GluR* mRNAs may have an impact on NMJ growth during development.

The roles of miRNAs at the NMJ have also been explored using mutations that affect the RISC complex. Argonaute 2 (Ago2) is expressed presynaptically at the NMJ and has been shown to be a positive regulator of bouton growth. *Ago 2* mutants have decreases in bouton number and in the number of

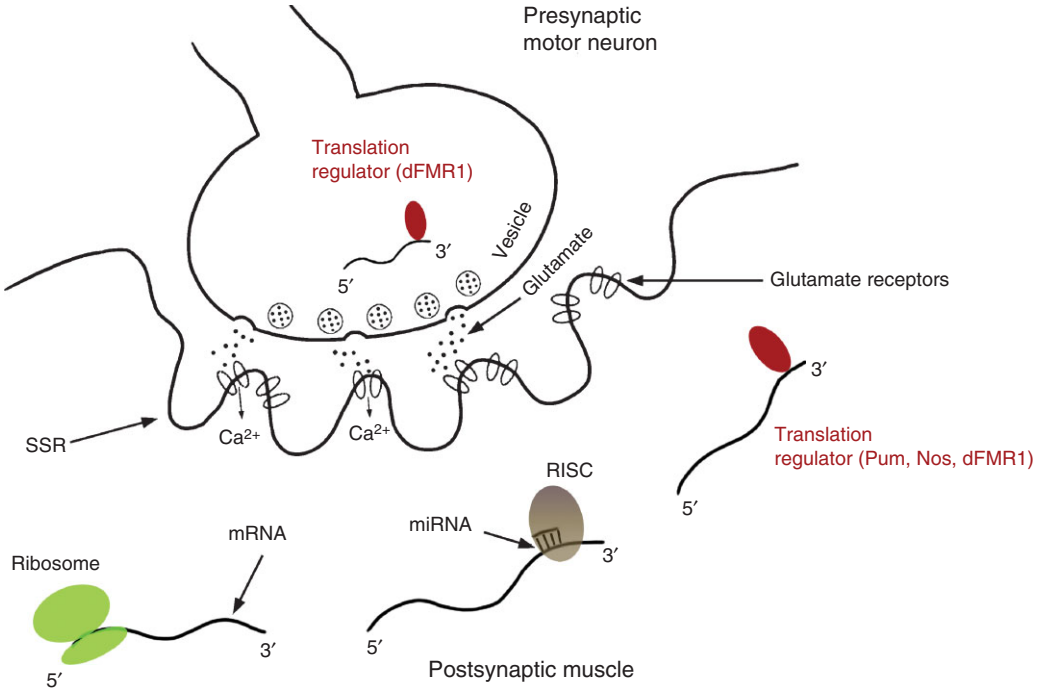

FIGURE 5 | A schematic diagram of the neuromuscular junction (NMJ) depicting some of the translational regulatory mechanisms that function in the postsynaptic muscle or in the motor neuron, possibly in its presynaptic terminal. In the presynaptic motor neuron, dFMR1 (indicated by the dark oval) binds to *futsch* mRNA and inhibits its translation. In the postsynaptic muscle, mRNAs are shown that are being actively translated (indicated by the ribosome), regulated by either microRNA and the RISC complex (indicated by base-paired complementary strand and light oval) or translational regulatory proteins (indicated by dark oval). miR-8 regulates translation of *enabled* and other target mRNAs. The translational repressors Pum and Nos regulate expression of GluRIIA and GluRIIB, respectively, and Pum binds directly to *GluRIIA* mRNA. dFMR1 represses the expression of both GluRII subunits. RISC, RNAi-silencing complex; SSR, subsynaptic reticulum.

arbor branches.[143] Ago2 functions predominantly in the siRNA pathway rather than in miRNA processing, although alternate miRNA biogenesis pathways may require Ago2.[144]

Two other *Drosophila* miRNAs have been demonstrated to regulate dendritic outgrowth and to repress translation of proteins that are also important for NMJ growth. It would be interesting to examine whether these miRNAs also have functions at the NMJ. In *Drosophila* embryos and larvae, GFP driven by the miR-124a promoter is expressed at high levels in the ventral nerve cord and motor neurons and at lower levels in dendritic arborization (DA) neurons in the peripheral nervous system (PNS).[145] Overexpression of miR-124a in DA neurons caused a reduction in the number of dendritic ends. miR-184 functions in ovaries to allow differentiation of the germline stem cells by reducing expression of the Decapentaplegic receptor, Saxophone (Sax).[146] *sax* mutants also have an NMJ phenotype characterized by reduced numbers of boutons and decreased synaptic strength.[147] It is not known whether miR-184 contributes to this *sax* phenotype.

Fragile X Mental Retardation Protein

Fragile X syndrome (FXS) is one of the most common forms of mental retardation in humans, affecting an estimated 1 in 4000 males and 1 in 8000 females. The disease is caused by mutations in a single gene, *FMR1* (for fragile X mental retardation 1).[148–150] FMR1 protein (FMRP) is widely expressed in fetal and adult tissues with enhanced expression in the brain and testes where major symptoms are manifested.[151] Loss of FMRP results in delayed dendritic spine maturation in both FXS patients and *Fmr1* knockout mice.[152–154] FMRP is a translational repressor[155,156] that interacts with sequences in the 3′ UTRs of its target mRNAs. Flies have only one ortholog of FMRP, called dFMR1, which regulates synaptic structure and physiology.[72]

At the *Drosophila* NMJ, dFMR1 is expressed presynaptically in the motor neurons and postsynaptically in the muscles. Analysis of *dfmr1* mutants revealed synaptic overgrowth characterized by an increase in the number of boutons, an increase in branching, and increased synaptic strength. Satellite boutons were also present in greater numbers than in wild type.[73] Overexpression of dFMR1 presynaptically produces a phenotype with fewer

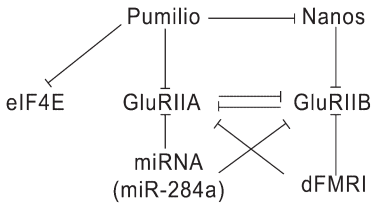

FIGURE 6 | A diagram depicting the actions of translational regulatory proteins on *GluRIIA*, *GluRIIB*, and *eIF-4E* mRNAs. GluRIIA is repressed by Pum, miR-284a, and dFMR1. However, only Pum has been shown to directly bind to the 3′ UTR of *GluRIIA* mRNA. Pum also binds to *eIF-4E* and *nos* mRNAs. GluRIIB expression is repressed by Nos, miR-284a, and dFMR1. GluRIIA and GluRIIB compete for synaptic occupancy, so that GluRIIA represses GluRIIB expression and *vice versa*.

boutons that are significantly larger than normal. One of the neuronal targets of FMRP is Futsch, the MAP-1b ortholog.[157,158] Futsch protein levels are upregulated in *dfmr1* mutants. dFMR1 repression of Futsch expression is important for NMJ development, because *futsch* mutations suppress the *dfmr1* NMJ phenotype.[72]

Like miRNAs, dFMR1 controls expression of the subunits of the postsynaptic ionotropic glutamate receptor.[159] NMJ GluRs can be divided into two classes: the larger amplitude, slower acting A class and the smaller amplitude, faster-acting B class. Each functional GluR consists of four subunits, three of which are shared among all receptors (GluRIIC, GluRIID, GluRIIE). The fourth subunit is either GluRIIA (A class) or GluRIIB (B class).[128,160] In *dfmr1* mutants, GluRIIA is increased and GluRIIB is decreased (Figure 6). Postsynaptic overexpression of dFMR1 caused a decrease in both GluRIIA and GluRIIB levels. Because GluRIIA mRNA has been localized to the postsynaptic region of the muscle fiber, it is possible that this repression of the GluRIIA subunit by dFmr1 occurs locally at synapses.[129] The contribution of dFMR1-regulated GluR expression to bouton growth has not been examined, although it is well documented that GluR levels affect NMJ growth during development.[140–142]

As described above, miR-124a could have roles at the NMJ, due to its high expression in motor neurons. miR-124a levels are regulated by dFmr1.[145] Thus, other than direct repression of target mRNAs, dFmr1 may also affect neuronal development at the NMJ by controlling levels of miR-124a. Another potential target of dFMR1 is Dlg, the PSD-95 ortholog. In mice, FMRP interacts with the 3′ UTR of *PSD-95* mRNA to regulate its stability.[161] Since Dlg plays a major role in synaptic structure and function at the larval NMJ,[6,18] dFMR1 might regulate bouton growth by repressing translation of *dlg* mRNA. Finally, interactions between FMRP and the miRNA pathway may further contribute to the roles that both of these systems play in synaptic growth. These interactions are discussed in more detail below.

Translational Repression by Pumilio and Nanos

The *Drosophila* Pumilio (Pum) protein is a member of the PUF RNA-binding protein family. Maternal Pum functions primarily as a translational repressor in embryonic patterning and germ cell proliferation and migration.[162,163] Pum recognizes sites called Nanos response elements (NREs) in its target mRNAs, including those encoding Hunchback, Cyclin B, and Para.[164–166] Most NREs are located in 3′ UTRs. In most cases, Nanos (Nos) functions as a corepressor necessary for Pum-mediated translational repression. However, Pum and Nos also have functions that are separate from those of their partners.[71,167,168]

Zygotic Pum also functions later in development at the larval NMJ. Pum is postsynaptically localized at the NMJ, and is also present in neuronal cell bodies.[70] Pum has distinct roles on the two sides of the synapse. In *pum* mutants, Type Ib boutons are much larger and their numbers are decreased, whereas the number of 1s boutons is increased. The 1b bouton phenotype can be fully rescued by neuronal expression of full-length Pum in the *pum* mutant background. Postsynaptic Pum expression in *pum* mutants has no effect on the 1b bouton phenotype, but it rescues the increase in 1s bouton numbers.[70]

The idea that local postsynaptic translation occurs at the NMJ emerged from the findings that puncta ('aggregates') of the translation factors eIF-4E and PABP appear at NMJ boutons after larval motor activity is induced by moving larvae from slurry (liquid) to solid food for a period of a few hours.[129] Larvae can remain relatively stationary while ingesting liquid food, but must actively burrow through solid food. GluRIIA levels are also increased by this protocol. It was speculated that the purpose of local postsynaptic translation at the NMJ is to allow rapid changes in synaptic strength and facilitate the growth of new boutons in response to increases in larval motor activity. These synaptic alterations might be able to occur more quickly if they are implemented through translation of mRNAs that are already localized to the postsynaptic SSR.

As Pum is a translational repressor[169] and is postsynaptically localized, Menon et al.[70] reasoned that it might control the levels of postsynaptic eIF-4E, which is limiting for translation in many systems. Indeed, it was observed that eIF-4E levels at the NMJ are very high in *pum* mutants (up to 12-fold

higher than in wild type), and that these aggregates are unchanged by increases in larval motor activity. eIF-4E is encoded by a single essential gene, so eIF-4E protein is also present within the cytoplasm of the muscle fiber. However, cytoplasmic eIF-4E levels are unchanged in *pum* mutants, indicating that translational repression by Pum only occurs at the synaptic sites where Pum is localized. Pum binds selectively to the 3′ UTR of *eIF-4E* mRNA, suggesting that it is a direct target. GluRIIA levels are also greatly increased in *pum* mutants, and Pum binds selectively to the 3′ UTR of *GluRIIA* mRNA as well.[71] These results are consistent with a model in which Pum normally represses translation of synaptically localized *eIF-4E* and *GluRIIA* mRNAs in larvae that are not moving vigorously. When larval motor activity increases, Pum (or a Pum cofactor) is partially inactivated. This would cause the levels of eIF-4E, GluRIIA, and other direct Pum targets to increase rapidly, because these proteins would be translated from preexisting mRNAs that had been translationally repressed by Pum. When larvae are forced to move they require more transmission at the NMJ, and this could be facilitated by eIF-4E induction, since eIF-4E might be limiting for translation of all postsynaptically localized mRNAs. The induction of GluRIIA, which produces receptor complexes that conduct more current, would also increase the ability of the NMJs to effectively depolarize the muscles. The mechanisms by which Pum might be inactivated after induction of larval motor activity are unknown, but there is evidence that Pum's postsynaptic functions are regulated by an aggregation-prone sequence within its unstructured N-terminal region.[170]

The regulation of eIF-4E and GluRIIA by Pum is part of a more complex circuit of translational regulation that operates at the NMJ. During early development, Pum and Nos work together to repress Hb and other targets. However, they work in opposition to each other at the NMJ. Pum binds to the 3′ UTR of *nos* mRNA, and Nos levels are increased in *pum* mutants. Nos represses expression of the alternate GluR subunit GluRIIB. Also, GluRIIA and GluRIIB compete with each other for occupancy in synaptic receptor clusters. Thus, when Pum levels are reduced, Nos is increased, leading to downregulation of GluRIIB, which amplifies the elevation of GluRIIA produced by loss of repression of its mRNA by Pum. Conversely, if Pum is increased, GluRIIA and Nos are both repressed, leading to increased expression of GluRIIB at the expense of GluRIIA[71] (Figure 6). The mechanism by which Nos represses GluRIIB without the involvement of Pum is unknown.

Pum is also involved in synaptic growth and plasticity in other types of neurons, both in *Drosophila* and in vertebrate systems. Pum can bind to the 3′ UTR of *dlg* mRNA. In the mushroom bodies of adult *Drosophila*, overexpression of Pum reduces the levels of Dlg and causes a defect in the elaboration of axonal projections.[171] However, Dlg levels at the NMJ are not affected by Pum. Hypomorphic *pum* mutants have been reported to have learning and memory defects.[172] Pum and Nos also affect dendritic arborization in *Drosophila* sensory neurons.[173] In dissociated mammalian hippocampal neurons, Pum is localized to granules in dendrites.[174] Knockdown of Pum by siRNA causes increases in dendritic arborization, while Pum overexpression reduces the size of the dendritic arbor.[175] Finally, a novel function of Pum in controlling translational repression by miRNAs was recently reported[176] and this is discussed below.

Orb2/CPEB2

Cytoplasmic polyadenylation element binding (CPEB) proteins can be divided into two subfamilies. The first CPEB subfamily functions mainly during oogenesis and early embryonic development[177] and includes the *Drosophila* CPEB ortholog Orb. CPEBs recognize specific sequences in the 3′ UTRs of their target mRNAs and control their translation. Orb is required for establishing anteroposterior and dorsoventral axes in early development by translationally activating *oskar* and *gurken* mRNAs, respectively.[178–182] The second CPEB subfamily includes vertebrate CPEB2-4 and *Drosophila* Orb2. This subfamily is more broadly expressed and has roles outside of the germline.[183,184]

Although no direct evidence yet links Orb2 to synaptic growth at the NMJ, it is reasonable to think that it may have a role there. *Drosphila* Orb2 is widely expressed in the nervous system from embryonic to adult stages. Specific localization of Orb2 at synaptic sites was observed in the CNS, suggesting that it might be involved in synaptic translation.[185] A study aimed at identifying mRNA targets of Orb2 identified a variety of genes involved in synaptic growth and stability at the NMJ, including *neuroligin*, *still life*, and *aPKC*.[186] It is unknown, however, if Orb2 regulates translation of these mRNAs in the neuromuscular system. Finally, Orb2 was identified in a screen for proteins likely to function in the dFmr1 pathway, suggesting that that Orb2 might regulate dFmr1-mediated synaptic growth.[187]

Interplay Among Translational Regulation Pathways

In addition to the separate action of each of these translational regulatory mechanisms on its respective targets, evidence suggests that these systems can regulate one another and/or work in tandem to control the same target mRNAs. The miRNA pathway and mammalian FMRP are intimately linked.[188,189] In *Drosophila*, *orb* mRNA translation is activated by Orb protein, forming a positive feedback loop. dFMR1 also binds *orb* mRNA and inhibits its translation, thus keeping the positive feedback loop in check.[190] The 3′ UTR of the mammalian tumor suppressor p27 mRNA has binding sites for Pum and for two miRNAs, miR-221 and miR-222. However, in order for the miRNAs to efficiently repress p27 translation, Pum must first bind and induce a conformational change in the mRNA.[176] A similar observation was made for translational regulation of the E2F3 oncogene.[191]

Collectively, these interactions suggest that a complex series of interconnected regulatory mechanisms control the translation of mRNAs that encode key regulators of synaptic growth and function at the NMJ. However, we still lack an overall understanding of how these mechanisms work together to ensure that mRNAs encoding synaptic regulators are translated at the correct times and places. We need to determine which mechanisms function in the postsynaptic SSR, and which act in the muscle and neuronal cytoplasm. Polyribosomes are localized to the SSR, but are all the necessary components for translation present in the SSR? This is not yet known. It is also unknown whether all these mechanisms function during the same developmental stages. Finally, it has not yet been directly demonstrated that local synaptic translation actually occurs at the NMJ. It would be valuable to develop an optical method to detect and localize synaptic translational events in wild-type and mutant larvae.

The genes described in this review encode proteins that control synaptic bouton growth through many different mechanisms. These include cytoskeletal dynamics, protein degradation, cell adhesion, and neuronal activity. NMJ growth and development is further fine-tuned at the level of protein synthesis by translational regulators that include miRNAs, FMRP, and Pum. The study of the larval NMJ is likely to continue to generate exciting new findings. It will also be of interest to examine the development and maintenance of the adult neuromuscular system, which is still poorly understood. We have highlighted molecular mechanisms employed at the *Drosophila* NMJ that are similar to those used at glutamatergic synapses in the vertebrate nervous system. Many vertebrate synaptic proteins have orthologs that are used for the development and function of the fly NMJ. Because of this, researchers can productively use forward genetic screens in *Drosophila* to find new synaptic components that are likely to be important for development and/or function of mammalian excitatory synapses. Insights gained from studies of the fly NMJ should provide information relevant to the development and function of synapses in many other systems.

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FURTHER READING

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