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Glutamate receptor composition at *Drosophila* neuromuscular junctions depends on developmental stage and muscle identity

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eLife Assessment

This study provides **important** findings on the expression of glutamate receptor (GluR) subunits across developmental stages and muscle types in Drosophila. It shows that adult muscle differs in GluR composition from larval body wall muscles, which have been the focus of most past studies. The study, while **convincing**, could be strengthened by acknowledging that it relies on heterogeneous methods and the absence of positive signals to infer receptor loss, which limits confidence in some of its claims. The findings illuminate how Drosophila excites muscles in diverse tissue types at different life stages, and are of interest to researchers across neuroscience.

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Abstract

The neuromuscular junction (NMJ) of larval *Drosophila* is widely used for studying synaptic transmission. Larval body wall muscles express five ionotropic glutamate receptor (iGluR) subunits that assemble into two tetrameric complexes, with subunit composition determining the strength and plasticity of synaptic transmission. Because NMJ function has been extensively characterized in larvae, it is often assumed that adult fly NMJs have similar molecular composition, despite substantial differences between life stages. Here, we systematically compare glutamate receptor expression across larval and adult *Drosophila* muscles. We find that adult leg and flight muscles exhibit different iGluR expression than larvae, lacking several receptors previously considered essential for viability and NMJ function. Adjacent muscles within the adult femur express distinct iGluRs, suggesting specialization of flexor and extensor muscles. Finally, the glutamate-gated chloride channel (GluClα) is expressed extrasynaptically in adult but not larval muscle fibers. Our results reveal unexpected heterogeneity in glutamate receptor expression across muscles and developmental stages, challenging assumptions about the uniformity of neuromuscular function and demonstrating the need for muscle-specific analyses in flies and other animals.

Introduction

Body movement relies on the release of chemical neurotransmitters from motor neurons onto muscles at the neuromuscular junction (NMJ). In vertebrate animals, most motor neurons release the excitatory neurotransmitter acetylcholine (Dale et al., 1936), which acts on nicotinic receptors to elicit contraction of skeletal muscle. In contrast, most invertebrate motor neurons release glutamate (Takeuchi and Takeuchi, 1964, 1963), which binds to ionotropic glutamate receptors (iGluRs) expressed in muscle (Schuster et al., 1991). However, muscles are not uniform effectors; they are highly specialized to control distinct body parts and behaviors ranging

from walking to flight (Hooper and Thuma, 2005; Schiaffino et al., 2025). Given this functional specialization, how is the expression and subunit composition of postsynaptic neurotransmitter receptors regulated to support the function of diverse muscles? Determining how receptors are tailored to the specific biophysical demands of individual muscles, and how this tuning shifts during development, is essential for understanding the molecular mechanisms that underlie adaptive motor control.

A powerful experimental system to investigate molecular, developmental, and physiological mechanisms of neuromuscular signaling is the larval NMJ of the fruit fly, *Drosophila*, which enables high-resolution analysis of identified motor neurons and muscles with electrophysiology and immunohistochemistry (He and Dickman, 2025). Flies, like humans, encode 16 GluR genes (Figure 1A; Li et al., 2016). Five ionotropic receptor (iGluR) subunits are expressed in larval muscle, where two subtypes mediate excitatory synaptic transmission (DiAntonio, 2006; Thomas and Sigrist, 2012). Similar to vertebrate AMPA-type receptors, fly iGluRs function as tetrameric complexes that require three obligate subunits (GluRIIC, GluRIID, and GluRIIE) along with a fourth, either GluRIIA or GluRIIB (Figure 1B; Han et al., 2024, 2015). The specific subunit composition dictates synaptic efficacy: GluRIIA-containing receptors generate large, stable synaptic currents, while GluRIIB complexes desensitize rapidly (DiAntonio, 2006; Thomas and Sigrist, 2012). Synaptic transmission at the larval NMJ is also regulated homeostatically: when postsynaptic GluRs are genetically or pharmacologically suppressed, the presynaptic motor neuron increases neurotransmitter release to maintain stable postsynaptic responses (He and Dickman, 2025). Distinct from this excitatory machinery, the inhibitory glutamate-gated chloride channel (GluClα) functions presynaptically in larval motor neurons to mediate presynaptic homeostatic depression, a mechanism that adaptively dampens neurotransmitter release at the NMJ (Li et al., 2021). Due to the extensive characterization of these mechanisms in the larva, it is often tacitly assumed that this canonical molecular architecture is preserved in the adult fly. This extrapolation overlooks the profound reorganization of the neuromuscular system during metamorphosis.

Compared to the NMJ of *Drosophila* larva, the physiology and molecular architecture of adult fly NMJs remain largely unexplored (with one notable exception, Rivlin et al., 2004). This gap is significant because the biomechanical context of movement changes fundamentally during metamorphosis (Agrawal and Tuthill, 2022). Larval body wall muscles mediate slow, rhythmic peristaltic locomotion by pulling against a hydraulic skeleton. In contrast, adult muscles drive a rigid exoskeleton to produce rapid behaviors such as flight and walking. Given these varied biophysical demands, it may be advantageous for specialized muscles to adapt the molecular composition of their synapses. Furthermore, with the emergence of comprehensive connectomes of the adult fly nervous system (Bates et al., 2025; Berg et al., 2025) and biomechanical body models (Vaxenburg et al., 2025; Wang-Chen et al., 2024), defining the molecular identity of neuromuscular synapses is a critical missing link required for integrative modeling of how the nervous system actuates the body.

Here, using genetic reporter lines, immunohistochemistry (IHC), and analysis of single-cell RNA sequencing data, we find that GluR expression in *Drosophila* is substantially more diverse than previously appreciated. We find that the receptor landscape in adult leg and flight muscles diverges radically from the larval pattern; unexpectedly, these muscles lack expression of GluR subunits that were previously considered essential for NMJ function and viability. In contrast, adult abdominal muscles retain the canonical larval receptor complement, consistent with the fact that the larval musculature is transformed into the abdominal musculature during metamorphosis (Currie and Bate, 1991; Kimura and Truman, 1990). We also identify an extrasynaptic population of glutamate-gated chloride channels (GluClα) specific to adult muscle, a molecular architecture that explains the hyperpolarizing glutamate response described in classical locust studies (Cull-Candy, 1976; Dudel et al., 1989; Lea and Usherwood, 1973). Collectively, these results overturn the assumption of NMJ uniformity, suggesting that synaptic composition is specialized to match the functional requirements of specific muscles.

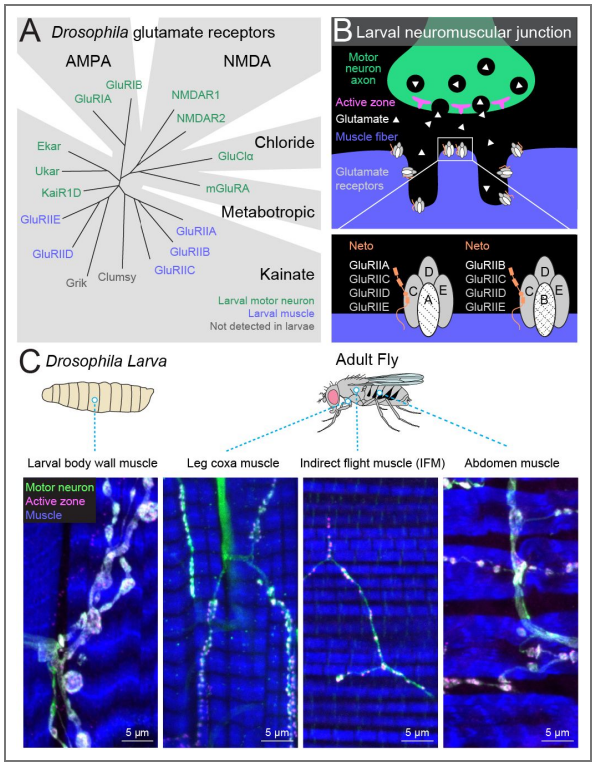

Figure 1. Glutamate receptors at *Drosophila* neuromuscular junctions (NMJs).

(A) Phylogeny of 16 glutamate receptors in the *Drosophila* genome and their reported expression at the larval NMJ (based on Li et al., 2016). (B) Schematic of the larval *Drosophila* fly NMJ (top) and composition of glutamate receptors (bottom). Larval GluRs occur as two distinct tetrameric complexes, each with the same auxiliary subunit (Neto). (C) Example fluorescence images of larval and adult NMJs. Motor neurons are labeled by driving GFP with vGlut-Gal4 (green). Active zones are labeled with an antibody against Brp (magenta). Muscle is labeled with phalloidin (blue).

Results

Larval and adult muscles express distinct GluRs

Consistent with past literature, GAL4 expression patterns and antibody labeling confirmed that all five GluRII subunits are expressed at NMJs throughout larval body wall muscle (Figures 2C, s1). We observed a similar expression pattern in the adult abdomen (Figure 2C, s2), although the labeling of *GluRIIB* and *GluRIIE* was weaker than in the larva. As expected, all larval and adult muscles expressed *Neto-β*, the primary auxiliary subunit required for iGluR receptor trafficking and function (Figure 2D). Expression of other Kainate-type receptors (*Grik*, *KaiR1D*, *Ukar*, *Ekar*), as well as AMPA, NMDA, and the sole metabotropic GluR were absent in all larval and adult muscles.

Surprisingly, we found that none of the primary GluRII subunits was strongly expressed in adult indirect flight muscles (Figures 2C, s2-s3, s5-s8). Leg coxa muscles expressed subsets of GluRII subunits that varied across specific muscle subtypes (Figure 2C, s5-8), though this expression was consistent across flies (Figure s9). Interestingly, the one iGluR that was broadly expressed throughout the adult (e.g., leg, proboscis, neck, haltere and indirect/direct flight muscles) was the kainate-type iGluR *Clumsy* (Figures 2C, s10). Larval muscles lacked *Clumsy* expression, and it was expressed only weakly in abdominal muscles. The *Clumsy* gene is phylogenetically related to GluRIIA/B/C (Li et al., 2016), but its function is poorly understood compared to the other GluRII subunits expressed in larval muscle.

Different leg muscles express distinct iGluRs

Our screen revealed that the expression patterns of iGluR-specific Gal4 lines in the adult leg were often patchy: different muscles appeared to express different iGluR subunits (Figures 2A, s5-s8). These expression patterns were consistent across flies (Figure s9). To examine muscle-specific iGluR expression in more detail, we focused on the femur of the fly's front leg (Figure 3A-B), for which we have previously characterized motor neuron physiology, muscle innervation, and muscle force production (Azevedo et al., 2020). Based on motor unit organization and tendon attachment, muscle fibers in the tibia can be grouped into four groups: a long tendon muscle, a tibia extensor, and two tibia flexors (Azevedo et al., 2024; Lesser et al., 2024).

To examine differences in iGluR expression between muscles, we compared Gal4 reporter expression and antibody labeling at NMJs in the femur for two GluR subunits: GluRIIB and GluRIIC. We chose these two subunits because their GAL4 expression was restricted to distinct muscle fibers (Figure 3C, E). We found that protein labeling was consistent with GAL4 expression: GluRIIB was expressed at NMJs of tibia extensor muscles but absent from other muscles, including the accessory tibia flexors (Figure 3C-D). In contrast, GluRIIC was expressed at NMJs of the more distal accessory tibia flexor muscles but was absent from the more proximal muscles (Figure 3E-F). The distal accessory tibia flexor fibers are innervated by the slow motor neurons that control slow, postural movements of the tibia (Azevedo et al., 2020).

We also observed differences in iGluR expression within a single muscle. For example, GluRIIB is expressed in proximal fibers of the tibia extensor but is absent from the more distal fibers (Figure 3C-D). Prior work has shown that the more proximal fibers are innervated by the fast extensor motor neuron (FETi), while the more distal fibers are innervated by the slow extensor motor neuron (SETi; Azevedo et al., 2024). These differences in GluRIIB expression within the tibia extensor muscle may reflect specialization of fast vs. slow twitch muscle fibers.

Overall, our results show that different muscles in the fly leg express different ensembles of GluR subunits. We also confirmed that all femur NMJs express the obligatory GluR accessory protein Neto (Figure 3B), affirming that all femur motor neurons are glutamatergic, despite their otherwise unexpected receptor expression. Given what is known about larval GluR subunit composition, this implies that at least some adult GluRs are composed of non-canonical GluR subunits. Further, the difference in GluR expression is interesting because our prior work has

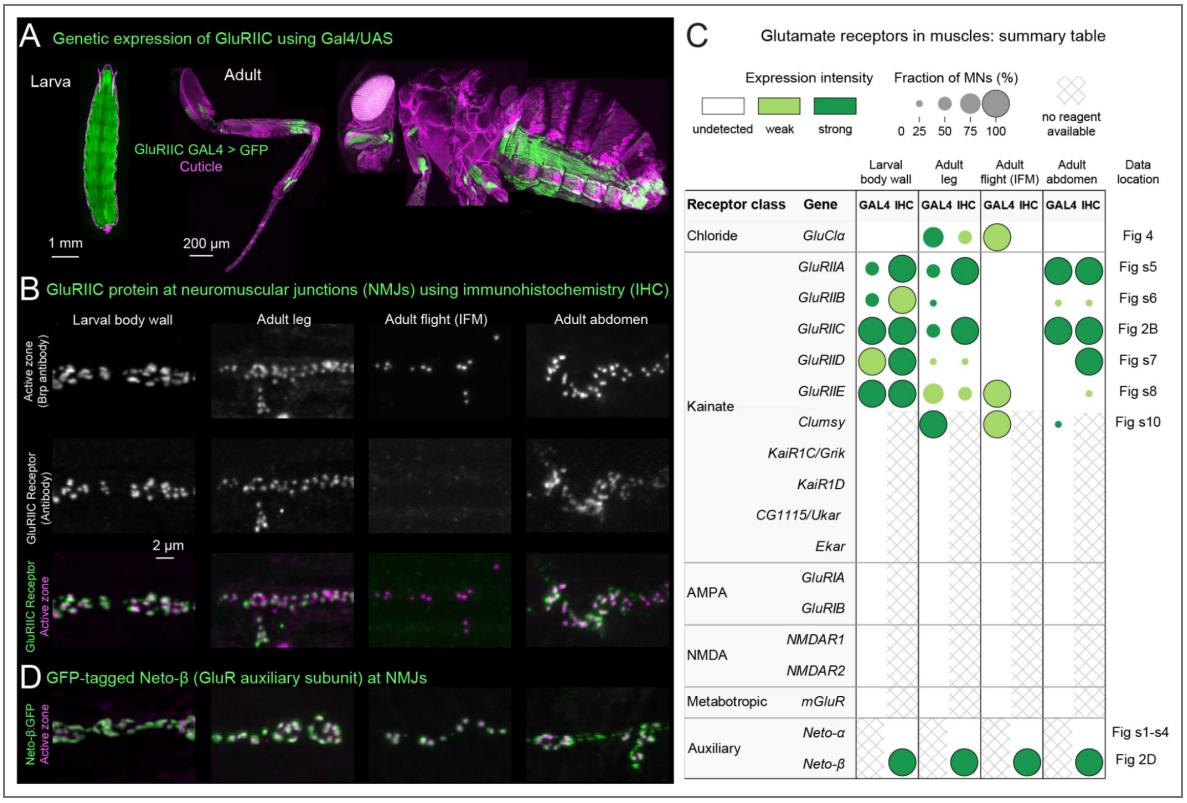

Figure 2. GluR subunit expression mapping in larval and adult muscles.

(A) Representative confocal images showing GFP expression driven by a GAL4 reporter line (GluRIIC-GAL4) in larval and adult muscles. (B) Representative images showing antibody staining of GluRIIC at larval and adult neuromuscular junctions. (C) Summary table of Gal4 expression/antibody staining for all GluRs in larval and adult muscles. Note that antibodies and tagged proteins are combined in a single immunohistochemistry (IHC) column. Note that this initial screen focused on the coxa segment of the adult leg. See supplemental figures for representative images and methods for a complete list of reagents screened. (D) Representative images showing the GluR auxiliary subunit *Neto-β* tagged with GFP at larval and adult neuromuscular junctions.

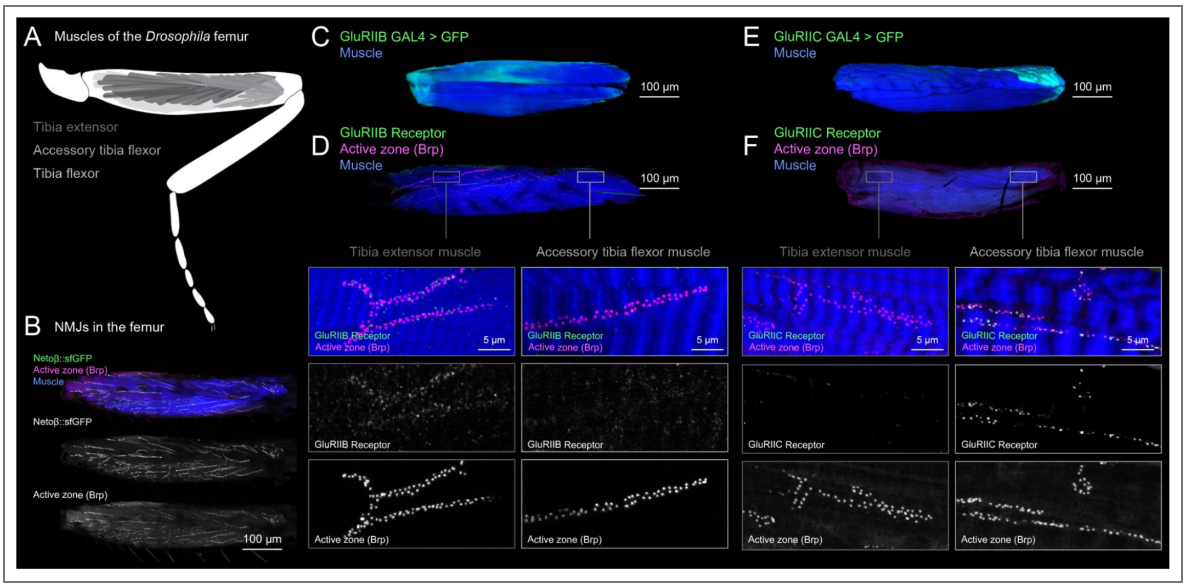

Figure 3. Differential GluRIIB vs GluRIIC subunit expression in adult muscle subtypes.

(A) Schematic of the fly's front leg with annotations of three key muscle groups in the femur. (B) Femur with Netoβ::sfGFP (green) in NMJs (Brp, magenta) in all femur muscle types (phalloidin, blue). (C) 3D rendering of a confocal stack of femur muscles (phalloidin, blue) and GluRIIB GAL4> UAS-GFP (green). GFP is expressed specifically in tibia extensor muscle fibers. (D) Representative antibody staining images showing presence of GluRIIB in a tibia extensor muscle (left) and absence of GluRIIB in an accessory tibia flexor muscle (right). (E) 3D rendering of a confocal stack of femur muscles (phalloidin, blue) and GluRIIC GAL4> UAS-GFP (green). GFP is expressed specifically in accessory tibia flexor muscle fibers. (F) Representative antibody staining images showing absence of GluRIIC in a tibia extensor muscle (left) and presence of GluRIIC in an accessory tibia flexor muscle (right).

demonstrated major differences in the physiology and force production of different muscle groups, and even different fibers within the same muscle (Azevedo et al., 2020). Thus, GluR subunit composition may be specialized to support diverse functions, even within a muscle.

RNA-seq data are consistent with adult muscle expression patterns

We next analyzed transcriptomic data of muscle cells from an existing single-nuclei RNA-seq dataset of adult *Drosophila* tissues (Li et al., 2022). Consistent with our results from GAL4 reporters and antibody staining, transcription of all GluRII genes was relatively low in clusters annotated as adult leg (Figures s11, s12) and indirect flight muscle (Figure s12), and only slightly higher in a cluster annotated as abdominal muscle. In comparison, and consistent with our results using GAL4 reporter lines, GluClα and Clumsy were both highly expressed in all three muscle clusters. One discrepancy was that *Ukar* and *Grik* had elevated transcription in multiple muscle clusters, which we did not observe with GAL4 lines. The Ukar GAL4 line had no detectable expression in any tissue, so this reporter line may not be functional. Overall, however, expression patterns from adult RNA-seq data were largely consistent with our main conclusions from GAL4 reporters and antibody staining.

Motor neurons express a distinct set of GluR genes compared to muscle

To determine whether GluR localization at the neuromuscular junction could be due to presynaptic expression, we also screened for GluR expression in motor neurons. We expressed GFP under the control of GluR-specific GAL4 lines and examined whole-mount expression in the central nervous system, as well as axons projecting to the legs, indirect flight muscles, and abdomen (Figure s13). As expected, we found that motor neurons expressed GluClα, which mainly functions as an inhibitory neurotransmitter in the *Drosophila* central nervous system (Liu and Wilson, 2013). We also observed that motor neurons expressed a subset of kainate, AMPA, NMDA, and mGluR receptors that was nonoverlapping with the expression patterns we observed in muscle. Overall, these results confirm that the patterns of GluR expression we document at neuromuscular junctions are not due to expression in presynaptic motor neurons.

Extrasynaptic glutamate-gated chloride channels in adult muscle

One surprising result from our survey of GluR expression was the broad expression of the glutamate-gated chloride channel, GluClα, in adult leg and flight muscles, but not in larval body wall or adult abdomen (Figure 2C). GluClα was also the GluR gene with the highest level of expression in leg muscles based on the single nuclei RNA-seq data (Figure 4A). Although GluClα has been shown to function presynaptically in larval motor neurons (Li et al., 2021), its presence and function in *Drosophila* muscle has not previously been investigated.

Whole-mount expression of GFP driven by two GluClα-Gal4 lines showed broad labeling of muscles throughout the flight and leg musculature (Figure 4B). However, using higher resolution imaging of an antibody against an endogenously-tagged GluClα:V5 protein (Sanfilippo et al., 2024), we found that puncta in leg and flight muscle did not cluster at synaptic active zones (Figure 4C). Rather, co-staining with phalloidin (Figures 4D, s14) revealed that the protein is distributed around the edges of the muscle fibers. Thus, unlike the other GluRs, GluClα localizes extrasynaptically in leg and indirect flight muscles. Although the function of extrasynaptic GluClα in muscle remains unclear, this result is consistent with classical electrophysiological studies in other invertebrates (e.g., locust, beetle, lobster) that described hyperpolarizing responses to extrasynaptic glutamate application (see Discussion).

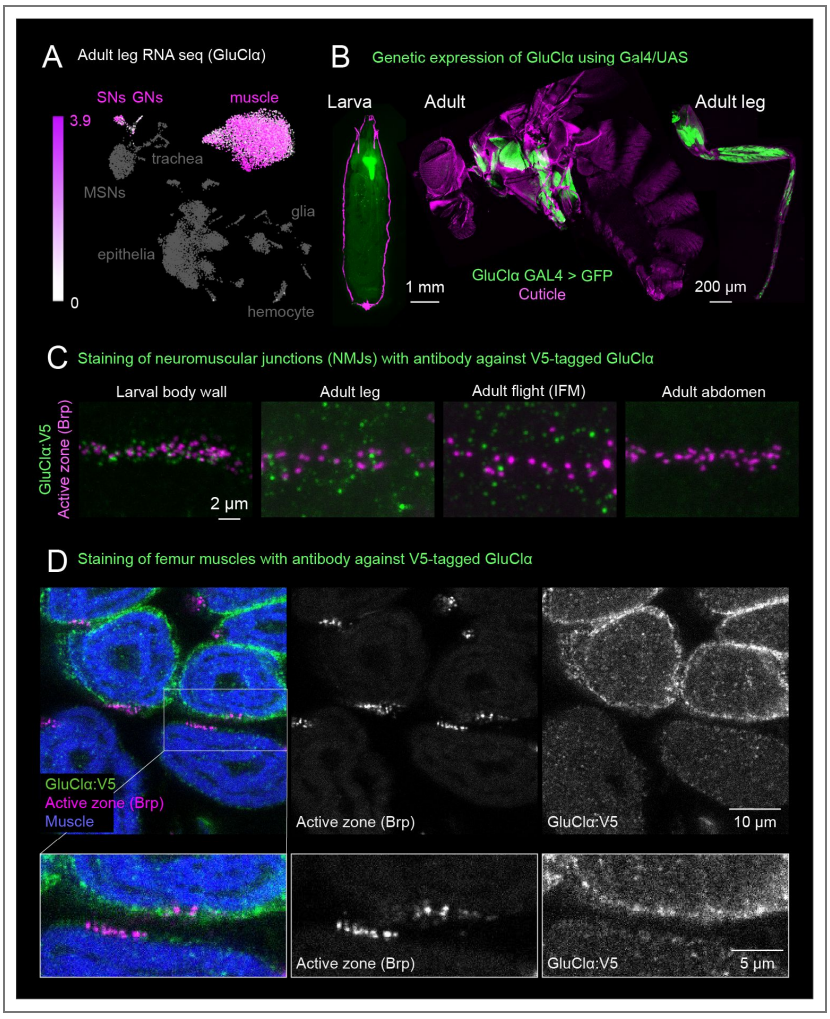

Figure 4. GluClα is expressed extrasynaptically in adult fly muscles.

(A) Single-nuclei RNA-seq data shows strong expression of GluClα in adult leg muscles. (B) A Gal4 reporter for GluClα labels muscles in adult legs but not larval muscles. Expression in the larva is mainly in the nervous system, as expected. (C) Representative images showing antibody staining of endogenously-tagged GluClα:V5 in larval and adult muscles. Co-staining with an antibody against Brp reveals that GluClα does not co-localize with active zones. Labeling in larval muscle is likely from motor neurons (Li et al., 2021). (D) Labeling of GluClα protein with a V5 tag shows extrasynaptic expression throughout the muscle fiber, whereas active zones (magenta) are localized.

Discussion

Here, we describe unexpected complexity in the molecular composition of glutamate receptors at neuromuscular junctions in *Drosophila melanogaster*. Our work suggests that glutamate receptor expression at *Drosophila* NMJs is far more heterogeneous than previously appreciated, with adult leg and flight muscles exhibiting receptor subunit combinations that diverge from the canonical expression patterns previously observed in larvae. Adult indirect flight muscles lack the five GluRII subunits considered essential for larval NMJ function, suggesting these specialized muscles rely on alternative receptor combinations for neuromuscular transmission. Conversely, adult abdominal muscles mostly retain larval-like receptor expression, reflecting their shared developmental lineage. The identification of extrasynaptic GluClα in adult muscles establishes the molecular identity of inhibitory glutamate responses described decades ago in other insects and suggests novel mechanisms for regulating muscle excitability.

Adult leg and wing muscles lack canonical larval glutamate receptors

Perhaps the most striking finding of our study is that some adult leg and flight muscles lack expression of the GluR subunits that have been characterized as essential for larval NMJ function. For example, we observed an absence of GluRIIA-E subunits in indirect flight muscles across multiple experimental approaches: GAL4 reporter expression, antibody staining, and single-cell RNA-seq. These iGluR subunits were previously identified through genetic studies showing that mutations disrupting GluRIIC (Marrus et al., 2004), GluRIID (Featherstone et al., 2005), or GluRIIE (Qin et al., 2005) cause lethality in larvae, while double mutants lacking both GluRIIA and GluRIIB are similarly nonviable (DiAntonio et al., 1999; Petersen et al., 1997). Our observation that adult indirect flight muscles seem to function without these “essential” subunits redefines what constitutes the minimal receptor complement for glutamatergic neuromuscular transmission in the fly.

We found that Neto, the obligate auxiliary subunit required for GluR receptor function (Han et al., 2024; Kim et al., 2012), is expressed in all larval and adult muscles, including the indirect flight muscles. This result suggests that glutamate is indeed the primary neurotransmitter at all adult NMJs, consistent with extensive evidence that *Drosophila* motor neurons release glutamate (i.e., they express the vesicular glutamate transporter, vGlut (Baek and Mann, 2009; Brierley et al., 2012; Daniels et al., 2008)). Thus, some adult muscles may use an alternative glutamate receptor composition that has yet to be characterized. Functional glutamate receptors without the previously assumed “essential” GluRIIC subunit have not been previously described. One past study (Han et al., 2015) explored many combinations of GluR subunits through functional reconstitution in a heterologous expression system, but they did not directly test many of the combinations we describe here, such as GluRIIB/IID/IIE in the tibia extensor muscles.

One possibility is that adult muscles express novel or uncharacterized glutamate receptor subunits from among the 16 iGluR genes encoded in the *Drosophila* genome, several of which have not been functionally characterized. A candidate that stands out is *Clumsy*. Our results showed that *Clumsy* is broadly expressed throughout the adult fly, including in muscles controlling the legs, wings, halteres, proboscis, and neck (Figure s10). In comparison, *Clumsy* is completely absent from larval muscle. *Clumsy* was originally identified as a mutant with impaired adult locomotion (Berger, 2008; Huen et al., 2001), though this work was never published in a peer-reviewed journal. The gene is phylogenetically related to the GluRIIs (Li et al., 2016), but its function is poorly understood compared to the other subunits expressed in larval muscle. For example, it is not known whether *Clumsy* interacts with *Neto-β*. More work is needed to understand the function of *Clumsy* at the neuromuscular junctions of adult *Drosophila*.

Adult abdominal muscles are similar to larvae

Our observation that adult abdominal muscles largely maintain the canonical larval GluRII receptor composition provides important developmental context for understanding receptor diversity. Adult abdominal muscles derive directly from larval body wall muscles through remodeling during metamorphosis, rather than through complete *de novo* muscle formation (Currie and Bate, 1991; Kimura and Truman, 1990). This developmental continuity is reflected in the persistence of the larval receptors, suggesting that the molecular identity established during larval stages is maintained through metamorphosis into muscles that mediate slower abdominal movements (e.g., abdominal pumping during flight (Lehmann and Heymann, 2005)).

In contrast, adult leg and flight muscles arise through *de novo* myogenesis during pupal development (Gunage et al., 2017), providing an opportunity for the developmental program to specify alternative receptor compositions tailored to the biomechanical demands of adult locomotion. The correlation between developmental origin and receptor composition suggests that muscle lineage may contribute to determining synaptic molecular architecture, with metamorphically-remodeled muscles maintaining larval characteristics and newly formed muscles adopting novel molecular identities.

Extrasynaptic glutamate-gated chloride channels in adult muscle

We identified robust expression of GluClα in adult leg and flight muscles, where the channels localize extrasynaptically along muscle fiber edges rather than at neuromuscular junctions. This finding provides molecular support for classical electrophysiological studies in locusts that described glutamate-gated hyperpolarizing responses (H-receptors) in leg muscles that were functionally and pharmacologically distinct from excitatory D-receptors at the NMJ (Cull-Candy, 1976; Dudel et al., 1989; Lea and Usherwood, 1973). These studies demonstrated that locust leg muscles exhibit two types of glutamate responses: fast depolarizing currents with 1-2 ms kinetics mediated by cation channels at synaptic sites, and slower hyperpolarizing currents with 20-50 ms kinetics mediated by chloride channels distributed extrasynaptically across the muscle surface. Similar observations were made in other invertebrate preparations, including yellow mealworm beetle coxa muscles (Saito and Kawai, 1985) and lobster gastric muscles (Lingle and Marder, 1981), suggesting that extrasynaptic inhibitory glutamate receptors may be a widespread feature of invertebrate muscle physiology. Our identification of GluClα in adult *Drosophila* muscles extends these findings to a genetically tractable organism and raises important questions about the functional role of extrasynaptic inhibitory receptors in motor control.

We hypothesize that ambient glutamate in the hemolymph, released either from synaptic spillover or from non-neuronal sources, could activate these channels to modulate muscle excitability and tone. By providing tonic hyperpolarization, extrasynaptic GluClα could increase the threshold for muscle activation, potentially preventing inappropriate or excessive contraction. GluClα could also function as a negative feedback controller, in the sense that excess glutamate built up during periods of intense motor activity could activate GluClα to reduce muscle excitability. Ambient glutamate levels in the hemolymph of *Drosophila* larvae are quite high (1-3 mM (Augustin et al., 2007)), while GluClα is fully saturated in oocytes at a glutamate concentration of ~100 μM (Kane et al., 2000). This feedback mechanism would be particularly important in adult muscles that must sustain precise force control during complex, energetically demanding behaviors like flight and walking.

Comparison to other animals

Our finding of muscle-specific and developmental regulation of postsynaptic receptor composition parallels glutamatergic synapse heterogeneity in the mammalian brain (Wichmann and Kuner, 2021) and neuromuscular system (Sanes and Lichtman, 1999). During mammalian development, fetal acetylcholine receptors containing the γ subunit are replaced by adult receptors containing the ε subunit at the neuromuscular junction, resulting in altered conductance

and channel kinetics (Cetin et al., 2020). Similarly, the distribution of acetylcholine receptors shifts from diffuse expression across the muscle surface to tight clustering at synaptic sites (Sanes and Lichtman, 1999).

While our results reveal that *Drosophila* muscles express extrasynaptic GluClα, mature vertebrate skeletal muscle fibers are not known to express inhibitory neurotransmitter receptors. Instead, they rely on the voltage-gated chloride channel ClC-1 to stabilize the resting membrane potential and prevent hyperexcitability (Pedersen et al., 2016). In vertebrate muscle, extrajunctional acetylcholine receptors are upregulated after injury (Hartzell and Fambrough, 1972), which is thought to increase muscle excitability and facilitate motor neuron reinnervation. Excitatory glutamate receptors are also upregulated after injury in locust leg muscles, while the inhibitory receptors remain unchanged (Cull-Candy, 1975).

Muscle-specific receptor tuning correlates with biomechanical demands

The heterogeneity in glutamate receptor expression we observed across different muscle types may reflect adaptation to specific biomechanical requirements. Larval body wall muscles mediate slow, rhythmic peristaltic waves against a hydrostatic skeleton, requiring sustained contractions with relatively slow temporal dynamics (0.5-2 Hz, (Heckscher et al., 2012)). In contrast, walking requires precise, graded force control to move jointed limbs against a rigid exoskeleton at higher frequencies (5-20 Hz, (Pratt et al., 2024)). As in other animals, different motor neurons and muscles within the fly leg are specialized for controlling rapid and powerful ballistic movement vs. slow postural stabilization (Azevedo et al., 2020). Different iGluR subunits exhibit a wide range of desensitization kinetics (DiAntonio et al., 1999). Our results raise the possibility that the expression of specific postsynaptic neurotransmitter receptors is tailored to the biomechanical function of different muscles and even fibers within a muscle (e.g., slow vs. fast-twitch).

Fly flight demands even higher frequency muscle contraction. To achieve this, the indirect flight muscles are asynchronous: the motor neurons spike at ~6 Hz while the stretch-activated muscles contract at >200 Hz (Dickinson and Tu, 1997). In contrast, direct flight muscles at the base of the wing hinge provide fine-tuned steering control through synchronous contraction with motor neuron spiking. While we found that the indirect flight muscles lack expression of canonical GluRs, the direct flight muscles do faintly express GluRIIA. The differential receptor expression between direct and indirect flight muscles may reflect their distinct functional roles: power production vs. steering may require unique receptor kinetics, conductances, and desensitization properties.

Another major finding from larval NMJ studies is presynaptic homeostasis, wherein reduction of postsynaptic GluRs triggers compensatory increases in presynaptic neurotransmitter release. Our discovery that adult leg and flight muscles lack canonical postsynaptic GluRII receptors raises critical questions about whether and how homeostatic mechanisms operate at these specialized synapses. Do muscles lacking canonical iGluR receptors retain the capacity for presynaptic homeostatic potentiation? If so, what postsynaptic sensors and retrograde signals mediate this plasticity? Alternatively, do highly specialized muscles trade plasticity for precision, relying on fixed synaptic properties that are established during development and remain relatively stable throughout adult life?

Limitations

Our study employed multiple complementary approaches to characterize receptor expression patterns. The convergence of results across GAL4 reporter expression, antibody staining, endogenous tagging, and single-cell RNA-seq data supports our conclusions about differences between larval and adult muscles. However, several technical limitations should be noted. GAL4 reporters may not capture all aspects of endogenous gene regulation. Antibody specificity, while validated in larval muscles, may differ in the adult. Single-cell RNA-seq provides a snapshot of the transcriptome but does not directly assess protein expression or localization. In the future,

electrophysiological recordings from adult NMJs and GluR mutant characterization will be essential to more directly assess synaptic transmission properties and to confirm the functional consequences of the receptor expression patterns we describe.

Conclusions

Our findings challenge the assumption that insights from larval NMJs generalize to adult flies and highlight the importance of examining neuromuscular function in appropriate developmental and anatomical context. More broadly, our results emphasize that developmental stage and tissue identity shape synaptic molecular architecture. As the field moves toward comprehensive, multi-scale models of motor control that integrate connectomics, biomechanics, and neural dynamics, it will be important to define the molecular and functional properties of synaptic transmission for different muscles. Understanding neuromuscular diversity and the principles that govern it will be essential for building accurate models of motor control and for understanding how the nervous system adapts its output to match the mechanical properties of the effectors it must control.

Materials and Methods

Sample preparation for confocal imaging of GAL4/UAS in hemisected adult flies

For imaging GFP expression in hemisected adult flies, we fixed whole female flies in fixative (4% paraformaldehyde in PBS). Next, flies were one by one transferred to a small drop of Tissue-Tek O.C.T., frozen on a glass slide on dry ice for 15 seconds, sliced along the anterior-posterior axis with a fine razor blade and transferred to a well with fixative for one minute, until the O.C.T. melted and dissolved. The tissue was then washed 3 times in PBS containing 0.2% Triton-X (PBST) over one hour. Tissue was cleared in FocusClear for 20 minutes and mounted in Mount Clear on a slide with four layers of 3M double-stick tape for spacers. Preps were imaged on a Confocal Olympus FV1000.

For imaging of whole larvae, we transferred larvae to a drop of Vectashield on a glass slide with double-stick tape spacers. We froze the larvae in Vectashield for 5 minutes to immobilize them, then covered the larvae with a coverslip, and immediately imaged them on a Leica DMI6000 widefield microscope (UW Keck Imaging Center). Images were processed in FIJI (Schindelin et al., 2012).

Immunohistochemistry and NMJ imaging

The tissue was fixed in the same manner as above. Femurs were bisected longitudinally in O.C.T. to allow reagent penetration. All fixing and staining steps were at room temperature with gentle nutation. Tissue was put into a blocking solution (5% goat serum, PBS, 0.2% Triton-X) for 1 hour, then primary antibody solution for 24 hours. Tissue was washed three times in PBST and incubated in blocking and secondary antibody solution (see concentrations in the table below). Finally, tissue was incubated overnight in Alexa Fluor 647-nm Phalloidin in a PBS solution with the following reagents to improve tissue penetrance: 1% triton X-100, 0.5% DMSO, 0.05 mg/ml escin, and 3% normal goat serum. Tissue was washed three times with PBST over three hours, once in PBS for one hour, and mounted on a slide in Vectashield. NMJ images were collected on a Leica SP8 confocal (UW Keck Imaging Center). Images were processed in FIJI (Schindelin et al., 2012).

Quantification of GAL4/UAS and IHC expression for tables in Figures 2 and s13

For characterizing GAL4/UAS expression in whole larva and whole flies, we fixed and mounted a minimum of six animals per genotype. For each tissue type, we collected two 20x confocal stacks from two representative flies per genotype. (In no cases did expression patterns vary significantly;

Key resources table.

Reagent	Source	Identifier
Mouse anti-Bruchpilot (nc82) antibody (1:50)	Developmental Studies Hybridoma Bank	RRID:AB_2314866
Chicken anti-V5 (1:300)	Fortis Life Sciences A190-118A	n/a
Rabbit anti-GFP (1:1000)	Thermofisher A-11122	RRID:AB_221569
Rat anti-FLAG (1:200)	Novus Biologicals NBP1-06712	n/a
Rabbit anti-GluRIIB (1:1000)	(Perry et al., 2017)	n/a
Rabbit anti-GluRIIC (1:100)	(Goel and Dickman, 2018)	n/a
Guinea pig anti-GluRIID (1:2000)	(Perry et al., 2017)	n/a
Rabbit anti-GluRIIE (1:2000)	Dickman lab, this study	n/a
Rabbit anti-ALFA (1:500)	Synaptic Systems N1581	n/a
Donkey anti-Rabbit Alexa Fluor 488 (1:1000)	Thermofisher A-21206	RRID:AB_2535792
Goat anti-Mouse Alexa Fluor 568 (1:500)	Thermofisher A-11004	RRID:AB_2534072
Goat anti-Rabbit Alexa Fluor 568 (1:500)	Thermofisher A-11011	RRID:AB_143157
Goat anti-Chicken Alexa Fluor 488 (1:250)	Thermofisher A11039	RRID:AB_2534096
Goat anti-Rat Alexa Fluor 568 (1:500)	Thermofisher A-11077	RRID: AB_2534121
Goat anti-Guinea Pig Alexa Fluor 568 (1:250)	Thermofisher A-11075	RRID: AB_2534119
Alexa Fluor Phalloidin Alexa Fluor 647 (1:50)	Thermofisher A22287	n/a
Tissue-Tek O.C.T	EMS 62550-01	n/a
Vectashield mounting medium	Vector Labs H-1000	n/a
FocusClear	CelExplorer FC-101	n/a
MountClear	CelExplorer MC-301	n/a

see Figure s9.) We examined the confocal stacks in FIJI and quantified the expression intensity and fraction of muscles qualitatively: three levels for intensity (undetected, weak, strong) and five levels for approximate fraction of muscles (0, 25%, 50%, 75%, or 100%).

For quantifying protein expression at neuromuscular junctions, we stained and mounted a minimum of six animals per genotype. We used a low power objective to identify general muscle regions (e.g. coxa, indirect flight muscle). We then zoomed in with a 60x microscope objective to find and collect images where an NMJ was in good view, with strong control stain (Brp) and in a focal plane close to the microscope objective for best resolution. For each staining condition and tissue type, we collected a minimum of four representative image stacks. As above, expression intensity and approximate fraction of NMJ expression was quantified qualitatively in FIJI.

To document muscle-specific GluR receptors within the femur, we mounted and collected confocal 20x stacks of ten femurs per experiment, then used the 60x objective with a 3x digital zoom to collect stacks of NMJs in specific muscle fibers. We analyzed the images qualitatively for receptor expression.

RNA-seq analysis

We accessed single-cell RNA-seq Fly Cell Atlas datasets through the SCope website (Davie et al., 2018; Li et al., 2022). Tissue types were determined based on Fly Cell Atlas annotation. Figure s11 shows the 10x Cross-tissue/Muscle system/Stringent dataset. Figure s12 shows the 10x Leg/Stringent dataset.

Tagged Neto and GluRIIB proteins

CRISPR/Cas9-mediated homology-directed repair (HDR) was performed by WellGenetics Inc. (Taipei City, 11570, Taiwan) to target the Neto (CG44328) locus to generate endogenously tagged Neto-α and Neto-β isoforms. The gRNA sequences GAACTTGAGGGGATGTACGC and GTCCCATTGACA TTTAAGGA were cloned into U6 promoter plasmids to target Neto-α and Neto-β, respectively. For both isoforms, tag cassettes were inserted immediately upstream of the C-terminal stop codon via homology-directed repair using pUC57-Kan donor vectors containing flanking homology arms. The Neto-α donor contained an mScarlet-PBacGFP cassette (mScarlet, 3xGly, 3xFLAG, and a floxed 3xP3-GFP), while the Neto-β donor contained an sfGFP-3xP3-RFP cassette (sfGFP and a floxed 3xP3-RFP). These donors were microinjected into w^{1118} embryos alongside the respective gRNA plasmids and hs-Cas9. Successful F1 transformants were identified by marker expression (3xP3-GFP or 3xP3-RFP) and validated by genomic PCR and sequencing. Subsequently, the selection markers were excised from the confirmed lines using piggyBac transposase.

To generate the endogenously tagged GluRIIB::ALFA allele, a gRNA (TTCGACGGGTTACTCATCGC) targeting the GluRIIB C-terminus was cloned into a pU6 plasmid by GenScript (Piscataway, NJ 08854). A single-stranded DNA (ssDNA) donor template was designed to insert an ALFA tag flanked by a proline linker and homology arms. To prevent Cas9 cleavage of the donor and the re-cleavage of the edited locus, two nucleotide mutations were introduced into the left homology arm to disrupt gRNA binding. The full reverse-complement ssDNA donor sequence was synthesized (TTTT TACTCCCCTACTTTTCAATTCGCCTGGTCTTTTTTTTCTTTTTCGCACTGGAAGCAGGTTCTG TGAGCCGTCG CCTCAGCTCTTCTTCCAGCCTACTTGGACTTGTAATTTGCTCCAAGGATGAGTAAC CCGTCGAAGTTGAATC CCGACTTTGGCGATAGGTGTGCAGCGGTTTTCT) by Integrated DNA Technologies (IDT; Coralville, IA 52241). The gRNA plasmid and ssDNA donor were co-injected into vas-Cas9 embryos (BDSC #51324) by Bestgene (Chino Hills, CA 91709). Correct insertions were identified via PCR screening using the following primers: GATTCGGTGGGTGGACTGTTC (forward) and CGTATTTCACTGAATGTTC GCAAGC (reverse). Successful editing was subsequently confirmed by Sanger sequencing and immunostaining.

GluRIIE antibody

To generate the anti-GluRIIE antibody, the peptide C-SASIYSRSRQSSMSVASVAQESQ was synthesized by Bio-Synthesis, Inc. (Lewisville, TX 75057, USA). This antigen was used for rabbit immunization and subsequent affinity purification of the sera by Cocalico Biologicals (Denver, PA

17517, USA).

Table of Fly Stocks

Fly Transgene	Full genotype	Source	Identifier
Wild type, Tuthill strain	+ ; + ; +	Tuthill lab	
vGlut-Gal4	P{w[+mC]=VGlut1-GAL4.D}1, w[*]	BDSC	BDSC_24635
GluClα-T2A-Gal4[MI14426]	y[1] w[*]; Mi{Trojan-GAL4.1}GluClalpha[MI14426-TG4.1]/TM3, Sb[1] Ser[1]	BDSC	BDSC_77841
GluClα-T2A-Gal4	w[*]; ; TI{T2A-GAL4}GluClα	(Kondo et al., 2020)	n/a
GluClα -smV5	w[*]; ; TI{TI}GluClalpha[smV5]/TM6B, Tb[1]	BDSC	BDSC_602298
GluRIIA::EGFP	w[*]; TI{TI}GluRIIA[EGFP.B]	BDSC	BDSC_99517
GluRIIA-T2A-Gal4	w[*]; TI{T2A-GAL4}GluRIIA	(Kondo et al., 2020)	n/a
GluRIIA-T2A-Gal4	w[*]; TI{2A-GAL4}GluRIIA[2A-GAL4]	BDSC	BDSC_84637
GluRIIB-T2A-Gal4[MI03631]	y[1] w[*]; Mi{Trojan-GAL4.2}GluRIIB[MI03631-TG4.2]; Dr[1]/TM3, Sb[1]	BDSC	BDSC_60333
GluRIIB::ALFA	w[1118]; GluRIIB::ALFA	Dickman Lab, this study	n/a
GluRIIC-T2A-Gal4[CR01324]	y[1] w[*]; TI{GFP[3xP3.cLa]=CRIMIC.TG4.1}GluRIIC[CR01324-TG4.1]/SM6a	BDSC	BDSC_83268
GluRIIC-T2A-Gal4	w[*]; TI{T2A-GAL4}GluRIIC	(Kondo et al., 2020)	n/a
GluRIID-T2A-Gal4	w[*]; ; TI{T2A-GAL4}GluRIID	(Kondo et al., 2020)	n/a
GluRIID-T2A-Gal4	w[*]; ; TI{2A-GAL4}GluRIID[2A-GAL4]/TM3, Sb[1]	BDSC	BDSC_84638
GluRIIE-T2A-Gal4	w[*]; ; TI{T2A-GAL4}GluRIIE	(Kondo et al., 2020)	n/a
GluRIIE-T2A-Gal4	w[*]; ; TI{2A-GAL4}GluRIIE[2A-GAL4]/TM3, Sb[1]	BDSC	BDSC_84639
GluRIIE-T2A-Gal4	y[1] w[*]; wg[Sp-1]/CyO; Mi{Trojan-	BDSC	BDSC_60332

	GAL4.1}GluRIIE[MI01909-TG4.1]/TM3, Sb[1]		
Clumsy-T2A-Gal4[CR71497]	y[1] w[*]; TI{GFP[3xP3.cLa]=CRIMIC.TG4.0}clumsy[CR71497-TG4.0]/SM6a	BDSC	BDSC_606523
Grik-Gal4[MB05324]	w[1118]; ; Mi{GFP[E.3xP3]=ET1}Grik[MB05324]	BDSC	BDSC_24332
KaiR1D-T2A-Gal4	w[*]; ; TI{T2A-GAL4}KaiR1D	(Kondo et al., 2020)	n/a
Ukar-T2A-Gal4[CR70473	y[1] w[*]; ; ; TI{GFP[3xP3.cLa]=CRIMIC.TG4.1}ukar[CR70473-TG4.1]/In(4)ci[D], ci[D] pan[ciD]	BDSC	BDSC_97180
Ukar-T2A-Gal4[CR01527]	y[1] w[*]; ; ; TI{GFP[3xP3.cLa]=CRIMIC.TG4.0}ukar[CR01527-TG4.0]	BDSC	BDSC_606620
Ekar-T2A-Gal4[MI02500]	y[1] w[1118]; ; ; Mi{DH.1}Ekar[MI02500-DH.GT-TG4.1]	BDSC	BDSC_93679
GluRIA-T2A-Gal4	w[*]; ; TI{T2A-GAL4}GluRIA	(Kondo et al., 2020)	n/a
GluRIA-T2A-Gal4	TI{2A-GAL4}GluRIA[2A-GAL4]	BDSC	BDSC_84640
GluRIB-T2A-Gal4	w[*]; ; TI{T2A-GAL4}GluRIB	(Kondo et al., 2020)	n/a
NMDAR1-T2A-Gal4	w[*]; ; TI{T2A-GAL4}NMDAR1	(Kondo et al., 2020)	n/a
NMDAR1-T2A-Gal4	w[*]; ; TI{2A-GAL4}Nmdar1[2A-GAL4]/TM3, Sb[1]	BDSC	BDSC_84669
NMDAR2-T2A-Gal4	w[*]; TI{T2A-GAL4}NMDAR2	(Kondo et al., 2020)	n/a
NMDAR2-T2A-Gal4	TI{2A-GAL4}Nmdar2[2A-C.GAL4] w[*]	BDSC	BDSC_84670
mGluRA-T2A-Gal4	w[*]; ; ; TI{T2A-GAL4}mGluRA	(Kondo et al., 2020)	n/a
mGluR-Gal4[NP6701]	y[*] w[*]; ; ; P{w[+mW.hs]=GawB}mGluRA[NP6701]	Kyoto Stock Center	DGRC 105323
mGluR-T2A-Gal4[MI02169]	y[1] w[*]; ; ; Mi{Trojan-GAL4.un}mGluR[MI02169-TG4.un]	BDSC	BDSC_77721

Netoβ::sfGFP	w[1118] Netoβ::sfGFP	Dickman lab, this study	n/a
Netoα::3xFlag	w[1118] Netoα::3xFlag	Dickman lab, this study	n/a
UAS-6xGFP	y[1] w[*]; wg[Sp-1]/CyO, P{Wee-P.ph0}Bacc[Wee-P20]; P{y[+t7.7] w[+mC]=20XUAS-6XGFP}attP2	BDSC	BDSC_52262
UAS-6xmCherry	y[1] w[*]; wg[Sp-1]/CyO, P{Wee-P.ph0}Bacc[Wee-P20]; P{y[+t7.7] w[+mC]=20XUAS-6XmCherry-HA}attP2	BDSC	BDSC_52268
vas-Cas9	W[1118]; ; PBac{y[+mDint2] GFP[E.3xP3]=vas-Cas9}VK00027	BDSC	BDSC_51324

Table of Genotypes

Figure Reference	Genotype
Figure 1C	P{w[+mC]=VGlut1-GAL4.D}1 / + ; ; ; P{y[+t7.7] w[+mC]=20XUAS-6XGFP}attP2 / +
Figure 2A	y[1] w[*]; TI{GFP[3xP3.cLa]=CRIMIC.TG4.1}GluRIIC[CR01324-TG4.1]/+; w[+mC]=20XUAS-6XGFP}attP2 / +
Figure 2B	Wild-type
Figure 2D	w[1118] Netoβ::sfGFP; + ; +
Figure 3B	w[1118] Netoβ::sfGFP; + ; +
Figure 3C	y[1] w[*]; Mi{Trojan-GAL4.2}GluRIIB[MI03631-TG4.2] / + ; P{y[+t7.7] w[+mC]=20XUAS-6XGFP}attP2 / +
Figure 3D	w[1118]; GluRIIB::ALFA; +
Figure 3E	y[1] w[*]; TI{GFP[3xP3.cLa]=CRIMIC.TG4.1}GluRIIC[CR01324-TG4.1]/+; w[+mC]=20XUAS-6XGFP}attP2 / +
Figure 3F	Wild type
Figure 4B	y[1] w[*]; + ; Mi{Trojan-GAL4.1}GluClalpha[MI14426-TG4.1] / P{y[+t7.7] w[+mC]=20XUAS-6XGFP}attP
Figure 4C-D	w[*]; ; TI{TI}GluClalpha[smV5]/TM6B, Tb[1]
Figure s1 - s4 top to bottom	w[*]; TI{TI}GluRIIA[EGFP.B] Wild type Wild type Wild type Wild type w[*]; ; TI{TI}GluClalpha[smV5]/TM6B, Tb[1] Netoα::mScarlet::3xFlag Netoβ::sfGFP
Figure s5A	w[*]; TI{T2A-GAL4}GluRIIA/ +; P{y[+t7.7] w[+mC]=20XUAS-6XGFP}attP2 / +
Figure s5B	w[*]; TI{TI}GluRIIA[EGFP.B]
Figure s6A	y[1] w[*]; Mi{Trojan-GAL4.2}GluRIIB[MI03631-TG4.2] / + ; P{y[+t7.7] w[+mC]=20XUAS-6XGFP}attP2 / +
Figure s6B	Wild type

Figure s7A	w[*]; ; TI{2A-GAL4}GluRIID[2A-GAL4] / P{y[+t7.7] w[+mC]=20XUAS-6XGFP}attP2
Figure s7B	Wild type
Figure s8A	y[1] w[*]; + ; Mi{Trojan-GAL4.1}GluRIIE[MI01909-TG4.1] / P{y[+t7.7] w[+mC]=20XUAS-6XGFP}attP2
Figure s8B	Wild type
Figure s9	y[1] w[*]; TI{GFP[3xP3.cLa]=CRIMIC.TG4.1}GluRIIC[CR01324-TG4.1]/+; w[+mC]=20XUAS-6XGFP}attP2 / +
Figure s10	y[1] w[*]; TI{GFP[3xP3.cLa]=CRIMIC.TG4.0}clumsy[CR71497-TG4.0]/+ ; w[+mC]=20XUAS-6XGFP}attP2 / +
Figure s14A	Top: w[*]; ; TI{TI}GluClalpha[smV5]/TM6B, Tb[1] Bottom: Wild type
Figure s14B	Left: w[*]; ; TI{TI}GluClalpha[smV5]/TM6B, Tb[1] Right: Wild type
Figure s14C	Top: w[*]; ; TI{TI}GluClalpha[smV5]/TM6B, Tb[1] Bottom: Wild type

Data availability

All data generated or analyzed during this study are included in the manuscript and supporting files. Original confocal stacks are available from the authors upon request. Single-nucleus RNA-seq data analyzed in this study are from the publicly available Fly Cell Atlas dataset (Li et al., 2022), accessible through SCope (*https://scope.aertslab.org*). Fly stocks and antibodies generated for this study are available upon request from the corresponding author.

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Additional information

Contributions

AS and JCT conceived the study. AS collected data, performed analyses, and drafted figures. CQ, YX, and DD generated transgenic fly stocks and antibodies. AS, CQ, DD, and JCT interpreted the results. AS and JCT wrote the paper with input from CQ and DD.

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Additional files

supplemental figures

References

Agrawal S, Tuthill JC (2022) The two-body problem: Proprioception and motor control across the metamorphic divide. *Current Opinion in Neurobiology* **74**:102546 https://doi.org/10.1016/j.conb.2022.102546 | PubMed

Augustin H, Grosjean Y, Chen K, Sheng Q, Featherstone DE (2007) Nonvesicular Release of Glutamate by Glial xCT Transporters Suppresses Glutamate Receptor Clustering In Vivo. *Journal of Neuroscience* **27**:111-123 https://doi.org/10.1523/JNEUROSCI.4770-06.2007 | PubMed

Azevedo A, Lesser E, Phelps JS, Mark B, Elabbady L, Kuroda S, Sustar A, Moussa A, Khandelwal A, Dallmann CJ, *et al.* (2024) Connectomic reconstruction of a female Drosophila ventral nerve cord. *Nature* **631**:360-368 https://doi.org/10.1038/s41586-024-07389-x | PubMed

Azevedo AW, Dickinson ES, Gurung P, Venkatasubramanian L, Mann RS, Tuthill JC (2020) A size principle for recruitment of Drosophila leg motor neurons. *eLife* **9**:e56754 https://doi.org/10.7554/eLife.56754 | PubMed

Baek M, Mann RS (2009) Lineage and Birth Date Specify Motor Neuron Targeting and Dendritic Architecture in Adult Drosophila. *Journal of Neuroscience* **29**:6904-6916 https://doi.org/10.1523/JNEUROSCI.1585-09.2009 | PubMed

Bates AS, Phelps JS, Kim M, Yang HH, Matsliah A, Ajabi Z, Perlman E, Delgado KM, Osman MAM, Salmon CK, *et al.* (2025) Distributed control circuits across a brain-and-cord connectome. *bioRxiv* https://doi.org/10.1101/2025.07.31.667571

Berg S, Beckett IR, Costa M, Schlegel P, Januszewski M, Marin EC, Nern A, Preibisch S, Qiu W, Shinya Takemura, *et al.* (2025) Sexual dimorphism in the complete connectome of the Drosophila male central nervous system. *bioRxiv* https://doi.org/10.1101/2025.10.09.680999

Berger J. (2008) Cloning and Characterization of Clumsy (clu) a Novel Presynaptic Glutamate Receptor that Regulates Synaptic Growth at the Larval Neuromuscular Junction in Drosophila Melanogaster. University of Wisconsin–Madison.

Brierley D j., Rathore K, VijayRaghavan K, Williams D w. (2012) Developmental origins and architecture of Drosophila leg motoneurons. *The Journal of Comparative Neurology* **520**:1629-1649 https://doi.org/10.1002/cne.23003 | PubMed

Cetin H, Beeson D, Vincent A, Webster R. (2020) The Structure, Function, and Physiology of the Fetal and Adult Acetylcholine Receptor in Muscle. *Frontiers in Molecular Neuroscience* **13**:581097 https://doi.org/10.3389/fnmol.2020.581097 | PubMed

Cull-Candy SG (1976) Two types of extrajunctional L-glutamate receptors in locust muscle fibres. *The Journal of Physiology* **255**:449-464 https://doi.org/10.1113/jphysiol.1976.sp011289 | PubMed

Cull-Candy SG (1975) Effect of denervation and local damage on extrajunctional L-glutamate receptors in locust muscle. *Nature* **258**:530-531 https://doi.org/10.1038/258530a0 | PubMed

Currie DA, Bate M. (1991) The development of adult abdominal muscles in Drosophila: myoblasts express twist and are associated with nerves. *Development* **113**:91-102 https://doi.org/10.1242/dev.113.1.91 | PubMed

Dale HH, Feldberg W, Vogt M. (1936) Release of acetylcholine at voluntary motor nerve endings. *The Journal of Physiology* **86**:353-380 https://doi.org/10.1113/jphysiol.1936.sp003371 | PubMed

Daniels RW, Gelfand MV, Collins CA, DiAntonio A. (2008) Visualizing glutamatergic cell bodies and synapses in Drosophila larval and adult CNS. *Journal of Comparative Neurology* **508**:131-152 https://doi.org/10.1002/cne.21670 | PubMed

Davie K, Janssens J, Koldere D, De Waegeneer M, Pech U, Aibar S, Makhzami S, Christiaens V, González-Blas C, Poovathingal S, *et al.* (2018) A Single-Cell Transcriptome Atlas of the Aging Drosophila Brain. *Cell* **174**:982-998.e20 https://doi.org/10.1016/j.cell.2018.05.057 | PubMed

DiAntonio A. (2006) Glutamate receptors at the Drosophila neuromuscular junction. *International Review of Neurobiology* **75**:165-179 https://doi.org/10.1016/S0074-7742(06)75008-5 | PubMed

DiAntonio A, Petersen SA, Heckmann M, Goodman CS (1999) Glutamate receptor expression regulates quantal size and quantal content at the Drosophila neuromuscular junction. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **19**:3023-3032 https://doi.org/10.1523/JNEUROSCI.19-08-03023.1999 | PubMed

Dickinson MH, Tu MS (1997) The Function of Dipteran Flight Muscle. *Comparative Biochemistry and Physiology Part A: Physiology* **116**:223-238 https://doi.org/10.1016/S0300-9629(96)00162-4

Dudel J, Franke C, Hatt H, Usherwood PN (1989) Chloride channels gated by extrajunctional glutamate receptors (H-receptors) on locust leg muscle. *Brain Research* **481**:215-220 https://doi.org/10.1016/0006-8993(89)90796-8 | PubMed

Featherstone DE, Rushton E, Rohrbough J, Liebl F, Karr J, Sheng Q, Rodesch CK, Broadie K. (2005) An essential Drosophila glutamate receptor subunit that functions in both central neuropil and neuromuscular junction. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **25**:3199-3208 https://doi.org/10.1523/JNEUROSCI.4201-04.2005 | PubMed

Goel P, Dickman D. (2018) Distinct homeostatic modulations stabilize reduced postsynaptic receptivity in response to presynaptic DLK signaling. *Nature Communications* **9**:1856 https://doi.org/10.1038/s41467-018-04270-0 | PubMed

Gunage RD, Dhanyasi N, Reichert H, VijayRaghavan K. (2017) Drosophila adult muscle development and regeneration. *Seminars in Cell & Developmental Biology* **72**:56-66 https://doi.org/10.1016/j.semcdb.2017.11.017 | PubMed

Han TH, Dharkar P, Mayer ML, Serpe M. (2015) Functional reconstitution of Drosophila melanogaster NMJ glutamate receptors. *Proceedings of the National Academy of Sciences* **112**:6182-6187 https://doi.org/10.1073/pnas.1500458112 | PubMed

Han TH, Vicidomini R, Ramos CI, Mayer ML, Serpe M. (2024) The gating properties of Drosophila NMJ glutamate receptors and their dependence on Neto. *The Journal of Physiology* **602**:7043-7064 https://doi.org/10.1113/JP287331 | PubMed

Hartzell HC, Fambrough DM (1972) Acetylcholine receptors. Distribution and extrajunctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity. *The Journal of General Physiology* **60**:248-262 https://doi.org/10.1085/jgp.60.3.248 | PubMed

He K, Dickman D. (2025) Building and modifying diverse synaptic properties: Insights from Drosophila. *Current Opinion in Neurobiology* **92**:102995 https://doi.org/10.1016/j.conb.2025.102995 | PubMed

Heckscher ES, Lockery SR, Doe CQ (2012) Characterization of Drosophila Larval Crawling at the Level of Organism, Segment, and Somatic Body Wall Musculature. *Journal of Neuroscience* **32**:12460-12471 https://doi.org/10.1523/JNEUROSCI.0222-12.2012 | PubMed

Hooper SL, Thuma JB (2005) Invertebrate muscles: muscle specific genes and proteins. *Physiological Reviews* **85**:1001-1060 https://doi.org/10.1152/physrev.00019.2004 | PubMed

Huen DS, Youles M, Sweeney S, Ponder BAJ, Davis GW, Ashburner M. (2001) Cloning of clumsy: a mutation affecting gait and ether sensitivity in Drosophila melanogaster. In: A. Dros. Res. Conf.. pp. 820A

Kane NS, Hirschberg B, Qian S, Hunt D, Thomas B, Brochu R, Ludmerer SW, Zheng Y, Smith M, Arena JP, *et al.* (2000) Drug-resistant Drosophila indicate glutamate-gated chloride channels are targets for the antiparasitics nodulisporic acid and ivermectin. *Proceedings of the National Academy of Sciences of the United States of America* **97**:13949-13954 https://doi.org/10.1073/pnas.240464697 | PubMed

Kim Y-J, Bao H, Bonanno L, Zhang B, Serpe M. (2012) Drosophila Neto is essential for clustering glutamate receptors at the neuromuscular junction. *Genes & Development* **26**:974-987 https://doi.org/10.1101/gad.185165.111 | PubMed

Kimura KI, Truman JW (1990) Postmetamorphic cell death in the nervous and muscular systems of Drosophila melanogaster. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **10**:403-411 https://doi.org/10.1523/JNEUROSCI.10-02-00403.1990 | PubMed

Kondo S, Takahashi T, Yamagata N, Imanishi Y, Katow H, Hiramatsu S, Lynn K, Abe A, Kumaraswamy A, Tanimoto H. (2020) Neurochemical Organization of the Drosophila Brain Visualized by Endogenously Tagged Neurotransmitter Receptors. *Cell Reports* **30**:284-297.e5 https://doi.org/10.1016/j.celrep.2019.12.018 | PubMed

Lea TJ, Usherwood PNR (1973) The site of action of ibotenic acid and the identification of two populations of glutamate receptors on insect muscle-fibres. *Comparative and General Pharmacology* **4**:333-350 https://doi.org/10.1016/0010-4035(73)90045-1 | PubMed

Lehmann F-O, Heymann N. (2005) Unconventional mechanisms control cyclic respiratory gas release in flying Drosophila. *Journal of Experimental Biology* **208**:3645-3654 https://doi.org/10.1242/jeb.01788 | PubMed

Lesser E, Azevedo AW, Phelps JS, Elabbady L, Cook A, Syed DS, Mark B, Kuroda S, Sustar A, Moussa A, *et al.* (2024) Synaptic architecture of leg and wing premotor control networks in Drosophila. *Nature* **631**:369-377 https://doi.org/10.1038/s41586-024-07600-z | PubMed

Li H, Janssens J, De Waegeneer M, Kolluru SS, Davie K, Gardeux V, Saelens W, David FPA, Brbić M, Spanier K, *et al.* (2022) Fly Cell Atlas: A single-nucleus transcriptomic atlas of the adult fruit fly. *Science* **375**:eabk2432 https://doi.org/10.1126/science.abk2432 | PubMed

Li X, Chien C, Han Y, Sun Z, Chen X, Dickman D. (2021) Autocrine inhibition by a glutamate-gated chloride channel mediates presynaptic homeostatic depression. *Science Advances* **7**:eabj1215 https://doi.org/10.1126/sciadv.abj1215 | PubMed

Li Y, Dharkar P, Han T-H, Serpe M, Lee C-H, Mayer ML (2016) Novel Functional Properties of Drosophila CNS Glutamate Receptors. *Neuron* **92**:1036-1048 https://doi.org/10.1016/j.neuron.2016.10.058 | PubMed

Lingle C, Marder E. (1981) A glutamate-activated chloride conductance on a crustacean muscle. *Brain Research* **212**:481-488 https://doi.org/10.1016/0006-8993(81)90482-0 | PubMed

Liu WW, Wilson RI (2013) Glutamate is an inhibitory neurotransmitter in the Drosophila olfactory system. *Proceedings of the National Academy of Sciences of the United States of America* **110**:10294-10299 https://doi.org/10.1073/pnas.1220560110 | PubMed

Marrus SB, Portman SL, Allen MJ, Moffat KG, DiAntonio A. (2004) Differential localization of glutamate receptor subunits at the Drosophila neuromuscular junction. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **24**:1406-1415 https://doi.org/10.1523/JNEUROSCI.1575-03.2004 | PubMed

Pedersen TH, Riisager A, de Paoli FV, Chen T-Y, Nielsen OB (2016) Role of physiological ClC-1 Cl− ion channel regulation for the excitability and function of working skeletal muscle. *The Journal of General Physiology* **147**:291-308 https://doi.org/10.1085/jgp.201611582 | PubMed

Perry S, Han Y, Das A, Dickman D. (2017) Homeostatic plasticity can be induced and expressed to restore synaptic strength at neuromuscular junctions undergoing ALS-related degeneration. *Human Molecular Genetics* **26**:4153-4167 https://doi.org/10.1093/hmg/ddx304 | PubMed

Petersen SA, Fetter RD, Noordermeer JN, Goodman CS, DiAntonio A. (1997) Genetic Analysis of Glutamate Receptors in Drosophila Reveals a Retrograde Signal Regulating Presynaptic Transmitter Release. *Neuron* **19**:1237-1248 https://doi.org/10.1016/S0896-6273(00)80415-8 | PubMed

Pratt BG, S-YJ Lee, Chou GM, Tuthill JC (2024) Miniature linear and split-belt treadmills reveal mechanisms of adaptive motor control in walking Drosophila. *Current biology: CB* **34**:4368-4381.e5 https://doi.org/10.1016/j.cub.2024.08.006 | PubMed

Qin G, Schwarz T, Kittel RJ, Schmid A, Rasse TM, Kappei D, Ponimaskin E, Heckmann M, Sigrist SJ (2005) Four different subunits are essential for expressing the synaptic glutamate receptor at neuromuscular junctions of Drosophila. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **25**:3209-3218 https://doi.org/10.1523/JNEUROSCI.4194-04.2005 | PubMed

Rivlin PK, St. Clair RM, Vilinsky I, Deitcher DL (2004) Morphology and molecular organization of the adult neuromuscular junction of Drosophila. *Journal of Comparative Neurology* **468**:596-613 https://doi.org/10.1002/cne.10977 | PubMed

Saito M, Kawai N. (1985) Developmental changes in the glutamate receptor at the insect neuromuscular synapse. *Developmental Brain Research* **18**:97-102 https://doi.org/10.1016/0165-3806(85)90253-6 | PubMed

Sanes JR, Lichtman JW (1999) Development of the vertebrate neuromuscular junction. *Annual Review of Neuroscience* **22**:389-442 https://doi.org/10.1146/annurev.neuro.22.1.389 | PubMed

Sanfilippo P, Kim AJ, Bhukel A, Yoo J, Pegah Mirshahidi, Pandey V, Bevir H, Yuen A, Parmis Mirshahidi, Guo P, *et al.* (2024) Mapping of multiple neurotransmitter receptor subtypes and distinct protein complexes to the connectome. *Neuron* **112**:942-958.e13 https://doi.org/10.1016/j.neuron.2023.12.014 | PubMed

Schiaffino S, Chemello F, Reggiani C. (2025) The Diversity of Skeletal Muscle Fiber Types. *Cold Spring Harbor Perspectives in Biology* **17**:a041477 https://doi.org/10.1101/cshperspect.a041477 | PubMed

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, *et al.* (2012) Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**:676-682 https://doi.org/10.1038/nmeth.2019 | PubMed

Schuster CM, Ultsch A, Schloss P, Cox JA, Schmitt B, Betz H. (1991) Molecular cloning of an invertebrate glutamate receptor subunit expressed in Drosophila muscle. *Science* **254**:112-114 https://doi.org/10.1126/science.1681587 | PubMed

Takeuchi A, Takeuchi N. (1964) The effect on crayfish muscle of iontophoretically applied glutamate. *The Journal of Physiology* **170**:296-317 https://doi.org/10.1113/jphysiol.1964.sp007332 | PubMed

Takeuchi A, Takeuchi N. (1963) Glutamate-induced Depolarization in Crustacean Muscle. *Nature* **198**:490-491 https://doi.org/10.1038/198490a0

Thomas U, Sigrist SJ (2012) Glutamate receptors in synaptic assembly and plasticity: case studies on fly NMJs. *Advances in Experimental Medicine and Biology* **970**:3-28 https://doi.org/10.1007/978-3-7091-0932-8_1 | PubMed

Vaxenburg R, Siwanowicz I, Merel J, Robie AA, Morrow C, Novati G, Stefanidi Z, Both G-J, Card GM, Reiser MB, *et al.* (2025) Whole-body physics simulation of fruit fly locomotion. *Nature* **643**:1312-1320 https://doi.org/10.1038/s41586-025-09029-4 | PubMed

Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Dürrbeck H, Buchner S, Dabauvalle M-C, Schmidt M, Qin G, *et al.* (2006) Bruchpilot, a Protein with Homology to ELKS/CAST, Is Required for Structural Integrity and Function of Synaptic Active Zones in Drosophila. *Neuron* **49**:833-844 https://doi.org/10.1016/j.neuron.2006.02.008 | PubMed

Wang-Chen S, Stimpfling VA, Lam TKC, zdil PG, Genoud L, Hurtak F, Ramdya P. (2024) NeuroMechFly v2: simulating embodied sensorimotor control in adult Drosophila. *Nature Methods* **21**:2353-2362 https://doi.org/10.1038/s41592-024-02497-y | PubMed

Wichmann C, Kuner T. (2021) Heterogeneity of glutamatergic synapses: cellular mechanisms and network consequences. *Physiological Reviews* https://doi.org/10.1152/physrev.00039.2020 | PubMed

Peer reviews

Reviewer #1 (Public review):

Summary:

The manuscript by Sustar et al. takes a methodical approach to document the types of glutamate receptor subunits that reside in Drosophila muscles, examining developmental stages spanning from larvae to adults. Prior work thoroughly documented the subunits operating in Drosophila larval body wall muscles. Most subsequent research focused on the glutamate receptor heterotetramers found in the body wall, composed of GluRIIA/C/D/E or GluRIIB/C/D/E subunits, along with auxiliary subunits like isoforms of Neto.

For the current work, the authors report that the larval muscle glutamate receptor composition is not universal for all Drosophila muscles. They examine the following muscle systems: larval body wall, adult abdomen, adult leg coxa, and adult indirect flight. They also briefly examine adult muscle structures associated with the proboscis, neck, and haltere. The authors find that the receptor subunits in the adult abdomen (mostly) match those in the larval body wall. This makes sense given that the adult abdominal muscles are derived from the larval body wall. Yet not much else matches the larval body wall. For example, all (or most) of the GluRII-type subunits are missing from the adult indirect flight muscles. Leg muscles have GluRII-type subunits, but they do not have all of them expressed prominently, and they are missing GluRIIB. Additionally, leg muscles express a glutamate-gated chloride channel, which could be a source of inhibitory glutamatergic transmission. Interestingly, when it comes to non-abdominal adult muscles, one general theme seems to be an active promoter (GAL4 driver) for the kainate-type glutamate receptor called Clumsy. The authors propose that Clumsy could be key to understanding how functional GluR complexes are assembled in adult insects.

Strengths:

(1) Documenting the types of glutamate receptors that operate in diverse insect muscle systems is important because it uncovers fundamental information.

(2) Much of the prior research focus has been on how the body wall muscle tetramers assemble and operate. It is a strength to demonstrate the other receptor solutions used by adult NMJs.

(3) The work uses GAL4 drivers and immunohistochemistry (when possible) in combination to draw conclusions.

(4) The muscle anatomical analyses are of high quality. This allows the research group to reach refined conclusions.

(5) The confocal-level images of synaptic active zones and their apposed glutamate receptor clusters are of high quality.

Weaknesses:

(1) There is a strawman argument that is used repeatedly to highlight the significance of the work. The argument implies that the field broadly assumes (or "tacitly" assumes) that the larval body wall glutamate receptor composition extrapolates to all muscles of the fly, including the adult. This reviewer cannot find evidence that this assumption or argument has been explicitly promulgated by others. More likely, others have not examined these muscles directly, and thus, they have not speculated one way or the other.

(2) Related - to the extent that there has been any tacit assumption about GluRIIC/D/E-anchored receptors being ubiquitous among adult muscles, tacit doubt was raised by Rivilin et al., 2004 (cited by the authors but not as a source of doubt) and by RNAseq datasets like FlyAtlas from 2022 (replicated in Figures s11 and s12). To be clear, the current analysis is better than a bulk transcript analysis from adult tissues. But rather than "overturning" a field or being paradigm-shifting, the current data seem confirmatory of FlyAtlas - and confirmatory of Rivlin et al., 2004, which explicitly concluded that larval and adult NMJs were different .

(3) One can draw expression-level conclusions from these data. But genetic tests (e.g., would clumsy losses of function impair leg muscles?) could help the authors and the field draw stronger conclusions about the roles of some of these glutamate receptor gene products. The current dataset falls short of definitively establishing the function of alternate glutamate receptor modules.

(4) The confocal synaptic images are of high quality. They are good enough that one could analyze how well Brp directly apposes a specific glutamate receptor subunit for all the associated imaging data underlying Figures S1-S8. No such analysis is done, but understanding what components seem to directly oppose the site of release could lead to better conclusions.

Overall Assessment and Discussion:

The data in this study are of high quality, and the results support the main conclusion: adult muscle glutamate receptor clusters do not recapitulate the "canonical" larval body wall clusters. This is important, and the data stand on their own. That is the most important part. This reviewer does have suggestions on how to put the current work in proper context; the current draft appears to overstate the novelty of the findings. Additionally, some sentences need editing for accuracy. None of those concerns impeach the excellent foundational data.

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Reviewer #2 (Public review):

Summary:

This manuscript presents a broad survey of glutamate receptor composition at the neuromuscular junction in Drosophila across developmental stages and muscle types. The topic is clearly important, and the central observation-that adult muscles differ substantially from the canonical larval NMJ-is interesting and potentially impactful. The dataset is extensive and will likely be of value to the community. However, in my view, there are significant limitations in how the data are generated and interpreted, which at present reduce the strength of the conclusions.

Strengths:

The study addresses a relevant and timely question and provides a large and systematic dataset. The finding that adult muscles diverge from larval NMJ organization is compelling and challenges a widely held assumption in the field. The breadth of approaches, including genetic reporters, immunohistochemistry, endogenous tagging, and transcriptomic data, is, in principle, a strong aspect of the work and allows for a broad overview of receptor expression across tissues and developmental stages. Even in its current form, the manuscript provides useful descriptive information that will be of interest to the community.

Weaknesses:

A major concern is the reliance on a heterogeneous combination of detection methods (GAL4 reporters, antibody staining, endogenous tagging, and RNA), which are treated largely as equivalent lines of evidence. These approaches differ substantially in what they measure and in their sensitivity and specificity. While convergence across methods can in principle be convincing, here this convergence is often inferred from the shared absence of signal. This is problematic because all methods used are susceptible to false negatives for different reasons. As a result, the repeated conclusion that specific GluR subunits are "absent" from adult muscles, including those previously considered essential, is not fully justified by the data presented.

This issue is not only theoretical. The manuscript itself seemingly contains examples where methods disagree, demonstrating that detection is incomplete and method-dependent. These discrepancies could be better integrated into the interpretation. Instead, negative results across methods are often taken as strong evidence for absence, which overstates the certainty of the findings.

In addition, antibody validation appears to rely largely on prior work in larval tissue. Given the structural and biochemical differences in adult muscles, it is not clear that staining performance is equivalent, particularly in cases where the signal is weak or undetected. This further complicates the interpretation of negative results.

More generally, the manuscript moves in several places from descriptive observations to functional or mechanistic implications that are not directly supported. The suggestion that adult muscles operate with fundamentally different receptor assemblies is intriguing, but remains speculative without functional validation. At a minimum, the distinction between observation and interpretation should be made more explicit.

I thus think that the current conclusions need to be more carefully constrained. Ideally, the study would be strengthened by at least one functional experiment, such as electrophysiological recordings from adult NMJs or perturbation of candidate receptors like GluClα or Clumsy. This would help to anchor the expression data in synaptic function.

In summary, this is an interesting and potentially important study, but the current manuscript somewhat overinterprets heterogeneous and partly indirect evidence. It will already be useful in its present form, but could be more convincing if the authors more rigorously account for methodological limitations and moderate their claims accordingly.

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Reviewer #3 (Public review):

The Sustar et al. manuscript catalogs glutamate receptor composition across distinct Drosophila NMJs: larval and adult abdominal NMJs, as well as NMJs on adult leg and flight muscles. This work is important and probably overdue. The larval NMJ is the exemplar NMJ in this system, and the identity of "essential" and "alternative" subunits at this stage is assumed by many to hold across developmental stages and NMJ types. Here, the authors show that there is surprising diversification among NMJ types and that the notion of essential/alternative subunits only holds true at larval NMJs.

The study will generate interest in the Clumsy GluR subunit, which has not been well-characterized at all, but is widely expressed at adult NMJs. They also find striking extrasynaptic expression of glutamate-gated chloride channel GluRClalpha in adult leg and flight muscles, raising questions about its role. The study is interesting, logical, and well-written. The figures are clear, and the discussion was particularly thoughtful. I have a couple of comments that the authors could consider.

(1) They cite Rivlin et al., (2004) in the Introduction as the sole previous study to investigate the molecular composition of adult NMJs, but do not mention this work again. In the Discussion, it would be helpful to compare/contrast their finding with those of the earlier work.

(2) Were these analyses done in adults of consistent ages? It seems possible that the GluR subunit composition could be different in very young adults or in aged flies. The age of the animals should be mentioned in the Methods.

(3) The broad expression of GluCl:V5 in adult leg and flight muscles is surprisingly robust and appears to light up the edges of all muscle fibers. Would the authors comment on the controls that were done to ensure that this staining is real and specific to animals carrying that V5 endogenous tag?

(4) The snRNAseq data in Figure S12 differ a bit from the IHC/GAL4 data summarized in the table in Figure 2. In particular, the data suggests that Ukar and Grik are widely expressed in adult muscles. Is there a reason not to include an "snRNA seq" column in Figure 2 alongside the data from GAL4 lines and IHC? To my mind, it is about as reliable as GAL4 lines that often capture only a subset of the full expression pattern. In this case, the snRNAseq data suggest that Ukar/Grik are likely at adult flight muscle NMJs, which might be important since NMJ was negative for everything except Neto-beta by IHC.

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