Trans-synaptic Teneurin Signalling in Neuromuscular Synapse Organization and Target Choice

Timothy J. Mosca, Weizhe Hong, Vardhan S. Dani, Vincenzo Favaloro & Liquin Luo

Synapse assembly requires trans-synaptic signals between the pre- and postsynapse, but our understanding of the essential organizational molecules involved in this process remains incomplete. Teneurin proteins are conserved, epidermal growth factor (EGF)–repeat–containing transmembrane proteins with large extracellular domains. Here we show that two Drosophila Teneurins, Ten-m and Ten-a, are required for neuromuscular synapse organization and target selection. Ten-a is presynaptic whereas Ten-m is mostly postsynaptic; neuronal Ten-a and muscle Ten-m form a complex in vivo. Pre- or postsynaptic Teneurin perturbations cause severe synapse loss and impair many facets of organization trans-synthetically and cell autonomously. These include defects in active zone apposition, release sites, membrane and vesicle organization, and synaptic transmission. Moreover, the presynaptic microtubule and postsynaptic spectrin cytoskeletons are severely disrupted, suggesting a mechanism whereby Teneurins organize the cytoskeleton, which in turn affects other aspects of synapse development. Supporting this, Ten-m physically interacts with α-Spectrin. Genetic analyses of teneurin and neuroligin reveal that they have differential roles that synergize to promote synapse assembly. Finally, at elevated endogenous levels, Ten-m regulates target selection between specific motor neurons and muscles. Our study identifies the Teneurins as a key bi-directional trans-synaptic signal involved in general synapse organization, and demonstrates that proteins such as these can also regulate target selection.

Vertebrate teneurins are enriched in the developing brain, localize to synapses in culture, and pattern visual connections. Both Drosophila Teneurins, Ten-m and Ten-a, function in olfactory synaptic partner matching and were further identified in neuromuscular junction (NMJ) defect screens with Ten-m also affecting motor axon guidance. We examine their roles and the underlying mechanisms involved in synapse development.

Both Ten-m and Ten-a were enriched at the larval NMJ (Fig. 1a and Supplementary Fig. 1a). Ten-a was detected at neuronal membranes: this staining was undetectable beyond background in ten-a null mutants (Supplementary Fig. 1b) and barely detectable after neuronal ten-a RNA interference (RNAi; Supplementary Fig. 1c), indicating that Ten-a is predominantly presynaptic. Partial co-localization was observed between Ten-a and the periactive zone marker Fasciclin 2 (ref. 12) as well as the active zone marker Bruchpilot (Fig. 1b, c), suggesting that Ten-a is localized to the junction between the periactive zone and the active zone. Ten-m appeared strongly postsynaptic and surrounded each bouton (Fig. 1a and Supplementary Fig. 1a, d). Muscle-specific ten-m RNAi eliminated the postsynaptic staining, but uncovered weak presynaptic staining (Supplementary Fig. 1e) that ubiquitously ten-m RNAi eliminated (Supplementary Fig. 1f). Thus, the Ten-m signal was specific and, although partly presynaptic, enriched postsynaptically. Consistently, muscle Ten-m colocalized extensively with Dlg (Fig. 1d) and completely with α-Spectrin (Fig. 1e) and is thus probably coincident with all postsynaptic membranes.

The localization of Ten-a and Ten-m suggested their trans-synaptic interaction. To examine this, we co-expressed Myc–tagged Ten-a in nerves using the Q system and haemagglutinin (HA)-tagged Ten-m in muscles using GAL4. Muscle Ten-m was able to co-immunoprecipitate nerve Ten-a from larval synaptosomes (Fig. 1f), suggesting that the Teneurins form a heterophilic trans-synaptic receptor pair at the NMJ.

To determine Teneurin function at the NMJ, we examined the ten-a null allele and larvae with neuron or muscle RNAi of ten-a and/or ten-m. Following such perturbations, bouton number and size were altered: the quantity was reduced by 55% (Fig. 2a–c, g and Supplementary Fig. 2).

Figure 1 | Teneurins are enriched at and interact across Drosophila neuromuscular synapses. a–e, Representative single confocal sections of synaptic boutons stained with antibodies against Ten-a (red) or Ten-m (green), horseradish peroxidase (HRP) to mark the neuronal membrane (blue), and a synaptic marker as indicated. α-Spectrin (f), Immunoblots (IB) of larval synaptosomes expressing neuronal Flag–Myc–tagged Ten-a (N) and muscle Flag–HA–tagged Ten-m (M) and immunoprecipitated (IP) using antibodies to HA. Ten-a is detected in the pull-down, indicating that nerve Ten-a and muscle Ten-m interact across the NMJ. This is not seen in control lanes. Owing to low expression, neither transgene product is detectable in input lysates, which are enriched in Bnp. Scale bar, 5 μm.

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and the incidence of large boutons markedly increased (Supplementary Fig. 2k). Both changes indicate impaired synaptic morphogenesis. The reduction in bouton number was probably cumulative through development, as it was visible in first instar ten-a mutants and persisted (Supplementary Fig. 2k). In the ten-a mutant, bouton morphogenesis was rescued by restoring Ten-a expression in neurons, but not muscles (Fig. 2d, g and Supplementary Fig. 2). Neuronal Ten-m overexpression could not substitute for the lack of Ten-a, revealing their non-equivalence (Supplementary Fig. 2e, l). Neuronal knockdown of Ten-a or Ten-m resulted in fewer synaptic boutons (Supplementary Fig. 2f–h, l), indicating that both have a presynaptic function, although presynaptic Ten-a has a more predominant role (Supplementary Fig. 2l). Moreover, knocking down postsynaptic Ten-m in the ten-a mutant did not enhance the phenotype (Fig. 2g). Thus, presynaptic Ten-a (and, to a lesser extent, Ten-m) and postsynaptic Ten-m are required for synapse development.

ten-a perturbation also caused defects in the apposition between presynaptic active zones (release sites) and postsynaptic glutamate receptor clusters (Supplementary Fig. 2h–k). Under electron microscopy, active zones are marked by electron-dense membranes and single presynaptic specializations called T-bars (Fig. 2i), which enable synapse assembly, vesicle release and Ca^{2+}-channel clustering. Teneurin disruption causes defects (Fig. 2m–r and Supplementary Fig. 3) in T-bar ultrastructure (Fig. 2m–o), membrane ruffling (Fig. 2p, q), and apposition to contractile tissue (Fig. 2p, q). Ten-a perturbation also impaired postsynaptic vesicle populations similarly required Teneurins for clustering at the bouton perimeter and proper density (Supplementary Fig. 4) further indicating organizational deficiency. These phenotypes resemble mutants with adhesion and T-bar biogenesis defects, suggesting a role for Teneurins in synaptic adhesion and stability. Synaptic vesicle populations similarly required Teneurins for clustering at the bouton perimeter and proper density (Supplementary Fig. 4). As these effects are not synchronous with active zone disruption, Teneurins are also required for synaptic vesicle organization.

Synapses lacking teneurin were also functionally impaired. The mean amplitude of evoked excitatory postsynaptic potentials (EPSPs) in larvae was decreased by 28% in the ten-a mutant (Fig. 2u, S). Spontaneous miniature EPSPs showed a 20% decrease in amplitude, a 46% decrease in frequency (Fig. 2u, S), and an altered amplitude distribution compared with control (Supplementary Fig. 5a). These defects resulted in a 20% reduction in quantal content (Fig. 2u), which could be partly due to...
fewer boutons and release sites. However, release probability may also be reduced, as suggested by an increased paired pulse ratio in ten-a mutants (Supplementary Fig. 5d, e). The decay kinetics of responses were faster in ten-a mutants, suggesting additional postsynaptic effects on glutamate receptors and/or intrinsic membrane properties (Supplementary Fig. 5b, c). Further, FM1-43 dye loading revealed markedly defective vesicle cycling in ten-a mutants (Supplementary Fig. 5f, h). Consistent with physiological impairment, teneurin-perturbed larvae exhibited profound locomotor defects (Supplementary Fig. 5i). In summary, Teneurins are required for multiple aspects of NMJ organization and function.

As a potential mechanism for synaptic disorganization following teneurin perturbation, we examined the pre- and postsynaptic cytoskeletons. In the presynaptic terminal, organized microtubules contain Futsch (a microtubule-binding protein)-positive ‘loops’, whereas disorganized microtubules possess punctate, ‘unbundled’ Futsch[9]. Each classification normally represented ~10% (often distal) of boutons (Fig. 3a, d and Supplementary Fig. 6). Upon teneurin perturbation, many more boutons had unbundled Futsch (Fig. 3b, c and Supplementary Fig. 6) whereas those with looped microtubules were decreased by 62–95% (Fig. 3d). Therefore, proper microtubule organization requires pre- and postsynaptic Teneurins. In contrast to mild active zone/glutamate receptor apposition defects, most boutons displayed microtubule organizational defects.

Teneurin perturbation also severely disrupted the postsynaptic spectrin cytoskeleton, with which Ten-m co-localized (Fig. 1e). Postsynaptic α-Spectrin normally surrounds the bouton (Fig. 3e). Perturbing neuronal or muscle Teneurins markedly reduced postsynaptic α-Spectrin without affecting Dlg (Fig. 3f–h and Supplementary Fig. 7). Postsynaptic β-Spectrin[7], Adducin[9] and Wsp were similarly affected (Supplementary Fig. 8). In muscle, α-Spectrin is coincident with and essential for the integrity of the membranous subsynaptic reticulum (SSR)[21,23]. Consistent with this, teneurin disruption reduced SSR width up to 70% (Supplementary Fig. 9d–g) and increased the frequency of ‘ghost’ boutons, which are failures of postsynaptic membrane organization[23].

Figure 3 | Teneurin perturbation results in marked cytoskeletal disorganization. a–c, Representative NMJs stained with antibodies to Futsch (green) and HRP (magenta). Arrowheads indicate looped organization. Arrows indicate unbundled Futsch. d, Quantification of the percentage of total boutons with looped or unbundled microtubules. e–g, Representative NMJs stained with antibodies to α-Spectrin (green), Dlg (red) and HRP (blue). Following teneurin perturbation, α-Spectrin staining is largely lost. Axonal α-Spectrin is unaffected by muscle teneurin RNAi (f). h, Quantification of α-Spectrin (green) and Dlg (red) fluorescence. A.U., arbitrary units. For all genotypes, n = 6 larvae, 12 NMJs. i, Immunoblots (IB) showing that α-Spectrin is detected in the Flag immunoprecipitates (IP) of larvae expressing muscle Flag-HA-tagged Ten-m but not in control larvae. Owing to low expression, Flag–HA–Ten-m is only detectable after enrichment by immunoprecipitation. j, Model showing the roles of Teneurins, Neurexin and Neuroligin at the NMJ. Arrow size represents the relative contribution of each pathway to the cellular process as inferred from mutant phenotypic severity. Scale bars, 5 μm. ***P < 0.001, NS, not significant.
function in the same pathway. Our finding that Ten-a and Ten-m co-immunoprecipitate from different cells in vitro\(^1\) and across the NMJ in vivo (Fig. 1f) further suggests a signal via a trans-synaptic complex. Teneurin function, however, may not be solely trans-synaptic. In some cases (vesicle density, SSR width), cell-autonomous knockdown resulted in stronger phenotypes than knocking down in synaptic partners (Supplementary Figs 3, 4, 9 and Supplementary Table 1). This suggests additional cell-autonomous roles in addition to trans-synaptic Teneurin signalling.

Signalling involving the transmembrane proteins Neurexin and Neuroligin also mediates synapse development. In Drosophila, Neurexin (nrx) and Neuroligin 1 (nlg1) mutations cause phenotypes similar to teneurin perturbation: reductions in bouton number, active zone organization, transmission, and SSR width. \(^{27,28}\) nlg1 and nrx mutations do not enhance each other, suggesting that they function in the same pathway.\(^{28}\) Consistently, we found that nrx and nlg1 mutants exhibited largely similar phenotypes (data not shown). To investigate the relationship between the teneurin genes and nrx and nlg1, we focused on the nlg1 null mutant. Both Nlg1 tagged with enhanced green fluorescent protein (Nlg1–eGFP) and endogenous Ten-m occupied a similar postsynaptic space (Supplementary Fig. 10a). Teneurin and nlg1 loss-of-function mutations also displayed similar bouton number reductions (Fig. 2e, g), vesicle disorganization (Supplementary Fig. 4), and ghost bouton frequencies (Supplementary Fig. 9). Other phenotypes showed notable differences in severity. In nlg1 mutants, there was a 29% failure of active zone/glutamate receptor apposition (Fig. 2k and Supplementary Fig. 10d), compared to 15% for the strongest teneurin perturbation. The cytoskeleton of nlg1 mutants, however, was only mildly impaired compared to that seen with teneurin perturbations (Fig. 3d, h and Supplementary Figs 6 and 7).

To examine further the interplay of teneurin and nlg1, we analysed ten-a nlg1 double mutants. Both single mutants were viable, despite their synaptic defects. Double mutants, however, were larval lethal. We obtained rare escapers, which showed a 72% reduction in boutons, compared to a 50–55% decrease in single mutants (Fig. 2e). Active zone apposition in double mutants was enhanced synergistically over either single mutant (Fig. 2k and Supplementary Fig. 10e). Cytoskeletal defects in the double mutant resembled the ten-a mutant (Fig. 3 and Supplementary Figs 6 and 7). These data suggest that teneurin genes and nrx and nlg1 act in partially overlapping pathways, cooperating to organize synapses properly, with Teneurins contributing more to cytoskeletal organization and Neurexin and Neuroligin to active zone apposition (Fig. 3).

In the accompanying manuscript, we showed that although the basal Teneurins are broadly expressed in the Drosophila antennal lobe, elevated expression in select glomeruli mediates olfactory neuron partner matching. At the NMJ, this basal level mediates synapse organization. Analogous to the antennal lobe, we found elevated ten-m expression at muscles 3 and 8 using the ten-m-GAL4 enhancer trap (Fig. 4a). We confirmed this for endogenous ten-m, and determined that it was contributed by elevated Ten-m expression in both nerves and muscles (Fig. 4b–g). Indeed, ten-m-GAL4 was highly expressed in select motor neurons, including MN3-1b, which innervates muscle 3 (ref. 29; Supplementary Fig. 11c). This elevated larval expression also varied along the anterior–posterior axis (Supplementary Fig. 12), and was specific for Ten-m, as Ten-a expression did not differ within or between segments (data not shown).

To test whether elevated Ten-m expression in muscle 3 and MN3-1b affects neuromuscular connectivity, we expressed ten-m RNAi using ten-m-GAL4. Wild-type muscle 3 was almost always innervated (Fig. 4h). However, after ten-m knockdown, muscle 3 innervation failed in 11% of hemisegments (Fig. 4i, j). This required Ten-m on both sides of the synapse, as the targeting phenotype persisted following neuronal or muscle RNAi suppression using tissue-specific GAL80 transgenes (Fig. 4j). ten-a RNAi did not show this phenotype (Fig. 4j), consistent with homophilic target selection via Ten-m. The phenotype

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**Figure 4** High-level Ten-m expression regulates muscle target selection. a, Representative images of hemisegment A3 stained with antibodies to Dlg (blue), phalloidin (red), and expressing GFP via ten-m-GAL4 (green). High-level expression is observed in muscles 3 and 8 and basally in all muscles. b, c, Muscle 3 (b) and 4 (c) NMJs show differential Ten-m (red) but similar Synaptotagmin 1 (Syt1; green) expression (from a ten-m muscle knockdown animal). d, Quantification of presynaptic Ten-m (red) and Syt1 (green) fluorescence at muscle 3 and 4 NMJs. MN, motor neuron. e, f, NMJs at muscles 3 (e) and 4 (f) show differential Ten-m (red) but similar Syt1 (green) expression in muscles (from a ten-m nerve knockdown). g, Quantification of postsynaptic Ten-m (red) and Syt1 (green) fluorescence at muscle 3 and 4 NMJs. h, i, Representative images stained with phalloidin (blue) and antibodies to HRP (green) and Dlg (red) to visualize motor neurons and muscles in control (h) or ten-m-GAL4 > ten-m RNAi larva (i). m, Muscle 3 mutant (i). a, b, m, Quantification of the total bouton percentage on muscles 6 (blue) and 7 (red). All genotypes contain H94-GAL4; additional transgenes are indicated (for details, see Methods). The Ten-m-mediated shift is abolished by neuronal or muscle GAL80 transgenes. Scale bars, 100 µm (a), 5 µm (b–l), 10 µm (k, l). In all cases, n = 12 larvae. ***P < 0.001, **P < 0.01, NS, not significant.

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was specific to muscle 3, as innervation onto the immediately proximal or distal muscle was unchanged (Fig. 4j). The low penetrance is probably due to redundant target selection mechanisms.\(^{30}\) Where innervation did occur, the terminal displayed similarly severe phenotypes to other NMJs (not shown). Thus, in addition to generally mediating synaptic organization, Ten-m also contributes to correct target selection at a specific NMJ.

To determine whether Ten-m overexpression could alter connectivity, we expressed Ten-m in muscle 6 (but not the adjacent muscle 7), and the motor neurons innervating both muscles using H94-GAL4. Normally, 60% of the boutons at muscles 6/7 are present on muscle 6 with 40% on muscle 7 (Fig. 4k, m). Ten-m overexpression caused a shift whereby 81% of boutons synapsed onto muscle 6 and only 19% onto muscle 7 (Fig. 4l, m). This shift also required both neuronal and muscle Ten-m, as neuronal or muscle GAL80 abrogated it (Fig. 4m). The effect was specific because Ten-a overexpression did not alter this synaptic balance (Fig. 4n), nor was it secondary to altered bouton

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number, which was unchanged (data not shown). Therefore, elevated Ten-m on both sides of the NMJ can bias target choice. This, combined with evidence that Ten-m can mediate homophilic interaction in vitro, supports a trans-synaptic homophilic attraction model at the NMJ as in the olfactory system.

We identified a two-tier mechanism for Teneurin function in synapse development at the Drosophila NMJ. At the basal level, Teneurins are expressed at all synapses and engage in hetero- and homophilic bi-directional trans-synaptic signalling to organize synapses properly (Fig. 3). Supporting this, Teneurins can mediate homo- and heterophilic interactions in vitro and heterophilic interactions in vivo (Fig. 1f). At the synapse, Teneurins organize the cytoskeleton, interact with γ-spectrin, and enable proper adhesion and release site formation. Further, elevated Ten-m expression regulates target selection in specific motor neurons and muscles via homophilic matching and functions with additional molecules to mediate precise neuromuscular connectivity. Teneurin-mediated target selection at the NMJ is analogous to its role in olfactory synaptic partner matching. As Teneurins are expressed broadly throughout the antennal lobe, it remains an attractive possibility that they also regulate synapse organization in the central nervous system.

**METHODS SUMMARY**

Details of *Drosophila* stocks, immunostaining, electron microscopy, functional assays, construction of epitope-tagged Teneurin constructs, immunoprecipitation, imaging and statistical analysis can be found in Methods.

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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2. Giagtzoglou, N., Ly, C. V. & Bellen, H. J. Cell adhesion, the backbone of the synapse: homophilic interactions bi-directional trans-synaptic signalling to organize synapses properly. *Cold Spring Harb. Perspect. Biol.* **1**, a003079 (2009).
3. Young, T. R. & Leamey, C. A. Teneurins: important regulators of neural circuitry. *Int. J. Biochem. Cell Biol.* **41**, 990–993 (2009).
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METHODS

Drosophila stocks. All Drosophila strains and controls were raised at 29 °C to maximize GAL4 expression. All mutants and transgenes were maintained over GFP balancer chromosomes to enable larval selection. Mhc-GAL4 or Mef2-GAL4 (ref. 31) was used to drive expression in all somatic muscles. Nnr2-GAL4 (ref. 32) and elav-GAL4 (ref. 33) were used to drive expression in all neurons. H94-GAL4 was used to drive expression in muscles 6, 13 and 4 and their corresponding (ref. 31) was used to drive expression in all somatic muscles. GAL4 described23. The following primary antibodies were used: mouse antibody to Wandering third instar larvae were processed as previously described. Phalloidin was used at 1:300. FITC-, Cy3- or Cy5-conjugated antibodies to HRP or Spectrin (mAb3A9, 1:250)47, mouse antibody to Fasciclin 2 (mAb1D4, 1:20)48, mouse antibody to Flag (M2, 1:500, Sigma-Aldrich), mouse antibody to Myc (SE10, 1:500, Santa Cruz Biotechnology), rat antibody to HA (3F10, 1:1,500, Roche). HRP-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:10,000. Blots were developed using the SuperSignal West Femto Maximum Sensitivity Substrate (ThermoScientific).

Imaging analysis. Larvae were imaged with a Zeiss LSM 510 Meta laser-scanning confocal microscope (Carl Zeiss) using either a ×63 1.4 NA or a ×4 1.0 NA objective. NMJ images were taken as confocal z-stacks with the upper and lower bounds defined by HRP staining unless otherwise noted. For all metrics, boutons were assessed in segment A3 at muscle 6/7 and muscle 4 on both the left and right sides of the larva. In mutants, intensity measurements were taken from each terminal on muscle 4. All phenotypes, however, were observed at all synapses regardless of muscle fibre or segment. For membrane organization, vesicle distribution and Teneurin colocalization, NMJ images were taken as single optical sections at the precise location of the axon as determined by HRP staining. Images were processed with the LSM software and Adobe Photoshop CS4. Bouton number, active zone/glutamate receptor apposition, fluorescent intensity and microtubule organization were quantified as previously described31. Targeting errors for each larva were quantified as the percentage of hemisegments from A1 to A7 at a single animal with a false-negative outcome. There was no difference in targeting errors based on body wall segment. Experiments using H94-GAL4 were conducted as described17, and their effects confirmed using Fasciclin 2 overexpression (control = 58.1% of boutons on muscle 6, 41.9% on muscle 7; Fas 2 overexpression = 73.0% on muscle 6, 27.0% on muscle 7; n = 8 animals for each, P < 0.0001)41.

In electron micrographs, parameters were quantified as previously described using ImageJ (NIH)32. T-bar defects were classified into one of five categories: normal (no discernible defect), double (two T-bars were observed in the same, contiguous active zone), detached (where the T-bar was clearly visible but was not explicitly connected to the membrane associated with the nearest PSD), apposed (where the T-bar was apposed to the SSR, but rather, the contractile tissue (of the muscle), misshapen (where an electron-dense T-bar was visible but did not conform to the ‘T’ shape). Often, the T-bars were ‘X’ shaped). For Fig. 2r, each defect is expressed as a percentage of the total number of T-bars observed in a particular genotype.

For Ten-m gradient calculation, single optical sections were taken through the centre of the NMJ on muscle 3 or muscle 4, as determined by HRP immunoreactivity. The GFP signal (ten-m-GAL4) or antibody signal was then measured using ImageJ (NIH). For each larva, measurements were taken on the right and left sides of each indicated segment. The fluorescence for each segment was expressed as a percentage of the fluorescence from segment A1 in the same animal, on the same side of the larva. For all larvae, segment A1 represented the maximal fluorescence. Statistical analysis. Statistical analysis used GraphPad Prism 5 (Graphpad Software). In all cases involving more than two samples, significance was calculated using ANOVA followed by a Dunnett post-hoc test to the control sample and a Bonferroni post-hoc test among all samples. For two-sample cases, an unpaired Student’s t-test was used to assess significance, unless otherwise indicated. In all cases, both methods provided similar significance measurements. In all figures, significance is with respect to control genotypes unless otherwise noted.

31. Lilly, B. et al. Requirement of MADS domain transcription factor D-MEF2 for muscle formation in Drosophila. Science 267, 688–693 (1995).
32. Sun, B., Xu, P. & Salvaterra, P. M. Dynamic visualization of nervous system in live Drosophila. Proc. Natl Acad. Sci. USA 96, 10438–10443 (1999).
33. Luo, L., Liao, Y. J., Jan, L. Y. & Jan, Y. N. Distinct morphogenetic functions of similar GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev. 8, 1787–1802 (1994).
34. Davis, G. W. & Goodman, C. S. Synapse-specific control of synaptic efficacy at the terminals of a single neuron. Nature 392, 82–86 (1998).
35. Wodarz, A., Hinz, U., Engelbert, M. & Knust, E. Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of Drosophila. Cell 82, 67–76 (1995).
36. Petersen, L. K. & Stowers, R. S. A Gateway MultiSite recombination cloning toolkit. PLoS ONE 6, e24531 (2011).
37. Loewen, C. A., Mackler, J. M. & Reist, N. E. Drosophila synaptotagmin I null mutants survive to early adulthood. Genesis 31, 30–36 (2001).
38. Dietzl, G. et al. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448, 151–156 (2007).
39. Lee, T. & Luo, L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 77, 587–598 (1994).
40. Parnas, D., Haghighi, A. P., Fetter, R. D., Kim, S. W. & Goodman, C. S. Regulation of postsynaptic structure and protein localization by the Rho-type guanine nucleotide exchange factor dPix. Nature 418, 340–344 (2002).
41. Byers, T. J., Dubreuil, R., Branton, D., Kiehart, D. P. & Goldstein, L. S. Drosophila synaptotagmin I null mutants survive to early adulthood. Genesis 31, 30–36 (2001).
42. Koh, Y. H., Popova, E., Thomas, U., Griffith, L. C. & Budnik, V. Regulation of DLG postsynaptic structure and protein localization by the Rho-type guanine nucleotide exchange factor dPix. Nature 418, 340–344 (2002).
43. Petersen, L. K. & Stowers, R. S. A Gateway MultiSite recombination cloning toolkit. PLoS ONE 6, e24531 (2011).
44. Zinsmaier, K. E., Eberle, K. K., Buchner, E., Walter, N. & Benzer, S. Paralysis and early death in cysteine string protein mutants of Drosophila melanogaster. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 151–156 (2007).
45. Malenckova, G. A., Eberle, K. K., Buchner, E., Walter, N. & Benzer, S. Paralysis and early death in cysteine string protein mutants of Drosophila melanogaster. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 151–156 (2007).
46. Verstreken, P., Ohyama, T. & Bellin, H. J. FM 1-43 labeling of synaptic vesicle pools at the Drosophila neuromuscular junction. Methods Mol. Biol. 440, 349–369 (2008).
47. Verstreken, P., Ohyama, T. & Bellin, H. J. FM 1-43 labeling of synaptic vesicle pools at the Drosophila neuromuscular junction. Methods Mol. Biol. 440, 349–369 (2008).
48. Van Vactor, D., Sink, H., Fambrough, D., Tsoo, R. & Goodman, C. S. Genes that control neuromuscular specificity in Drosophila. Cell 73, 1137–1153 (1993).
49. O’Neill, E. M., Rebay, I., Tjian, R. & Rubin, G. M. The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. Cell 78, 137–147 (1994).
50. Patel, N. H., Schaffer, B., Goodman, C. S. & Holmgren, R. The role of segment polarity genes during Drosophila neurogenesis. Genes Dev. 3, 690–904 (1989).
51. Byers, T. J., Husain-Chishti, A., Dubreuil, R. R., Branton, D. & Goldstein, L. S. Sequence similarity of the amino-terminal domain of Drosophila beta spectrin to alpha actinin and dystrophin. J. Cell Biol. 109, 1633–1641 (1989).
52. Ben-Yaacov, S., Le Borgne, R., Abramson, I., Schweisguth, F. & Schejter, E. D. Wasp, the Drosophila Wiskott-Aldrich syndrome gene homologue, is required for cell fate decisions mediated by Notch signaling. J. Cell Biol. 152, 1–13 (2001).
53. Stewart, B. A., Atwood, H. L., Renger, J. J., Wang, J. & Wu, C. F. Improved stability of Drosophila larval neuromuscular preparations in haemolymph-like physiological solutions. J. Comp. Physiol. A Neuroethol. Physiol. 175, 179–191 (1994).
54. Martin, A. R. A further study of the statistical composition on the end-plate potential. J. Physiol. (Lond.) 130, 114–122 (1955).
55. Lennicka, G. A., Spencer, G. M. & Keshishian, H. Effect of reduced impulse activity on the development of identified motor terminals in Drosophila larvae. J. Neurobiol. 54, 337–345 (2003).
56. Verstreken, P., Ohyama, T. & Bellin, H. J. FM 1-43 labeling of synaptic vesicle pools at the Drosophila neuromuscular junction. Methods Mol. Biol. 440, 349–369 (2008).
57. Markstein, M., Pitsouli, C., Villalta, C., Celniker, S. E. & Perrimon, N. Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. Nature Genet. 40, 476–483 (2008).
58. Higashi-Kovtun, M. E., Mosca, T. J., Dickman, D. K., Meinertzhagen, I. A. & Schwarz, T. L. Importin-β11 regulates synaptic phosphorylated mothers against decapentaplegic, and thereby influences synaptic development and function at the Drosophila neuromuscular junction. J. Neurosci. 30, 5253–5268 (2010).