

RESEARCH REPORT

# When Guidance Meets Synaptic Function

The Emerging Role of Unc-5 in *Drosophila* Development

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## Abstract

The transmembrane protein Unc-5 was initially characterised for its role in axon guidance. However, it has recently been shown to locally promote distinct forms of neurotransmission at 40% of release sites, termed active zones (AZs), in the adult *Drosophila* neuromuscular junction. This observation either represents an independent, novel function of Unc-5 in the adult nervous system, or unveils a previously unidentified function of Unc-5 that also manifests during development. We used a combination of immunohistochemistry and electrophysiology, together with the reinvestigation of classic loss-of-function mutants, to investigate the role and regulation of synaptic Unc-5 during embryonic and early larval development.

We find that Unc-5 localises to 60% of AZs in mature embryonic synaptic boutons and to 80% of glutamate receptor puncta. Genetic depletion of the vesicle release SNARE protein Syntaxin induced a dramatic increase in Unc-5 localisation, with Unc-5 associating with over 95% of AZs. Finally, functional analysis of Unc-5 loss-of-function escapers, revealed reduced spontaneous neurotransmission across multiple muscle fibres and impaired synaptic bouton maturation in early larvae.

Together, these findings identify a conserved synaptic role for Unc-5 in the establishment and regulation of neurotransmission during synaptic bouton and AZ maturation and show that Unc-5 levels can be regulated through manipulation of the canonical synaptic release machinery. AZ heterogeneity does not simply emerge from stochastic differences between mature release sites, but can be actively established during development by molecular programmes that spatially organise the release machinery, a process that appears to be maintained into adulthood and ageing.

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## Glossary

**Active zone (AZ)** A specialized presynaptic membrane site where synaptic vesicles are positioned for neurotransmitter release.

**BRP (Bruchpilot)** A presynaptic active-zone protein used as a marker of release sites [7].

**GluR** Glutamate receptor located on the postsynaptic muscle membrane at the *Drosophila* neuromuscular junction.

**mEJP (miniature excitatory junction potential)** A small postsynaptic potential produced by the spontaneous release of a single synaptic vesicle at the neuromuscular junction.

**MOC (Manders' overlap coefficient)** A fluorescence-colocalization measure used here to quantify the overlap between two labelled signals.

**NMJ (neuromuscular junction)** The synapse between a motor neuron and a muscle fibre. The *Drosophila* NMJ is the experimental model used in this study [8].

**SNARE complex** A group of proteins that drives synaptic vesicle fusion with the presynaptic membrane.

**Syntaxin** A presynaptic SNARE protein involved in synaptic vesicle fusion and examined here as part of the release machinery.

**Unc-5** A transmembrane receptor best known for its role in axon guidance and investigated here for its contribution to synaptic development and organization.

**VGLUT** Vesicular glutamate transporter that loads glutamate into synaptic vesicles and is used here as a marker of glutamatergic synaptic boutons.

# 1 Introduction

## 1.1 Digging in: uncovering the rules of synaptic assembly

Neuronal communication is essential for the development and function of the nervous system, allowing neurons to transmit information to other cells and coordinate processes such as movement, sensory perception and behaviour. A clear example is the neuromuscular junction (NMJ), where motor neurons control muscle activity through specialised synaptic structures. The *Drosophila* NMJ has been extensively characterised as a model of synaptic development and maturation [4].

At the NMJ, this communication is primarily chemical. The presynaptic terminal forms a synaptic bouton in which neurotransmitter-containing synaptic vesicles are stored. Upon release, vesicles fuse with the presynaptic membrane and discharge their content into the synaptic cleft, where the neurotransmitter binds to receptors on the muscle membrane. This release does not occur anywhere on the membrane, but at specialised sites called active zones (AZs), where vesicles are positioned for fusion [7]. Receptors on the muscle side cluster directly opposite the AZs, forming a highly organised site of communication between neuron and muscle.

Motor neurons at the *Drosophila* NMJ primarily use glutamate, a major excitatory neurotransmitter that activates glutamate receptors on the muscle. Because these receptors cluster opposite AZs, glutamate receptor clusters provide a convenient readout of the postsynaptic side of each synapse and glutamatergic transmission is a useful system to study how synaptic communication is established and regulated.

Release at AZs can occur in two ways. Evoked neurotransmission is triggered by a stimulus, which causes the coordinated release of many vesicles and produces a measurable postsynaptic response. Spontaneous neurotransmission, in contrast, consists of the release of individual vesicles independently of stimulation. Both rely on vesicle fusion but differ in what triggers it [5].

Spontaneous release was long considered an unregulated consequence of stochastic vesicle fusion. However, work at the *Drosophila* NMJ has shown that miniature release can influence synaptic structure and that its disruption produces specific developmental phenotypes [2,5]. More recently, spontaneous release was shown to be spatially heterogeneous across individual AZs, suggesting that it is locally regulated rather than simple background activity [1]. This raises the question of which molecular factors set this local regulation and how they are established during development. The *Drosophila* NMJ is well suited to address it, since synapses can be studied throughout development and AZs can be examined individually.

## 1.2 Unc-5: from axon guidance to synaptic function

One candidate for such regulation is Unc-5, a cell-surface receptor best known for its role in axon guidance. In *Drosophila*, Unc-5 was initially characterised as a receptor contributing to repulsive axon guidance [3], allowing growing axons to use guidance cues to navigate towards appropriate targets.

More recently, Unc-5 has been detected at a subset of AZs at the adult *Drosophila* NMJ. Rather than being present at every release site, it is localised to only some AZs, and studies from the McCabe laboratory indicate that Unc-5 levels can regulate the frequency of spontaneous neurotransmitter release [1]. This suggests that Unc-5 could contribute to the local regulation described above, beyond its role in axon guidance. It also raises a developmental question: when is this synaptic function of Unc-5 established?

### 1.3 Defining the role of Unc-5 during synaptic development

If Unc-5 regulates spontaneous neurotransmission in the adult NMJ, it is important to know whether its localisation and associated function are already in place as synapses develop. In this study, we therefore investigated whether Unc-5 contributes to the development and organisation of synaptic sites at the embryonic *Drosophila* NMJ. We first examined how Unc-5 is distributed during synaptic maturation and whether it is associated with AZs and glutamate receptor clusters. We then investigated its relationship with the molecular machinery of synaptic vesicle release and assessed the consequences of Unc-5 loss on synaptic structure and spontaneous neurotransmission.

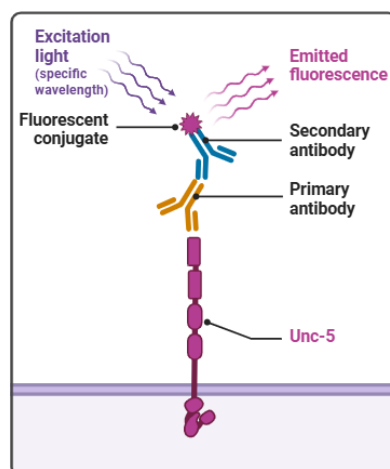
Together, these approaches allowed us to determine whether the association between Unc-5 and spontaneous neurotransmission observed at the adult NMJ is already established during synaptic development.

## 2 Experimental approach

### 2.1 Embryo collection and immunofluorescence

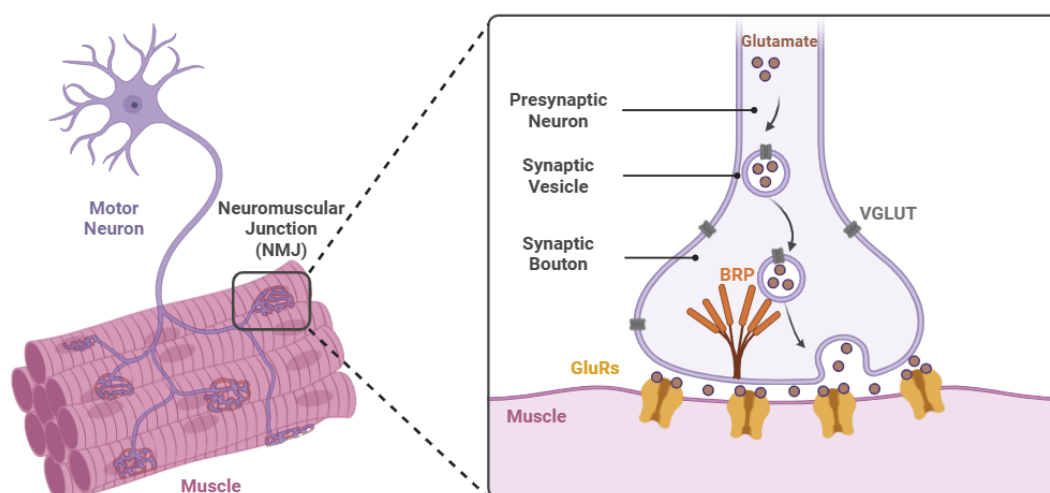
To investigate how synapses develop over time, *Drosophila* embryos were collected at different developmental stages. This allowed us to compare NMJs at different points during their maturation. In particular, embryos at stage 16 (13–16 h) and stage 17 (16–24 h) were analysed.

Once collected, embryos were fixed to preserve their cellular and molecular organization at the developmental stage at which they were collected. The embryos were then processed by immunofluorescence to visualize specific proteins within the tissue (Figure 1).



**Figure 1. Principle of the immunofluorescence staining procedure.** Unc-5 is shown here as an example: a primary antibody recognizes the protein of interest and a fluorescently labelled secondary antibody binds to it, allowing the target to be visualized by fluorescence microscopy. The same staining principle was applied to each protein analysed in this study, using primary antibodies specific to the corresponding target.

To relate Unc-5 to the different components of the synapse, we combined its staining with markers of the pre- and postsynaptic compartments (Figure 2). Vesicular glutamate transporter (VGLUT) labels glutamatergic synaptic vesicles within the presynaptic bouton, Bruchpilot (BRP) marks presynaptic active zones [7], and glutamate receptors (GluRs), which cluster on the muscle membrane opposite the release sites, label the postsynaptic side. Together, these markers provide a framework for analysing Unc-5 localisation within developing synapses.



**Figure 2. Organization of the *Drosophila* neuromuscular junction.** Motor neurons form synaptic boutons along muscle fibres. VGLUT labels glutamatergic synaptic vesicles, BRP marks presynaptic active zones and GluRs are clustered on the muscle membrane opposite the release sites. These markers provide a framework for analysing Unc-5 localization within developing synapses.

Fluorescence microscopy was used to visualize the labelled proteins and compare their localization and relative fluorescence intensity between developmental stages.

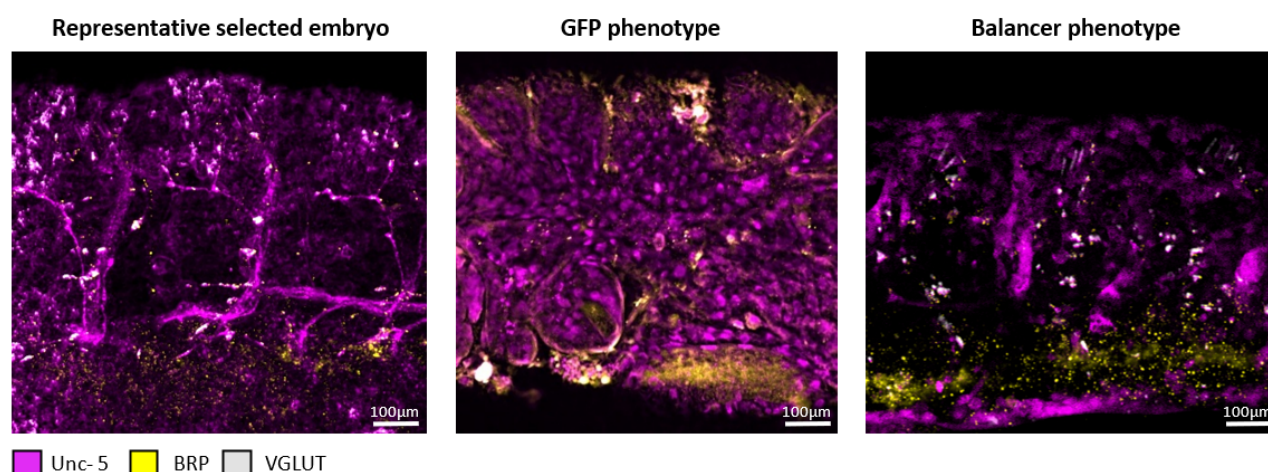
## 2.2 Genetic analysis

To visualise Unc-5 by immunofluorescence, we used an Unc-5 allele tagged with smV5, a short protein sequence introduced into the Unc-5 gene and recognised by an anti-V5 antibody. This allows endogenous Unc-5 to be detected without an antibody against the protein itself.

Because a single cross produces embryos of different genotypes, embryos had to be selected before analysis. A GFP marker was used as a visible criterion: embryos displaying GFP fluorescence were excluded, and embryos lacking it, which carry the desired Unc-5::smV5 genotype, were selected.

Mutant lines (Unc-5 and Syntaxin) were also used for the loss-of-function experiments and were maintained over balancer chromosomes. Balancers preserve mutations of interest in fly stocks while making it possible to identify genotypes among the offspring. Embryos with an inappropriate genotype, some of which display abnormal development (Figure 3), were identified and excluded, and embryos with normal development were selected for analysis.

This selection is essential: only embryos with the correct genotype can reveal the true effect of the genetic manipulation on synaptic structure. Including embryos carrying the balancer would risk attributing a phenotype to the loss of a protein when it in fact reflects the balancer itself. We therefore selected against a balancer phenotype that was consistently observed across multiple genotypes carrying the same balancer, ensuring that each observation can be confidently linked to the genotype studied.



**Figure 3. GFP and balancer-associated phenotypes used for embryo selection.** Left: a representative embryo of the type typically selected for analysis, carrying the Syntaxin deletion but showing neither GFP signal nor balancer-associated phenotype. Second from left: GFP-positive embryo, identified by the presence of fluorescent GFP signal. Right: embryo displaying the characteristic developmental phenotype associated with the balancer, including visible malformation of the central nervous system. These visible markers were used to distinguish and exclude inappropriate genotypes during genetic selection.

## 2.3 Image analysis and quantification

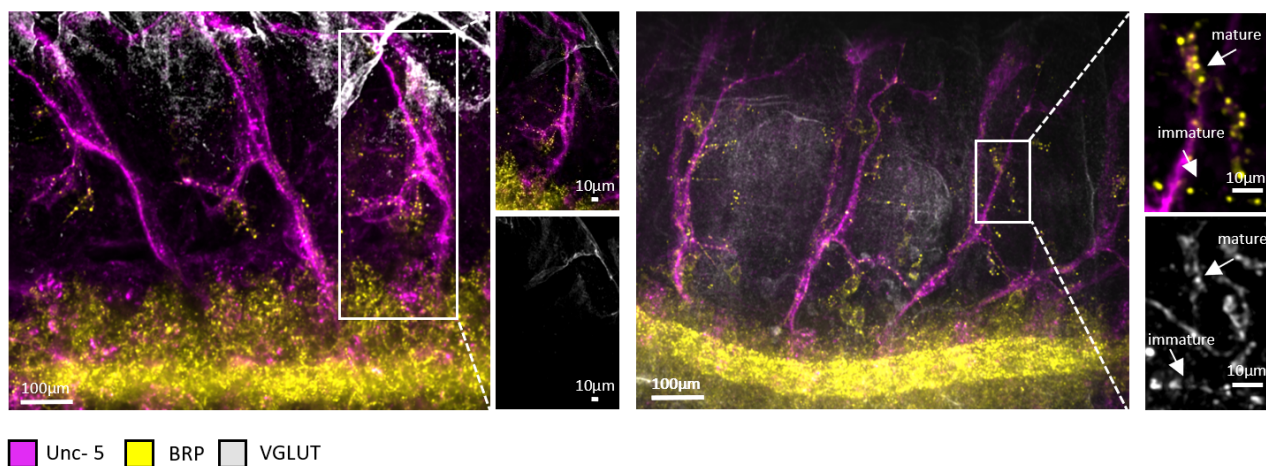
Fiji was used to quantify fluorescence data, using manual and automated approaches. Manually, active zones were identified as discrete BRP puncta and Unc-5 was scored as overlapping or not with each punctum. The proportion of Unc-5-positive AZs was calculated per embryo, and the proportion of Unc-5-positive GluR puncta per NMJ. Automatically, Manders' overlap coefficient (MOC) was calculated per bouton for Unc-5 relative to BRP and GluRs [8]. VGLUT and BRP intensity was measured per bouton using integrated density, with mature boutons expressed relative to the mean of immature boutons; the same values were used to correlate Unc-5 intensity with VGLUT and BRP intensity. Normality was assessed and was not met, so comparisons used the Mann-Whitney U test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ns = not significant).

## 3 Results

### 3.1 Unc-5 becomes selectively enriched during synaptic maturation

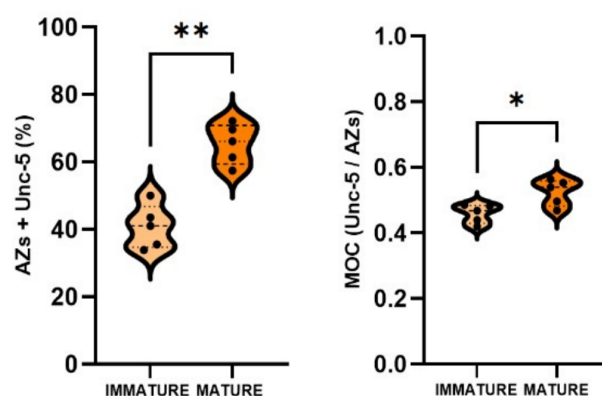
We first compared developing NMJs at stage 16 (13-16 h) and stage 17 (16-24 h) to determine how synaptic organization changes during development. At stage 16, VGLUT staining was not clearly detectable and distinct synaptic boutons could not yet be reliably identified. By stage 17, VGLUT-positive boutons were clearly visible, indicating the emergence of a more developed synaptic organization [4]. This developmental difference provided a basis for examining whether Unc-5 localization also changes as synapses mature.

We next examined the distribution of Unc-5 at these two developmental stages. At stage 16, Unc-5 appeared relatively homogeneous across the synaptic regions analysed. In contrast, at stage 17, Unc-5 was no longer uniformly distributed but appeared enriched at specific synaptic sites, revealing a more heterogeneous pattern of localization (Figure 4).



**Figure 4. Immunostaining of stage 16 (13-16 h) and stage 17 (16-24 h) *Drosophila* embryonic NMJs showing synaptic boutons, AZs and Unc-5.** Compared with stage 16, VGLUT staining at stage 17 reveals the formation of synaptic boutons. During maturation, Unc-5 becomes increasingly associated with AZs and displays a more heterogeneous distribution.

To determine whether this heterogeneous distribution was associated with synaptic maturation, we compared Unc-5 localization with BRP, a marker of presynaptic active zones. Unc-5 was detected at approximately 40% of AZs in immature boutons and 60% in mature boutons (Figure 5). This increase suggests that Unc-5 becomes increasingly associated with active zones as synapses mature.



**Figure 5. Quantification of Unc-5 localization at active zones using manual and automated MOC quantification [8].** Unc-5 was detected at approximately 40% of AZs in immature boutons, increasing significantly to approximately 60% in mature boutons.

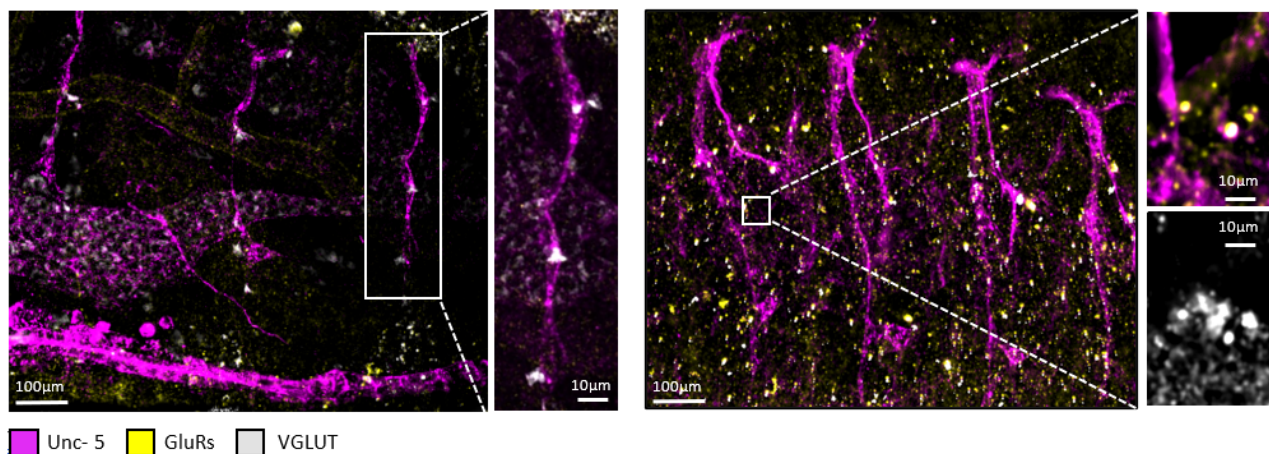
Together, these observations indicate that Unc-5 distribution changes during NMJ maturation. Rather than remaining uniformly distributed, Unc-5 becomes enriched at a subset of synaptic sites as boutons mature, suggesting that its localization is associated with the progressive organization of the synaptic machinery.

We then asked whether this specific localization of Unc-5 was associated with a postsynaptic apposition. Glutamate receptors (GluRs), which detect glutamate released by the motor neuron, were used to identify postsynaptic receptor clusters.

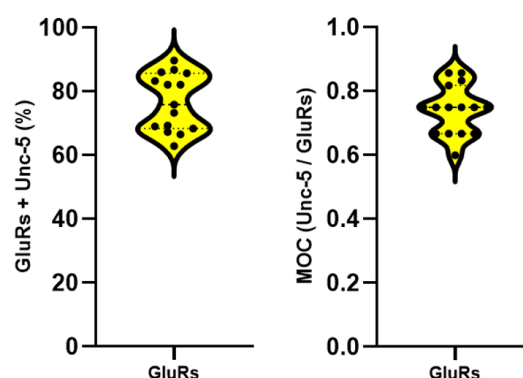
Unc-5 was associated with approximately 80% of the GluR puncta analysed, while some GluR puncta showed little or no detectable Unc-5. This heterogeneous association suggests that Unc-5 is not uniformly distributed across postsynaptic sites either (Figures 6 and 7).

Because bouton maturity could not be reliably assessed from GluR localization alone, we did not

distinguish immature and mature boutons in this analysis. Nevertheless, the association between Unc-5 and a subset of GluR puncta further supports the idea that Unc-5 has a specific localization within developing synapses, extending beyond the presynaptic active zone. This higher colocalization with GluRs compared with BRP suggests that Unc-5 is disproportionately enriched at more mature release sites, which are characterised by more clustered glutamate receptor fields.



**Figure 6. Immunostaining of stage 16 (13-16 h) and stage 17 (16-24 h) *Drosophila* embryonic NMJs showing synaptic boutons, GluRs and Unc-5.** Compared with stage 16, VGLUT staining at stage 17 reveals the formation of synaptic boutons, while Unc-5 displays a more heterogeneous distribution.



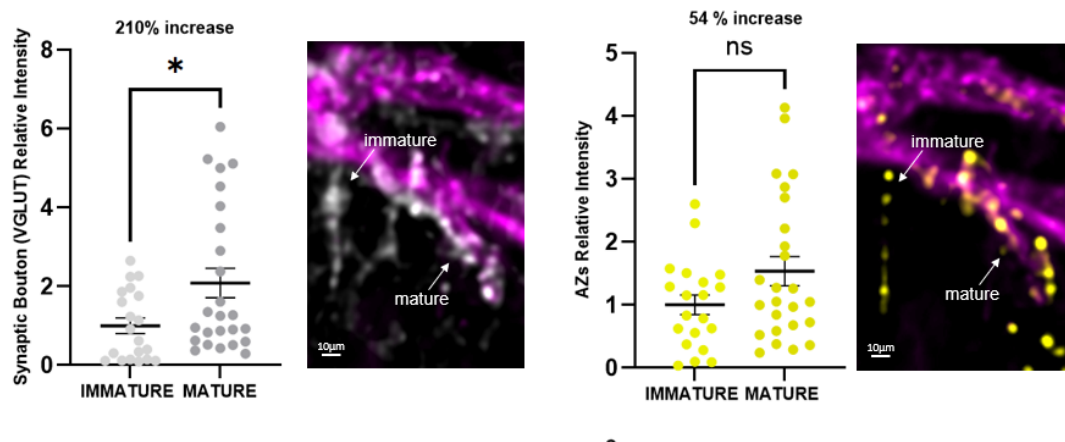
**Figure 7. Quantification of Unc-5 localization at GluRs using manual and automated MOC quantification [8].** Unc-5 was associated with approximately 80% of analysed GluR puncta, while some puncta showed little or no detectable Unc-5.

### 3.2 Unc-5 localization tracks synaptic maturation

We next asked whether synaptic maturation was accompanied by changes in the intensity of synaptic markers. VGLUT and BRP fluorescence intensity were quantified in immature and mature boutons. VGLUT intensity increased significantly during maturation, with mature boutons showing approximately 210% higher intensity than immature boutons. BRP intensity showed a similar tendency, with approximately 54% higher intensity in mature boutons, although this difference was not statistically significant (Figure 8).

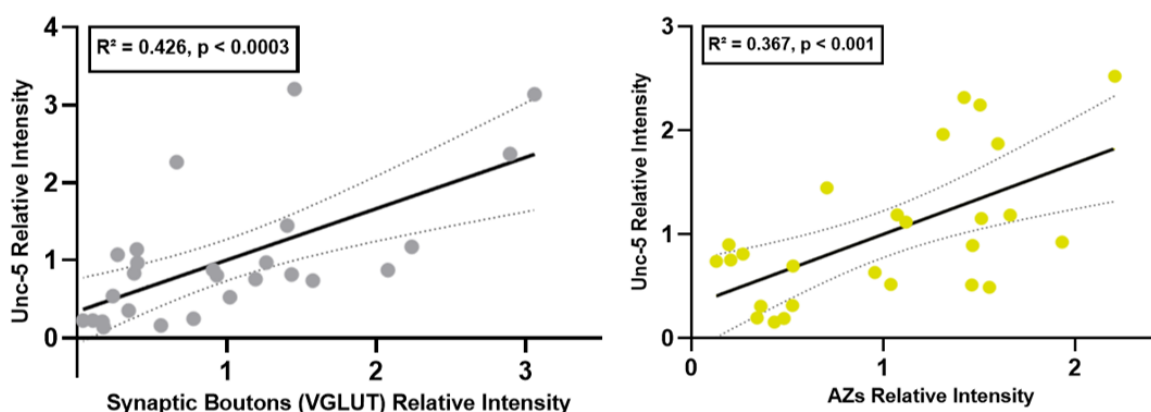
This asymmetry between the two markers likely reflects their distinct roles at the synapse. VGLUT is a synaptic vesicle protein, so its stronger increase is consistent with the accumulation of synaptic vesicles expected during bouton maturation. BRP, in contrast, is an active-zone protein, and its more

limited change in overall intensity is consistent with previous work showing that BRP abundance at individual active zones continues to increase gradually with synaptic development, even after the initial growth phase of the active zone [6], being a slower and more incremental accumulation than the vesicle pool captured by VGLUT.



**Figure 8. Relative intensity of VGLUT and BRP in immature and mature boutons.** Quantification across multiple boutons showed a significant 210% increase in VGLUT intensity between immature and mature boutons and a non-significant 54% increase in BRP intensity. Representative immunostaining illustrates this difference in intensity between a typical immature and mature bouton.

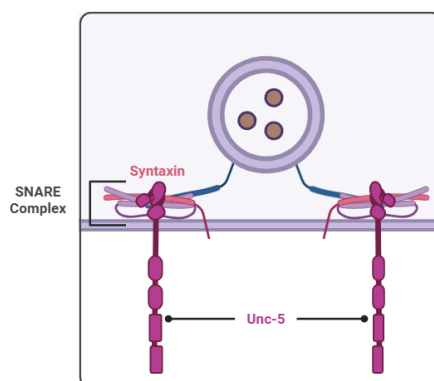
The variability in marker intensity among mature boutons led us to investigate whether it was associated with Unc-5 intensity. Linear regression revealed a significant positive correlation between VGLUT and Unc-5 intensity ( $R^2 = 0.426$ ,  $p < 0.0003$ ). A similar trend was observed for BRP ( $R^2 = 0.367$ ,  $p < 0.001$ ) (Figure 9). The association between Unc-5 and VGLUT and BRP intensity suggests that Unc-5 localization is linked to the molecular maturation of individual boutons, supporting a role for Unc-5 in the development and specialization of synaptic sites.



**Figure 9. Unc-5 relative intensity as a function of synaptic bouton (VGLUT) and AZ (BRP) relative intensity in mature boutons.** Unc-5 intensity correlated significantly with VGLUT and BRP intensity.

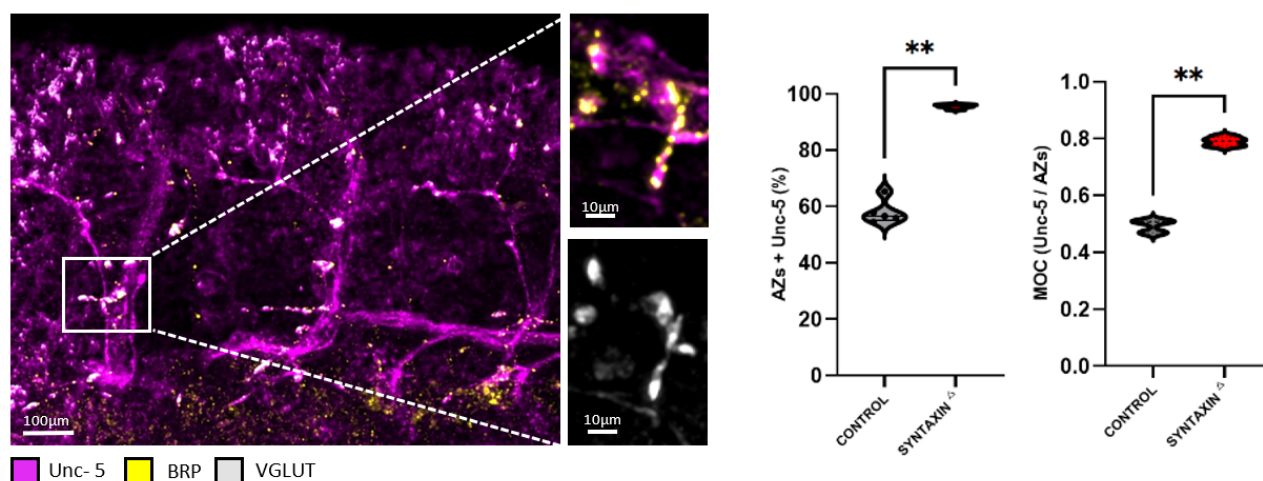
### 3.3 Synaptic release machinery shapes Unc-5 organization

We next investigated whether Unc-5 localization was influenced by the synaptic release machinery by analysing Unc-5 and GluR localization in Syntaxin mutant embryos. Syntaxin is a component of the SNARE complex, which mediates synaptic vesicle fusion at the presynaptic membrane (Figure 10).



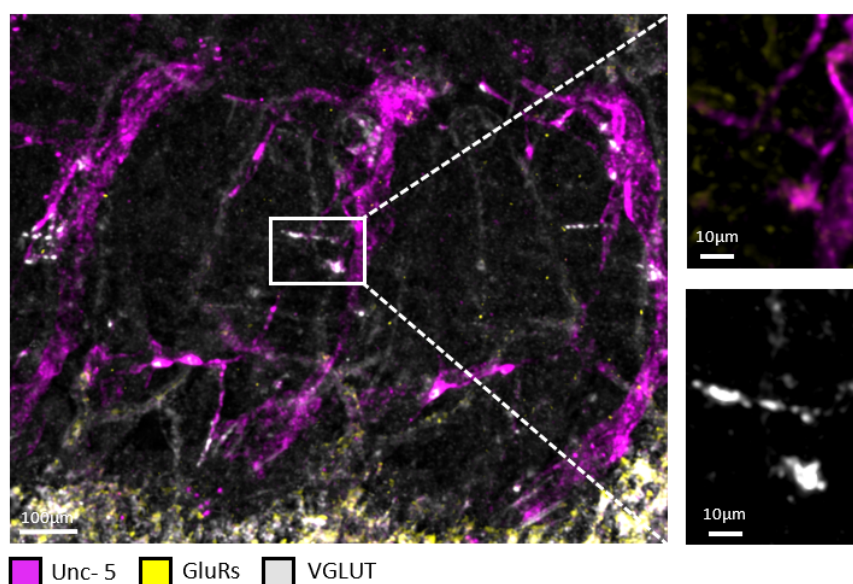
**Figure 10. Schematic representation of the potential association between Unc-5 and the synaptic release machinery.** Syntaxin is a component of the SNARE complex that mediates synaptic vesicle fusion at the presynaptic membrane.

To determine whether the synaptic release machinery influences Unc-5 localization, we examined stage 17 embryos following Syntaxin depletion. Compared with controls, Syntaxin depletion increased the proportion of active zones associated with Unc-5 (Figure 11).



**Figure 11. Immunostaining and quantification of stage 17 (16-24 h) *Drosophila* embryonic NMJs following Syntaxin depletion, showing synaptic boutons, AZs and Unc-5.** Genetic depletion of Syntaxin results in Unc-5 losing its characteristic heterogeneous distribution. Both manual and automated quantification show a consistent, significant reduction in Unc-5 heterogeneity at AZs compared with controls.

The effect of Syntaxin depletion was also examined at the postsynaptic side of the NMJ. In control embryos, GluRs formed discrete puncta associated with developing synaptic boutons. Following Syntaxin depletion, GluR staining appeared less organized, with receptors failing to form the characteristic clusters observed in controls (Figure 12).



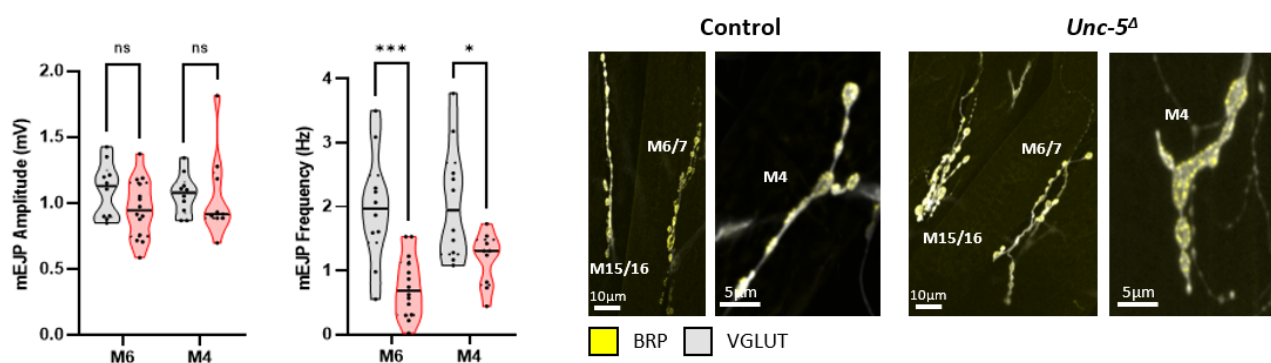
**Figure 12. Immunostaining of stage 17 (16-24 h *Drosophila* embryonic NMJs with Syntaxin depletion showing synaptic boutons, GluRs and Unc-5.** Syntaxin depletion disrupts postsynaptic glutamate receptor organization.

These observations indicate that Syntaxin depletion disrupts the normal organization of postsynaptic glutamate receptors. Together with the altered Unc-5 localization observed at active zones, the results suggest that Syntaxin-dependent synaptic organization is associated with the coordinated development of both presynaptic and postsynaptic compartments. Together, these results demonstrate that Unc-5 heterogeneity at active zones is enabled by Syntaxin, and that its depletion collapses this selectivity.

### 3.4 Unc-5 loss disrupts synaptic development and spontaneous neurotransmission

To determine whether the developmental role of Unc-5 was also reflected at the functional level, Unc-5 loss-of-function escapers were examined at early larval stages. These rare homozygous mutant individuals can survive beyond embryogenesis due to maternal contribution of functional Unc-5 RNA and/or protein deposited in the egg, partially compensating for the loss of zygotic Unc-5. Escapers represented approximately 3% of the expected mutant progeny.

Compared with controls, mutant escapers displayed visibly altered synaptic morphology, including malformed and incompletely developed synaptic boutons, consistent with impaired bouton maturation. Electrophysiological recordings in specific muscles were used to assess spontaneous neurotransmission. The frequency of miniature excitatory junction potentials (mEJPs) was significantly reduced in Unc-5 mutants, whereas mEJP amplitude was not significantly different from controls (Figure 13). Thus, Unc-5 loss was associated primarily with a reduction in the frequency of spontaneous neurotransmitter release rather than with a significant change in the amplitude of individual postsynaptic responses.

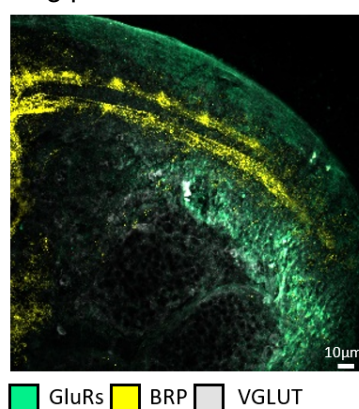


**Figure 13. mEJP amplitude and frequency in muscles 4 and 6 (M4 and M6), together with synaptic morphology in *Unc-5* loss-of-function escapers.** *Unc-5* loss reduces mEJP frequency without a significant change in amplitude and is associated with malformed, incompletely developed boutons in representative immunostaining of muscles 4, 6/7 and 15/16.

Together, the morphological and electrophysiological phenotypes provide functional support for a role of *Unc-5* in synaptic development, linking its loss to impaired bouton maturation and reduced spontaneous neurotransmitter release. This is consistent with previous findings that spontaneous release itself contributes to the maintenance of synaptic structure [2], and that *Unc-5* regulates the frequency of spontaneous neurotransmission at the adult NMJ [1]. Our results extend this role to embryonic development, suggesting that the association between *Unc-5* and spontaneous release described in the adult NMJ is already established and functionally required during synaptic maturation.

## 4 Additional experiments and technical limitations

A co-staining of BRP and GluRs was also attempted to further investigate the relationship between presynaptic and postsynaptic compartments and potentially explain the difference in *Unc-5* association between the two (Figure 14). However, the available antibody combinations presented a technical limitation, as the secondary antibody could recognize both primary antibodies, preventing the two signals from being reliably distinguished. Nevertheless, successful staining of both markers was obtained, although the embryos analysed were not sufficiently mature for reliable assessment of bouton maturation. This experiment demonstrated the feasibility of the approach and provides a basis for further optimization of the co-staining protocol.



**Figure 14. Co-staining of presynaptic and postsynaptic markers in a developing *Drosophila* NMJ.** BRP is shown in yellow, GluRs in green and VGLUT in grey. The absence of a clear VGLUT signal indicates that the embryo was at an earlier developmental stage than expected, preventing reliable assessment of bouton maturation. The image demonstrates the feasibility of detecting BRP and GluRs simultaneously.

Several broader limitations should also be considered. First, screening embryos from 12 h or 24 h lays was labour-intensive, as embryos at different developmental stages had to be individually staged and characterised. This could be reduced by preselecting embryos, using more restrictive laying windows, or dissecting embryos directly, although the latter would require additional training. Such approaches would limit the staging and screening burden associated with staining large, developmentally mixed populations at once.

Second, the mutants used in this study were classic germline mutants, affecting Unc-5 or Syntaxin throughout the organism. More recent approaches, such as CRISPR-induced cell-type-specific knockouts, would allow more targeted manipulation, for example restricting Syntaxin loss to motor neurons. Such a strategy would help isolate the observed phenotypes specifically to motor neuron function, rather than reflecting broader, organism-wide effects of the mutation.

Third, Unc-5 is expressed in both glia, the second major cell type in the nervous system, alongside neurons, and neurons. The colocalization observed between Unc-5 and VGLUT or BRP was interpreted as neuronally derived, but this has not been formally established at the embryonic stage; some of the Unc-5 signal could originate from glial cells. Evidence for a predominantly neuronal origin of synaptic Unc-5 is currently stronger in the adult NMJ than in the embryo and this distinction remains to be clarified in future work.

## 5 Conclusion: what we have uncovered

This study provides evidence that Unc-5 is already associated with synaptic development during embryonic NMJ maturation, rather than being exclusively associated with neurotransmission at the adult NMJ. As boutons mature, Unc-5 changes from a relatively homogeneous distribution to a heterogeneous localization, becoming enriched at a subset of active zones and glutamate receptor-associated sites, and its intensity correlated with that of synaptic proteins, further linking Unc-5 to the molecular maturation of individual boutons.

The altered Unc-5 distribution following Syntaxin depletion indicates that its localization is influenced by the synaptic release machinery. Conversely, Unc-5 loss was associated with impaired bouton maturation and reduced spontaneous neurotransmitter release, providing functional evidence that Unc-5 contributes to developing synapses.

Overall, these findings support a model in which Unc-5 contributes to the establishment and maturation of developing synapses by becoming selectively enriched at specific synaptic sites. Its localization and functional effects during early development suggest that the synaptic role identified at the adult NMJ is not an entirely new function, but may represent a role that is already established during synaptic development.

## 6 Looking ahead: from synaptic assembly to neuronal resilience

If Unc-5 heterogeneity is actively established during development rather than arising stochastically, this raises the question of which molecular programmes drive this process and whether the same programmes act to maintain it later in life. The link between Unc-5 and the synaptic release machinery observed here offers one entry point into this question, but leaves open how AZ identity, once established, is preserved or lost as synapses age. Spontaneous neurotransmitter release and synaptic structure are known to be maintained together into adulthood [2], suggesting that the developmental programmes uncovered here may extend beyond synapse formation to synapse maintenance. This provides a basis for further investigating the roles and interactions of these proteins. Understanding how synapses acquire their identity may therefore be a necessary first step toward understanding how and why they lose it.

## 7 Acknowledgments

I would like to thank my supervisor, Samuel Vernon, for his guidance, patience, and for teaching me the techniques used throughout this project. I am also grateful to Professor Brian McCabe and the members of the McCabe Laboratory for welcoming me into the team, and to the Laidlaw Scholars Program for this opportunity.

## 8 References

1. Vernon, S. W., Ruchti, E., Paolantoni, C., Smith, R. C., Van Campenhoudt, M. & McCabe, B. D. (2026). *Spontaneous neurotransmitter release is regulated by Unc-5*. bioRxiv. doi:10.64898/2026.06.02.729526.
2. Banerjee, S., Vernon, S., Jiao, W. et al. (2021). *Miniature neurotransmission is required to maintain Drosophila synaptic structures during ageing*. **Nature Communications** **12**, 4399. doi:10.1038/s41467-021-24490-1.
3. Keleman, K. & Dickson, B. J. (2001). *Short- and long-range repulsion by the Drosophila Unc5 Netrin receptor*. **Neuron** **32**, 605–617. doi:10.1016/S0896-6273(01)00505-0.
4. Broadie, K. S. & Bate, M. (1993). *Development of the embryonic neuromuscular synapse of Drosophila melanogaster*. **Journal of Neuroscience** **13**, 144–166. doi:10.1523/JNEUROSCI.13-01-0144.1993.
5. Andrae, L. C. & Burrone, J. (2018). *The role of spontaneous neurotransmission in synapse and circuit development*. **Journal of Neuroscience Research** **96**, 354–359. doi:10.1002/jnr.24154.
6. Cunningham, K. L., Sauvola, C. W., Tavana, S. & Littleton, J. T. (2022). *Regulation of presynaptic Ca<sup>2+</sup> channel abundance at active zones through a balance of delivery and turnover*. **eLife** **11**, e78648. doi:10.7554/eLife.78648.
7. Wagh, D. A., Rasse, T. M., Asan, E. 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. doi:10.1016/j.neuron.2006.02.008.
8. Manders, E. M. M., Verbeek, F. J. & Aten, J. A. (1993). *Measurement of co-localization of objects in dual-colour confocal images*. **Journal of Microscopy** **169**, 375–382. doi:10.1111/j.1365-2818.1993.tb03313.x.