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# Chapter 9

# Glutamatergic Synthesis, Recycling, and Receptor Pharmacology at *Drosophila* and Crustacean Neuromuscular Junctions

**Joshua S. Titlow and Robin L. Cooper**

## Abstract

Invertebrate glutamatergic synapses have been at the forefront of major discoveries into the mechanisms of neurotransmission. In this chapter we recount many of the neurophysiological advances that have been made using invertebrate model organisms, from receptor pharmacology to synaptic plasticity and glutamate recycling. We then direct your attention to the crayfish and fruit fly larva neuromuscular junctions, glutamatergic synapses that have been extraordinarily insightful, the crayfish because of its experimental tractability and *Drosophila* because of its extensive genetic and molecular resources. Detailed protocols with schematics and representative images are provided for both preparations, along with references to more advanced techniques that have been developed in these systems. The chapter concludes with a discussion of unresolved questions and future directions for which invertebrate neuromuscular junction preparations would be particularly well suited.

**Key words** Neuromuscular junction, Glutamatergic synapse, Invertebrate, Crayfish, *Drosophila*

## 1 Overview of Glutamate Activity at Neuronal Synapses

Glutamate (Glu) is one of the most common neurotransmitters in animals as it is known to be present in some of the most primitive animal species [1–3] and is one of the most abundant transmitters in the central nervous system (CNS) of vertebrates [4]. Various receptor subtypes have evolved to provide a wide range of responses to Glu, from fast acting ion channels (ionotropic) to slow acting second messenger cascades (metabotropic), and excitatory as well as inhibitory responses. The types of receptors show a wide diversity across the animal kingdom [3, 5] and are even present in roots of some plants to respond to environmental Glu [6]. Classically, receptors have been defined by their pharmacological profile with agonist and antagonist binding affinities [7]. More recently receptors have been taxonomically defined by gene and protein sequence homology. On the presynaptic side, neurons employ various

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# Glutamatergic synthesis, recycling, and receptor pharmacology at *Drosophila* and crustacean neuromuscular junctions

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## Abstract

Invertebrate glutamatergic synapses have been at the forefront of major discoveries into the mechanisms of neurotransmission. In this chapter we recount many of the neurophysiological advances that have been made using invertebrate model organisms, from receptor pharmacology to synaptic plasticity and glutamate recycling. We then direct your attention to the crayfish and fruit fly larva neuromuscular junctions, glutamatergic synapses that have been extraordinarily insightful, the crayfish because of its experimental tractability and *Drosophila* because of its extensive genetic and molecular resources. Detailed protocols with schematics and representative images are provided for both preparations, along with references to more advanced techniques that have been developed in these systems. The chapter concludes with a discussion of unresolved questions and future directions for which invertebrate neuromuscular junction preparations would be particularly well suited.

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**Running Head:** Invertebrate glutamatergic synapse models

## 1. Overview of glutamate activity at neuronal synapses

Glutamate is one of the most common neurotransmitters in animals as it is known to be present in some of the most primitive animal species [1-3] and is one of the most abundant transmitters in the CNS of vertebrates [4]. Various receptor subtypes have evolved to provide a wide range of responses to glutamate, from fast acting ion channels (ionotropic) to slow acting second messenger cascades (metabotropic), and excitatory as well as inhibitory responses. The types of receptors show a wide diversity across the animal kingdom [3, 5] and are even present in roots of some plants to respond to environmental glutamate [6]. Classically, receptors have been defined by their pharmacological profile with agonist and antagonist binding affinities [7]. More recently receptors have been taxonomically defined by gene and protein sequence homology. On the presynaptic side, neurons employ various mechanisms to incorporate glutamate and organize its release. Glutamate can be taken into cells by plasma membrane transporters (GLUT or excitatory amino acid transporters- EAAT) or indirectly by a transporter for other amino acids such as glutamine [8].

Through intracellular biochemical reactions amino acids and intermediate compounds can be converted to glutamate. Intracellular glutamate is packaged into synaptic vesicles against a concentration gradient through vesicular transporters [VGLUT; 9, 10]. The process of glutamate release, re-uptake, and repackaging to be released again is dependent on many molecular functions. The recycling process can be estimated by kinetic rates; however, there are various pathways depending on the synaptic circuit. In the CNS of vertebrates, glutamate recycling occurs directly through GLUT and indirectly through glial glutamate-glutamine-glutamate pathways, making it difficult to discreetly measure the various rates in intact systems. Glutamate can also be taken up into neurons that use GABA as a transmitter since glutamate is converted to GABA in GABA-ergic neurons [11, 12].

Invertebrate neuromuscular junction (NMJ) preparations have played an important role in fostering our understanding of neurotransmission at glutamatergic synapses. The aim of this chapter is to consolidate the knowledge of invertebrate NMJs and discuss the experimental potential of invertebrate synapses going forward. In doing so we highlight the important similarities and differences in the molecular mechanisms underlying invertebrate NMJ and mammalian glutamatergic transmission, including pharmacological and physiological characteristics of glutamate receptors. We then provide a brief description of protocols for the crayfish and fruit fly larva neuromuscular junctions and conclude with some ideas for future research directions with these systems.

## 2. Glutamatergic transmission at invertebrate neuromuscular junctions

Various invertebrate models have been used to investigate the regulation and developmental aspects of glutamate receptors and their action on cells [13-18]. Likely due to the ease of experimental setup and long viability in a minimal saline, invertebrate neuromuscular junctions (NMJs) of insects and crustaceans lead the way in obtaining pharmacological profiles with a battery of compounds that would later be screened on isolated neural preparations of vertebrates to address similar physiological questions [19-25]. Thus, early on, due to the simplicity of NMJs for physiological recordings and observation these specimens served as models for understanding potential actions for vertebrate systems. Invertebrate NMJs were not necessarily a model for vertebrate NMJs, as acetylcholine (Ach) had already been touted as a transmitter for the heart [26] and NMJs in frog and mammals [27]; however an assay to demonstrate Ach was the active substance for vertebrate NMJs the leech skeletal muscle preparation was used [28]. Likely a need to replicate findings from the frog NMJ for Ach drove similar questions about glutamate's action on the crustacean and locust NMJs, such as quantal responses [29, 30] and desensitization with prolonged application. Since Ach did not have an action at the crustacean NMJs, other potential transmitters known in the vertebrate CNS were tried from homogenized CNS samples of dog and guinea-pig on NMJs of the limbs as well as the hindgut of crayfish. This lead to further studies into the specific compounds that activated or inhibited transmission at crustacean NMJs on the limbs

and gut [31]. Rapid progress followed in primarily crayfish preparations to determine the specific compounds that resulted in muscle contraction and inhibition. Ach and Ach antagonists were shown not to have a direct effect on NMJ preparations and would not block the actions of L-glutamate [31-35].

An historical review detailing the discovery of GABA [36] walks one through the intriguing science from a compound termed 'inhibitory factor', which was isolated from homogenized bovine brain tissue, to the observed effects and postulation that GABA was an active synaptic compound [32, 37, 38]. It was shown by Kuffler and Edwards [39], Boistel and Fatt [40], and later proved by Kravitz [41-44] that indeed GABA does exhibit inhibitory action as a neurotransmitter released from lobster motor neurons on the opener muscle of the walking leg. The discovery that GABA is released from nerves at the crustacean NMJs was of interest since GABA could block the response of glutamate. It was later shown that GABA not only had reception on the contractile muscle directly but presynaptically on the excitatory motor neuron which released glutamate [45].

After the initial discoveries demonstrated that amino acids were the compounds released from the motor neurons innervating crustacean muscle, a focus then turned to examining which various amino acids could have an effect on the NMJ responses in various crustacean and insect preparations. Past reviews by Usherwood [1] and [2] mention various species used for investigating glutamatergic NMJs. Of crustaceans the crab [46-48], lobster [49], shrimp [50, 51], and heart of the isopod [52] were some of the preparations used. As for insects the cockroach [46], locust [53], moth [54], cabbage looper caterpillar [55], and blowfly [56] have been used for physiological studies. Other invertebrates such as an acorn barnacle [57] and snails [46] were also used.

Various agonist and antagonist as well as modulators of transmission were uncovered using invertebrates as experimental organisms over the years. The rationale to focus on crustaceans was most likely due to accessibility of the animal, viability and ease to examine the responses from nerve stimulation, which was occurring even before the neurotransmitters were identified. In addition, there is a long history of anatomical characterization for these preparations going as far back as the 1880's [58] with observations that nerve stimulation could cause muscle contractions that lead to facilitation in force development [59, 60, see review on the history of experimentation using the opener muscle of crayfish: 61]. When one considers that Sidney Ringer [62, 63] had only developed a saline for maintaining the viability of the frog heart preparations around the same time crayfish were being used to demonstrate muscle contraction from stimulating nerves over long periods of time in isolation, the crayfish offered further hope in addressing the properties of synaptic transmission. It was not until Van Harreveld [64] developed a saline for crayfish that prolonged physiological studies were practical. Synaptic physiology and dissection of the pharmacology and function of glutamate and GABA receptors grew steadily afterwards using the crayfish and other crustaceans [65-67].

Using various stimulation paradigms of the motor nerve, short-term facilitation (STF) [68] and long-term facilitation (LTF) was first demonstrated at crustacean NMJs [69] and later long-term potentiation (LTP) was shown to be present in mammalian CNS preparations [70]. These findings directed investigations to determine if the mechanisms were due to purely presynaptic or postsynaptic modifications in the receptor density or receptor subtypes to account for the effects. Pharmacological profiling of crustacean NMJs continued in the early days [19, 71-76] providing assays to determine mechanisms for modulation of the response to glutamate with a wide variety of compounds. Shank and Freeman [77] demonstrated that aspartate produced a cooperative effect with glutamate at lobster NMJs. This was also confirmed to occur at NMJs in a Hermit crab [78]. L-proline was shown to act as a glutamate antagonist [79] which is surprising as proline increases in the hemolymph with cold stress in insects [80]; thus, it would appear to further limit NMJ function in response to cold. The effects of other compounds such as piperidine dicarboxylates [81], 5-methyl-1-phenyl-2-(3-piperidinopropylamino)-hexane-1-ol (MLV-5860) [82], chlorisondamine and TI-233 [83, 84], spermidine [85], streptomycin and similar antibiotics [86], quisqualic acid [87], stizolobic acid [88], AMPA, N-methyl-D-aspartate (NMDA) and (1S,3R)-1-

aminocyclo-pentane-1.3-dicarboxylic acid (t-ACPD) [89] were also discovered. The glutamate receptor subtype on the body wall muscles of the crayfish and many crustaceans is primarily classified as quisqualate sensitive [~100 times increased responsiveness than glutamate; 90] and ionotropic [19, 91] with $Na^+$ being the predominate ion, in addition to some $Ca^{2+}$ influx and $K^+$ efflux when opened at resting membrane levels [92].

During synaptic transmission glutamate induces a rapid current influx that produces a rapid depolarization of the muscle membrane followed by a much slower decay in the synaptic potential. The amplitudes of the excitatory synaptic responses varies greatly at crustacean NMJs as there are a variety of synaptic responses from spiking muscles to graded excitatory postsynaptic potentials (EPSPs) that can arise from high- and low-output synapses [93-97]. The non-spiking EPSPs show a slow decay which is partly due to desensitization of the receptors [92, 98-101] and if the muscle is bathed in glutamate the receptors will fully desensitize, blocking transmission [98, 102]. The presence of high extracellular calcium ions is known to decrease the rate of desensitization by glutamate [103, 104] and concanavalin A [a plant lectin; 105] can not only partially decrease desensitization on its own but it can also block the effect of $Ca^{2+}$ on the receptors [103]. Thus, the desensitization effect of $Ca^{2+}$ is extracellular on the receptors or membrane. As far as we are aware this has not been addressed in insect NMJs.

The potential for presynaptic glutamatergic autoreceptors has also been investigated at the crustacean and insect NMJs. Since presynaptic glutamatergic receptors occur in the mammalian CNS [106] it would not be surprising to also predict they might occur at NMJs in the invertebrates. The use of a metabotropic agonist t-ACPD on NMJs of the crayfish provided confounding results with some preparations being enhanced and others depressed [89]. Since some preparations showed an effect there may well be presynaptic autoreceptors for glutamate in the crustacean preparations [89]. It would be of interest to examine high output as well as low output NMJs for differences in effects to t-ACPD as well as other potential metabotropic agonists and antagonists.

While the crustaceans were being examined for glutamatergic actions at the NMJs and pharmacological profiling, the NMJ of locust legs served as an insect counterpart. This preparation was used likely due to accessibility and being a relatively large insect preparation for physiologists at the time. There is a rich history of physiology and pharmacology using the locust preparation (Anderson et al., 1976; Cull-Candy and Parker, 1983; Gration et al., 1981; Patlak et al., 1979). Similarly, the locust NMJ paralleled the crayfish NMJ in physiology and pharmacological profiling as well as in desensitization with glutamate. A literature search in PUBMED.GOV using the key words “Insect glutamate neuromuscular junction” returned 319 hits. The first 142 references and most following ones focused exclusively on *Drosophila* which indicates the recent research focus among the vast array of insect species present. As with the crayfish and other crustacean preparations, the locust model fell short in being able to genetically manipulate the expression of glutamate receptor subunits and proteins involved with synaptic transmission. Though these model systems are still valuable for addressing particular physiological questions, the era of molecular biology has given way to the more genetically amenable *Drosophila melanogaster* as a model for synaptic studies using the neuromuscular junction.

## 3. Glutamate receptors in the *Drosophila* neuromuscular junction

Sophisticated gene manipulation, extensive collections of mutant lines, and relatively simple, inexpensive maintenance make *Drosophila melanogaster* an excellent experimental system for neurobiology. The *Drosophila* larva NMJ in particular has been steadily revealing the physiological mechanisms of synaptic transmission for over 40 years [23]. It is a rare system where individual synapses from identified neurons can easily be manipulated in the context of development or plasticity *in vivo*. In partially dissected preparations the glutamatergic synapses lie directly on the muscle cell surface, providing uninhibited optical access for single molecule localization, super resolution, and other advanced microscopy techniques in combination with electrophysiology. Physiologically relevant salines that allow prolonged viability have been a breakthrough for physiologists in the *Drosophila* field [107-109]. Optogenetic stimulation and calcium imaging are

also well established in this system [110, 111]. The purpose of this section is to describe what is known about glutamatergic neurotransmission at the *Drosophila* NMJ, while pointing out essential similarities and differences between it and mammalian neural synapses. We then discuss recent discoveries in glutamate receptor pharmacology and synaptic plasticity at the *Drosophila* NMJ, and finish the section with an overview of molecular mechanisms that are required for proper glutamate receptor localization. For detailed information on experimental paradigms and other molecular factors that have been described in the larva NMJ there is an entire book and several comprehensive review articles [112-114].

*Pharmacological properties of glutamate receptors in the Drosophila larva NMJ*

Pharmacological and genetic analysis have provided a clear picture of the ionotropic glutamate receptor (iGluR) subtypes present at the *Drosophila* larva NMJ. The field unanimously asserts that the iGluRs present at the post synaptic density are heterotetramers composed of three common subunits (GluRIIC, GluRIID, and GluRIIE) and an interchangeable forth subunit (either GluRIIA or GluRIIB) [15, 115-117]. Though these iGluR subunits most closely resemble vertebrate AMPA and kainate receptors at the amino acid sequence level, *Drosophila* iGluRs exhibit distinct differences in their pharmacological profile. Most notable is that AMPA type iGluRs expressed at the *Drosophila* NMJ are not especially sensitive to AMPA, kainate, or NMDA, but respond to quisqualate [118, 119]. The molecular difference underlying species specific agonist activity may have been detected in a recent study that reported the crystal structure of GluRIIB bound to glutamate. Though the volume of the GluRIIB ligand binding cavity is similar to the vertebrate ligand binding cavity, the presence of Tyr481, through interactions with Asp509 and Arg429, appears to prevent binding of the common ligands [120]. A similar finding was later made for the GluRIIA glutamate complex, which also exhibits a pharmacological profile that diverges from the vertebrate iGluR [7]. Importantly, heterologous expression approaches were achieved in both studies that enable functional reconstitution of the iGluR complex, providing the opportunity to test different gene products with single channel resolution *in vitro*, quickly transfer those gene products into the organism with *Drosophila* gene editing [121], and verify the hypotheses *in vivo* at the larva NMJ.

Another difference in *Drosophila* larva NMJ receptor pharmacology is sensitivity to toxins. Lobster and cricket NMJs as well as at mammalian hippocampal pyramidal neurons are blocked by the Joro spider toxin (JSTX) [122-124](Abe et al., 1983; Kawai, 1991; Kawasaki & Kita, 1996), but to our knowledge JSTX does not block *Drosophila* glutamate receptors. Philanthotoxin-433 (PhTx), however, a non-competitive open channel glutamate receptor blocker derived from wasp venom, has proven to be a powerful pharmacological tool for investigating glutamatergic transmission at the *Drosophila* larva NMJ. When injected into the larva or applied directly to the exposed NMJ, PhTx induces presynaptic compensation within ~10 minutes [125]. This form of synaptic plasticity, referred to as homeostatic plasticity [126], is achieved through an increase in quantal content, and is also observed in GluRIIa mutants [127]. Not only is this form of plasticity observed at hippocampal glutamatergic synapses, some of the key molecular components are conserved, including presynaptic calcium channels [128, 129] and postsynaptic mTOR signaling [130, 131]. A notable mechanistic aspect of homeostatic plasticity at the larval NMJ is that it requires retrograde signaling from the postsynaptic muscle cell to the motor neuron. Retrograde signaling appears to be a widespread mechanism that has emerged throughout nervous system evolution to regulate various forms of synaptic plasticity. Cell-specific control of gene expression in pre- and post-synaptic compartments has made the *Drosophila* NMJ a convenient system to address the location of action for many molecules.

Two metabotropic glutamate receptors are found in the *Drosophila* genome though only one was found to be functional [mGluR; 132, 133]. *Drosophila* mGluR has 45% and 43% amino acid sequence homology with its mammalian homologs, mGluR3 and mGluR2 respectively, and it was responsive to several mammalian mGluR agonists and antagonists in a mammalian heterologous expression system, showing negative coupling to the adenylate cyclase pathway [132]. At the larval NMJ, mGluR is expressed predominantly in the presynaptic compartment where it has a role in activity-dependent plasticity [134]. mGluR mutants exhibited normal baseline synaptic

transmission but significantly enlarged bouton size and reduced bouton number. A relatively limited panel of pharmacological agents have been tested in this system *in vivo*, and it is also not yet known whether these receptors have a role in rapid activity-dependent structural modifications at the NMJ.

*Physiological properties of glutamatergic neurotransmission at the Drosophila larva NMJ*

Simple electrophysiological accessibility to a genetically specified synapse is a valuable feature of the *Drosophila* larva NMJ. In the larva filet preparation, motor synapses on the dorsoventral longitudinal muscles lie directly on the cell surface. These muscles are large (~100um x 300um), isopotential, and do not exhibit active membrane properties under normal culturing conditions. Muscles 6 and 7 are the most often used and best characterized [135], but they do exhibit an important drawback, which is that they are each innervated by two separate motor neurons. The larval muscles also receive innervation from aminergic neurons [136].

Ionic currents in the larva muscle have been well characterized through genetic and pharmacological analysis. Iontophoresis of L-glutamate at the synaptic termini was used to determine that the excitatory transmitter at the larva NMJ is glutamate [137, though for inhibitory effects of L-glutamate see: 138]. iGluRs in the larva NMJ rapidly desensitize in the presence of excessive extracellular glutamate [137, 139]. Synaptic potentials can be investigated by electrical stimulation of the segmental nerves innervating dorsoventral longitudinal muscles. A non-specific cation synaptic current can be recorded intracellularly throughout the muscle in response to nerve stimulation or as a result of endogenous activity if the nerves are not severed from the brain. Single quantal events can also be observed in intracellular recordings from the muscle. Kinetics of the evoked potentials have been analyzed using ion exchange, common reagents for blocking ion channels, and through analysis of ion channel mutants that were isolated from genetic screens. As described in the synaptic plasticity section below, transmission at the NMJ is extremely sensitive to extracellular $Ca^{2+}$ levels [140]. Passive membrane properties of the muscles are well characterized. An inward $Ca^{2+}$ current and outward $K^+$ current are readily observable under two-electron voltage clamp. The $K^+$ current is sensitive to tetraethyl ammonium and is significantly reduced in *either-a-go-go* and *shaker* mutants, which code for potassium channels known to be responsible for the inward rectifying and transient A current respectively [141]. Peron et al., [142] provide a detailed overview of the other ion channel genes expressed at the larva NMJ.

*Synaptic plasticity at the Drosophila larva NMJ*

Activity-dependent synaptic plasticity has been extensively studied at the *Drosophila* larva NMJ. Throughout larva development the muscle size increases exponentially and requires equivalent expansion of the synaptic field. Through genetic analysis it was determined that synaptic expansion during NMJ development is an activity-dependent process that also requires trophic factors associated with tissue development (reviewed in Menon et al., 2013). The mature NMJ synapse at the last stage of larva development has also been shown to exhibit several forms of short and long-term synaptic plasticity resembling long-term potentiation (LTP) and long-term depression (LTD) that are investigated in mammalian brain preparations. Here we describe the characteristics of acutely inducible forms of synaptic plasticity at the larva NMJ.

Different forms of activity-dependent synaptic plasticity can be assessed at the larva NMJ simply by adjusting the stimulus parameters. Similar to long term facilitation (LTF) in crustaceans and long term potentiation (LTP) in mammals, nerve evoked synaptic potentials in the *Drosophila* larva NMJ can be enhanced by trains of high frequency stimuli, referred to as post-tetanic potentiation (PTP, Zhong and Wu, 1991). This form of activity-dependent plasticity is evoked by stimulus frequencies between 5-20Hz, low extracellular $Ca^{2+}$ (0.2mM), is cAMP-dependent, and lasts on average for 158sec [143]. Lower stimulus frequencies (0.1-1Hz) induce a form of depression called low frequency short term depression [144], whereas higher frequencies (40-60Hz) induce short term depression in low extracellular calcium conditions (<1mM). Paired pulse facilitation is another form of short term plasticity that provides a robust readout of synaptic physiology at the larva NMJ [140]. Currently there is no widely accepted example of nerve induced stimulation that induces long term

synaptic changes resembling LTP. Given that NMDA-like iGluRs have not been identified at the larva NMJ it is unlikely that a strictly homologous LTP phenomenon exists. However activity-dependent synaptic phenomena resembling the cellular changes in LTP have been identified. Increasing synaptic activity through elevated temperature or induced crawling can cause NMJ growth and potentiated transmitter release [145]. Spaced potassium depolarization, in dissected but intact NMJ preparations, also induces synapse formation and potentiated transmitter release [146]. Both phenomena require translation and the latter process requires transcription. Taken together, the physiological changes that are consolidated with new structures that require changes in gene expression, activity-dependent plasticity at the larva NMJ is a legitimate experimental system for investigating the molecular mechanisms of long term information storage in glutamatergic synapses. Wnt signaling, BMP signaling, miRNAs, and CamKII have already been implicated in long term facilitation at the NMJ [146-149], and others are sure to follow.

*Molecular mechanism of glutamate receptor localization at the Drosophila larva NMJ*

The *Drosophila* larva NMJ has been especially valuable for determining how glutamate receptors are localized to synaptic sites. Success in this field is due in large part to ease of imaging the larva filet preparation and reliable, commercially available antibodies for labelling glutamate receptors and other synaptic markers [150]. Live imaging of fluorescent protein tagged glutamate receptors in the *Drosophila* larva has provided unparalleled insight into the dynamics of glutamate receptor assembly *in vivo* [17, 151]. Genetic analysis has also provided extensive insight into glutamatergic synapse formation. We identified at least 41 separate studies that reported a change in larva NMJ glutamate receptor level as a result of loss of function gene mutation (**Table 1**). A more recent reverse genetic screen, which focused specifically on PDZ containing genes, determined that null mutations in 42.8% (48 of the 112 non-lethal mutations) of the PDZ containing genes disrupt GluRIIa localization *in vivo* [152]. These results indicate that glutamate receptor localization is an amazingly complex process that is regulated by several convergent molecular pathways. Physiological state of the NMJ is also important, as spontaneous neurotransmission is required for proper iGluR localization [153, 154]. Localization of the presynaptic active zones and the postsynaptic receptor array are tightly correlated; however, it does appear that spontaneous vesicle fusion events and evoked events do not always use the same synaptic sites [155]. Thus, synaptic sites have varying probabilities of transmission which may also have to do with differences in synaptic complexity [155, 156].

The mechanism for iGluR localization appears to be post translational, as RNA fluorescence *in situ* hybridization (FISH) shows very little overall enrichment for iGluR mRNA at the post synaptic density [157], though studies have shown that mRNA and RNA binding proteins are present in close proximity to the synapse [158, 159]. With recent application of single molecule FISH to the larva NMJ [160] it will be possible to determine how different aspects of glutamatergic transmission are locally regulated at the mRNA level.

## 4. Glutamate recycling in *Drosophila* and crayfish NMJs

It is apparent that the glutamatergic synapses at the invertebrate NMJs function similarly to other chemical synapses, although some of the synaptic ultrastructure may differ [95-97]. Generally, transmitter is packaged into clear core synaptic vesicles within the presynaptic nerve terminal via vesicular transporters (VGLUT) [161, 162] and vesicles exist in various states, from being docked and readily releasable to being sequestered in reserve pools [161, 163-166]. The vesicle pools are dynamic with stimulation dependent recruitment [18, 167] and can be depressed with repetitive stimulation [168, 169]. As with other synaptic preparations of NMJs in vertebrates and invertebrates there are low- and high-output type synapses and differing muscle phenotypes [slow, intermediate and fast; 170, 171]. The general characteristics are that the low output synapses have few docked vesicles but can show dramatic facilitation due to reserve vesicles and recruitment to active zone sites on synapses, whereas the high output synapses fuse many vesicles and produce large EPSPs but fatigue quickly due to a limited reserve pool [169].

The process of glutamate uptake through the presynaptic plasma membrane transporter (GLUT/EAAT) and repackaging in the vesicles (VGLUT) [9, 10] in *Drosophila* and crayfish models serve as models for vertebrate glutamatergic synapses as they are pharmacologically similar. TBOA blocks reuptake via GLUT [18, 172-174] and Bafilomycin A1(B1793) blocks vacuolar ATPase which drives VGLUT [18, 162].

Although novel proteins and functional significance associated with vesicle and glutamate receptor dynamics are continuously being discovered at *Drosophila* NMJs, homologs are sought in analogous glutamatergic synaptic sites in vertebrates and in synapses which are not glutamatergic [175-178]. The ability to examine the effect of overexpression or knock down is rapidly able to be addressed using the *Drosophila* model. Temporally regulated expression with Gal80 has promoted this model over others to separate acute molecular mechanisms from developmental issues in synaptogenesis. Diseases inflicting glutamatergic synapses in humans are also modeled at the *Drosophila* NMJ [179]. The glutamatergic synapses at the *Drosophila* NMJ show effects of aging and disuse with a loss of presynaptic vesicles and prolonged recovery due to stressors of activity [180], which are similar to those shown in crustaceans [181] and mammals [182-184].

## 5. Interesting side notes

An interesting phenomenon that occurs at crayfish and *Drosophila* NMJs is that $CO_2$ blocks glutamate receptors directly, independent of decreased intracellular or extracellular pH induced by $CO_2$ exposure [185-187]. $CO_2$ also rapidly paralyzes honeybees [188]. Lower extracellular and intracellular pH to 5.0 still allows synaptic transmission to occur but in the presence of $CO_2$ the synaptic transmission is rapidly blocked and removing $CO_2$, even though intracellular pH is still reduced, reverses the receptor block. Hypoxia or displacement of $O_2$ with $N_2$ does not mimic the rapid effect of $CO_2$. Interestingly $CO_2$ was used as an anesthetic for human and animal surgeries in early medicine [189]. Vertebrate NMDA receptors on cerebellar neurons are inhibited by protons even within a physiological pH range [190]. The open channel blocker MK-801 decreases its affinity in low pH suggesting that possible low $Ca^{2+}$ flux with low pH results in causes inward currents through the NMDA receptors to decrease [191, 192]. The effect of protons on the NMDA receptors may be extracellular [193] but such detail as to the potential mechanism of action on the quisqualate glutamate receptors of the invertebrates has not been investigated.

Invertebrate models, with the exception of *Drosophila,* have not previously been genetically amenable to investigate molecular mechanisms of synaptic transmission at NMJs. Some clever alternative approaches have enabled successful molecular investigation in these systems. Because many crustacean motor axons are large enough for pressure injection or iontophoresis, chemical compounds, small proteins, siRNA, or mRNA have been injected directly into the presynaptic terminal to examine functionality [194]. Gene transfection in primary cell culture has also been an effective approach to study molecular mechanisms of synaptic transmission in invertebrates [195]. With CRISPR/Cas9 mutagenesis, it is now possible to perform genetic analysis in crustacean organisms that are not typical genetic model organisms [196], making it possible that we will see a resurrection in the use of crustacean NMJs systems for glutamatergic synapse biology.

## 6. Protocols for the crayfish and *Drosophila* larva neuromuscular junction preparations

To help visualize the invertebrate neuromuscular junction preparations we provide a brief overview and pertinent references for the crayfish leg and fruit fly larva neuromuscular junction protocols, instead of giving step-by-step protocols for each preparation which are referenced in the sections below. The aim here is to introduce the experimental procedure for accessing these synapses and highlight some of the key advantages and limitations.

*Crayfish neuromuscular junction preparations*

The crayfish and lobster offer several types of NMJ preparations, many of which have been described in numerous publications for teaching modules or detailed research based protocols.

The crayfish NMJ preparations tend to have a better viability than lobster or crab models over longer periods in a defined saline for teaching labs and for addressing experimental questions. The muscle phenotypes and innervation profiles of the commonly used crayfish and lobster muscles have been described [96, 171, 197, 198]. Depending on the synaptic responses to be investigated in the crayfish model one can readily choose a low output tonic-like NMJ or a high output phasic-like NMJ on a single muscle fiber that is dually innervated (i.e., the walking leg extensor muscle), or muscle fibers that are mostly innervated by one type of innervation profile (tonic-like or phasic-like) that also correlates with the muscle phenotype. Abdominal muscles which are tonic-like are as follows: superficial extensor lateral, superficial extensor medial, and superficial flexor muscles. The abdominal muscles which are phasic-like are as follows: deep extensor lateral, deep extensor medial, and deep flexor muscles. The anatomical arrangement of the abdominal muscles are highlighted in Sohn et al., [199, 200], and dissection procedures are shown in video format in Baierlein et al., [201]. The opener muscle of the walking leg contains regional variation in the innervation and muscle fiber profiles even though the muscle is innervated by a single excitatory motor neuron. However, the innervation and muscle phenotype generally displays a tonic-like profile. The dissection and physiological procedures for the opener muscle is shown schematically in **Figure 1A** and video format [61]. The dissection and recording procedures for the walking leg extensor with the dually innervated muscle fibers of high- and low-output synapses is also shown in video format [202]. An advantage to using the walking legs is that the animal will autotomize the leg when pinched at the base so four or more preparations can be obtained from one animal and if the animal is left alive for some time the legs will regenerate.

The tonic-like innervation profile is one that will show smaller EPSP amplitudes but will rapidly facilitate in amplitude in a stimulation frequency dependent manner. The synaptic responses are fatigue resistance and generally contain larger varicosities than the thin filiform like nerve terminals of the phasic-like innervation. The high-output phasic innervation usually produces large amplitude EPSPs and will fatigue relatively quickly compared to the tonic innervation. Intracellular recordings in the axons of the motor neurons are a key asset to the crustacean preparations. Substances such as peptide fragments, ionic indicators and direct measures of the action potential shape to address presynaptic contributions to synaptic physiology have been conducted in the crayfish opener preparation [194, 203-205]. The caveat in working with NMJs is the fact the muscle can contract. If one is only examining the presynaptic terminal then the glutamate receptors on the muscle fibers can be desensitized by adding glutamate (1 to 10 mM) to the bath. However, in measuring functional synaptic responses the intracellular electrode in a contracting muscle fiber may be dislodged. This can usually be prevented by maintaining the muscle in a taut position when pinning the preparation in a recording dish.

Synaptic responses can readily be measured with standard intracellular recording techniques. However, due to the large size of some muscle fibers two electrode voltage clamp is not as feasible compared to larval *Drosophila* muscles due to space clamp issues. In addition, in larger muscles the minis can be difficult to detect, which is in part due to lower input resistance but also a decrement in the electrical responses due to cable properties of the muscle membrane. To directly measure quantal responses from select regions of a motor nerve, focal macropatch recordings offer excellent resolution of single quantal events. The single quantal responses can be used to address synaptic efficacy and shapes of the synaptic responses related to glutamate receptor function [206].

In investigating proteins involved in synaptic structure the crayfish preparations offers tissue with sufficient material for Western blots and *in situ* staining. The crayfish nerve terminals have shown to be immunocytochemically similar to *Drosophila* in terms of some antibody staining (i.e., synaptotagmin staining, [207]) but not for HRP antibody staining. The vesicular uptake of FM1-43 is similar in crayfish and *Drosophila* NMJs; however, the vital fluorescent dye, 4-14-(diethy1amino)styryll-N-methylpyridiniumio dide (4-Di-2-Asp; [208]), obtained from Molecular Probes (Eugene, OR) works extremely well for crayfish NMJs but not for *Drosophila* (**Figure 1B;**

[209]). In addition, a dilute methylene blue stain made in crayfish saline can also be used to highlight the innervation of the muscle.

Unlike rodent brain slices or cultured rodent neurons the crayfish and *Drosophila* preparations function well at room temperature without any special considerations of an incubator and gas mixtures for maintaining the pH of the media. In addition, the preparations function well within a temperature range of 18-22 degrees C. The buffers added to the salines used for the *Drosophila* and crayfish are stable. Even though the crayfish NMJs can last several hours in minimal saline, attempts to culture intact NMJs for days have not been successful with the crayfish NMJ preparations.

*The Drosophila larva filet preparation*

The *Drosophila* larva filet preparation can be used for electrophysiology, optogenetics, live or fixed imaging. To perform the procedure one needs very fine insect pins, a petri dish filled partially with a solid elastomer, a simple physiological saline [107], forceps, micro dissection scissors, and a dissecting microscope. Prior to dissection a larva is rinsed, dried, and pinned dorsal side up in the head and tail region, as shown in **Figure 2A**. After submerging the larva in saline, a shallow incision is made along the dorsal midline of the larva and the internal organs are carefully removed. The internal organs can be removed in a single step by first cutting the trachea attachments to the bodywall along each segment. Additional pins can then be placed in the four corners to gently spread the carcass, as shown in the second panel of **Figure 2A**. Alternatively, the preparation can be dissected on a glass slide fitted with magnetic tape and insect pins attached to paper clips that can be easily maneuvered [210, 211]. At this point the specimen could be fixed for immunohistochemistry, imaged on an upright fluorescence microscope with water dipping objecting, or prepared for electrophysiology. The schematic in **Figure 2A** shows a basic configuration to evoke excitatory junction potentials (eEJPs). A small glass capillary suction electrode is placed on a severed nerve and a sharp glass capillary intracellular electrode is placed into a muscle fiber of the same segment. Resting membrane potential of the muscle should be larger than -60mV. Frequent (> 1Hz) miniature excitatory junction potentials (mEJPs) should be observed with amplitudes larger than 1mV. Supra threshold electrical stimulation of the nerve should evoke excitatory junction potentials larger than 20mV. Once this fundamental procedure can be reliably performed, one can embark on the more exotic techniques that have led to the discoveries described in Section 3, e.g., two-electrode voltage clamp, paired pulse and high frequency stimulation, calcium imaging, optogenetic or thermogenetic activation, and FM 1-43 labelling. Several detailed protocols and videos have been published on the larva filet preparation [212-214].

The larva filet preparation can be a powerful tool in laboratories that aren't equipped with electrophysiology or advanced microscopy equipment. A standard epiflourescent microscope with a camera is all that is required to assess synaptic morphology at the larva NMJ. Immunohistochemistry protocols for the NMJ are relatively simple (< 24hrs total, < 1hr hands on time) and involve standard reagents (phosphate-buffered saline, Triton-X, formaldehyde, glycerol). Antibody markers for the larva NMJ are also inexpensive and robust. Antibodies raised against the horseradish peroxidase (HRP) enzyme, which are commercially available with a wide selection of conjugated fluorophores, specifically label the axon terminals. An antibody against the discs large protein (Dlg1) reliably marks the post synaptic density. And endogenous GFP-tagged proteins are publicly available for thousands of *Drosophila* genes. An example microscopy image of the larva NMJ with each of the markers and a GFP-tagged GluRIIA is shown in **Figure 2B**. The specimen was prepared using the technique described above and standard immunohistochemistry procedures [215, 216]. Quantitative analysis of axon terminal and glutamatergic synapse morphology in various mutant backgrounds [217-219] has provided a wealth of understanding about the molecular mechanisms of synapse development and plasticity.

There are some caveats for the larva filet preparation. It does not involve exotic culturing techniques but the dissection does require a fair bit of skill, especially for physiology and live

imaging experiments. Not only does the tissue have to remain healthy and be consistent from preparation to preparation, but it should be well restrained to minimize movement from muscle contractions. Supplemented media can maintain the NMJ preparation in culture for over 24hrs without substantial physiological changes if the cells are not disturbed. However prolonged recording, stimulation, or imaging can cause the preparation to run down over time, e.g., decreased resting membrane potential and decreased mEJP and eEJP amplitudes. A source of variability in NMJ phenotypes that must be carefully accounted for is developmental plasticity. The structure and function of the NMJ is very plastic and subtle changes in the environment can have significant physiological effects, therefore culturing conditions must be rigorously controlled when using *Drosophila* larva.

## 7. Summary and future directions for invertebrate and vertebrate glutamate synapses

There are various topics we feel are worth continuing as well as novel directions where using the invertebrate glutamatergic synapse preparations could have implications for mammalian glutamatergic synapses or even chemical transmission in general. The effects of pH and the idea that molecular $CO_2$ may block the pore of the glutamate receptor could have direct implications for pH sensing and regulation throughout the animal kingdom. Details of potential mechanisms of action for putative presynaptic glutamatergic auto-receptors in influencing synaptic transmission still need to be determined, as well as the possibility that such presynaptic receptors reside on non-glutamatergic presynaptic neurons as a means of detecting volume transmission. Differences in the postsynaptic array of glutamate receptors have not been addressed in the context of synaptic output or the rate of spontaneous events. There are many accessory proteins now known to be present pre- and post-synaptically but their functional roles will take some time to determine and how they are regulated. The influence of an animal's diet and metabolism on glutamate receptor function is an area that was prominent earlier in pharmacological studies but today there are so many herbal supplements containing plant and algae extracts known to have an action on glutamate receptors yet careful monitoring of long term consequences in low level concentrations have not been addressed. The common monosodium glutamate (MSG) added to food as a supplement, domoic acid from red algae and kainic acid from seaweed are a few of the compounds that are well known to have consequences in humans and other animals. On a clinical note, one treatment for epileptic seizures involves manipulation of VGLUT through the action of acetoacetate, a metabolite of fat, which competes with $Cl^-$ for the binding site on VGLUT and hinders glutamate transport [220]. The natural body metabolite homocysteine, which can act as an agonist and an antagonist on glutamate receptors, is now gaining attention. *Drosophila* and possibly other crustacean systems could provide a well-defined system to investigate the physiological effects of disorders related to glutamatergic transmission or outstanding questions about chemical transmission in general.

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| Table 1. Factors affecting GluR localisation at the *Drosophila* larva NMJ | | | |
|---|---|---|---|
| **Gene name** | **GluR subtype** | **LoF effect on iGluR levels** | **Reference** |
| PDZ containing genes | GluRIIA | 42 decrease, 6 increase | [152] |
| Filamin | GluRIIA | decrease | [177] |
| diablo | GluRIIA | increase | [221] |
| Lk6 kinase | GluRIIA | decrease | [176] |
| Monensin sensitivity 1 | GluRIIA | increase | [222] |
| Neuropilin and toll-like protein | GluRIIA | increase | [223] |
| kismet | GluRIIA | decrease | [224] |
| activin | GluRIIA/B | decrease | [225] |
| Neurologin 3 | GluRIIA | decrease | [226] |
| Staufen | GluRIIA | decrease | [159] |
| wingless | GluRIIA | increase | [227] |
| reverse polarity | GluRIIA | increase | [227] |
| Mgat1 | GluRIIB | decrease | [228] |
| slowpoke | GluRIIA/B | decrease | [229] |
| Tbc1d15-17 | GluRIIA | decrease | [230] |
| Akt1 | GluRIIA | decrease | [231] |
| Lethal giant larvae | GluRIIB | increase | [232] |
| longitudinal lacking | GluRIIA/B, III | decrease | [233] |
| Tor and eIF2a | GluRIIA | decrease | [133] |
| calcium/calmodulin-dependent serine protein kinase | GluRIIA | decrease | [234] |
| Neuroligin 2 | GluRIIB, III | decrease | [235] |
| Neurexin | GluRIIA | decrease | [236] |
| Neuroligin 1 | GluRIID | decrease | [237] |
| Metro | GluRIID | decrease | [238] |
| twinfilin | GluRIIA | decrease | [239] |
| Rho GTPase activating protein at 100F | GluRIIA | increase | [240] |
| CamKII | GluRIIA | increase | [241] |

| Table 1. Factors affecting GluR localisation at the *Drosophila* larva NMJ | | | |
|---|---|---|---|
| nanos | GluRIIA/B | decrease/increase | [242] |
| dystroglycan | GluRIIA | decrease | [243] |
| Protein-O-mannosyl transferase 1 | GluRIIB | decrease | [244] |
| dorsal | GluRIIA | decrease | [245] |
| mind the gap | GluRIIC/D | mislocalised | [246] |
| β2 and β6 proteasome | GluRIIB | increase | [247] |
| coracle | GluRIIA | decrease | [248] |
| discs large 1 | GluRIIB | decrease | [249] |
| pumilio | GluRIIA | increase | [250] |
| p21-activated kinase | GluRIIA | decrease | [251] |
| survival of motor neuron | GluRIIA | decrease | [252] |
| G protein α s subunit | GluRIIA | decrease | [253] |
| Rho-type guanine nucleotide exchange factor | GluRIIA | decrease | [254] |
| Actin 57b | GluRIII | decrease | [255] |
| nesprin | GluRIIA | decrease | [256] |

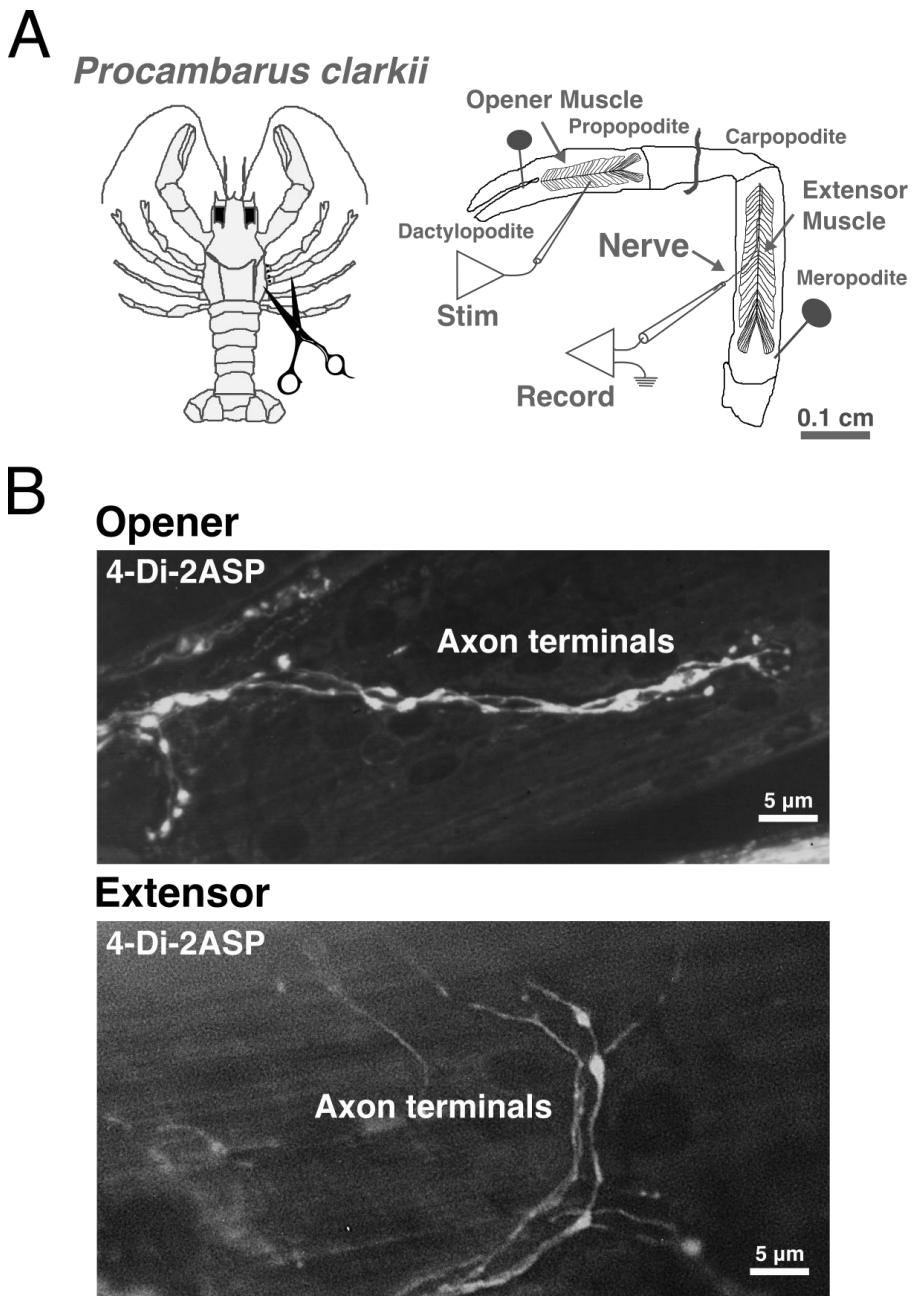


Figure 1. Overview of the crayfish walking leg preparation. (A) The walking leg is readily obtained by pinching at the base of the leg above the autotomy plane to minimize bleeding when the leg is removed. After pinning the leg down, the ventral aspect of the opener preparation is accessible by removing the closer muscle and if one would like to stimulate the excitatory nerve to the opener this can be accomplished by finding the branch in the meropodite region (See video of dissection Cooper and Cooper, 2009). (B) Staining of the living motor nerve terminals with 4-Di-2ASP highlights the innervation, making it easier to place a focal macropatch electrode over a desired region of the terminal. The top panel illustrates innervation on the opener muscle with the two motor nerve terminals. One is the excitor and one is the inhibitor, but the two cannot be differentiated with the 4-Di-2ASP staining. However they can be selectively stimulated by separating the nerve in the meropodite region. The lower panel illustrates innervation on the leg extensor muscle with the large varicosities of the tonic excitatory motor nerve and the thin filiform terminals of the phasic terminal.

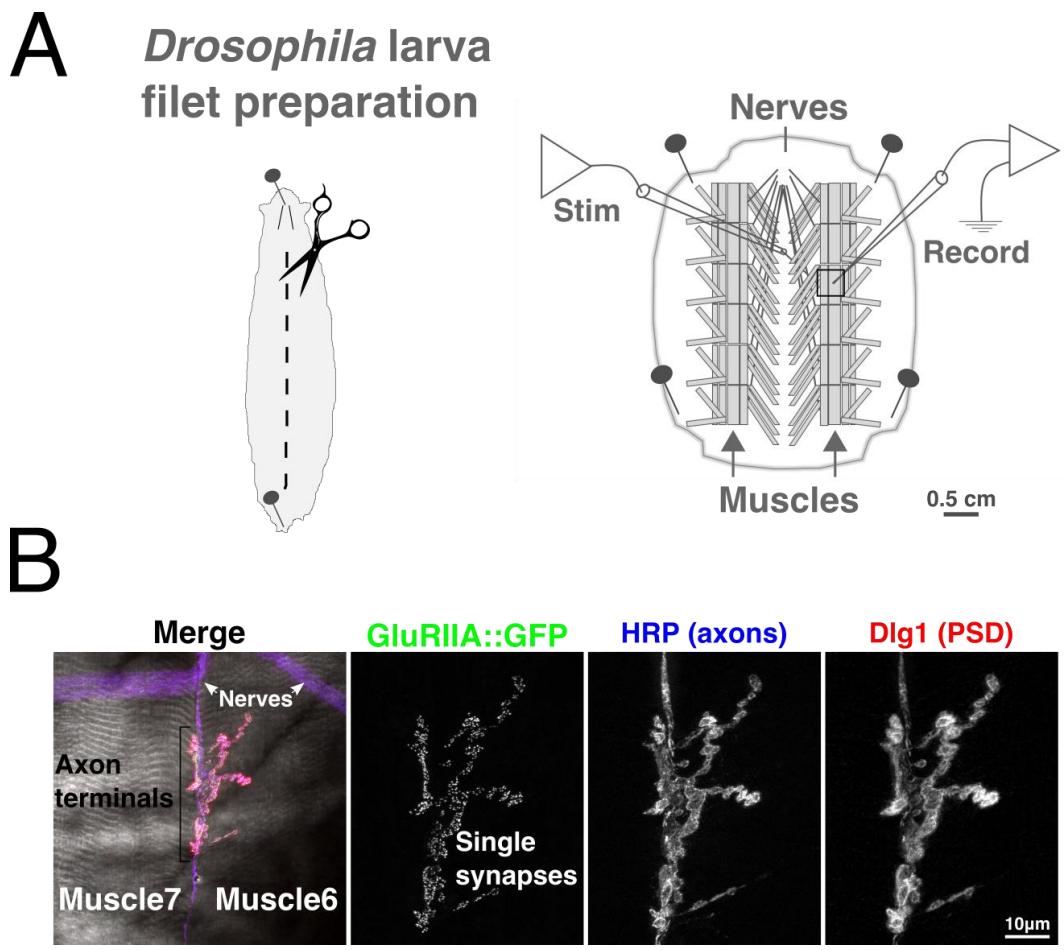


Figure 2. Overview of the *Drosophila* larva filet preparation. (A) Schematics for dissecting the larva for direct access to the neuromuscular junction (NMJ). After pinning the larva in an elastomer-lined dish with saline, a shallow incision is made along the midline, then viscera and central nervous system are removed, making the nerves and bodywall muscles easily accessible to electrodes or a fluorescence light source for microscopy. (B) Anatomy of a fixed larva NMJ as visualised through a 60x objective. Fluorescence markers are superimposed onto a DIC brightfield image in the Merge panel to show the muscle ultrastructure. Nerve fibers that innervate the bodywall muscles are also seen in this image. Glutamate receptors are observed by an endogenous GFP-tagged GlurIIA construct. Commercially available reagents are available for labelling the axons (anti horse-radish peroxidase (HRP)) and the post synaptic density (PSD- discs large antibody (Dlg1)).