Development/Plasticity/Repair

Structural and Functional Synaptic Plasticity Induced by Convergent Synapse Loss in the Drosophila Neuromuscular Circuit

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Throughout the nervous system, the convergence of two or more presynaptic inputs on a target cell is commonly observed. The question we ask here is to what extent converging inputs influence each other’s structural and functional synaptic plasticity. In complex circuits, isolating individual inputs is difficult because postsynaptic cells can receive thousands of inputs. An ideal model to address this question is the Drosophila larval neuromuscular junction (NMJ) where each postsynaptic muscle cell receives inputs from two glutamatergic types of motor neurons (MNs), known as 1b and 1s MNs. Notably, each muscle is unique and receives input from a different combination of 1b and 1s MNs; we surveyed multiple muscles for this reason. Here, we identified a cell-specific promoter that allows ablation of 1s MNs postinnervation and measured structural and functional responses of convergent 1b NMJs using microscopy and electrophysiology. For all muscles examined in both sexes, ablation of 1s MNs resulted in NMJ expansion and increased spontaneous neurotransmitter release at corresponding 1b NMJs. This demonstrates that 1b NMJs can compensate for the loss of convergent 1s MNs. However, only a subset of 1b NMJs showed compensatory evoked neurotransmission, suggesting target-specific plasticity. Silencing 1s MNs led to similar plasticity at 1b NMJs, suggesting that evoked neurotransmission from 1s MNs contributes to 1b synaptic plasticity. Finally, we genetically blocked 1s innervation in male larvae and robust 1b synaptic plasticity was eliminated, raising the possibility that 1s NMJ formation is required to set up a reference for subsequent synaptic perturbations.

Key words: co-innervation; compensation; neuromuscular junction; neurotransmission; plasticity; synaptic growth

Significance Statement

In complex neural circuits, multiple convergent inputs contribute to the activity of the target cell, but whether synaptic plasticity exists among these inputs has not been thoroughly explored. In this study, we examined synaptic plasticity in the structurally and functionally tractable Drosophila larval neuromuscular system. In this convergent circuit, each muscle is innervated by a unique pair of motor neurons. Removal of one neuron after innervation causes the adjacent neuron to increase neuromuscular junction outgrowth and functional output. However, this is not a general feature as each motor neuron differentially compensates. Further, robust compensation requires initial coinnervation by both neurons. Understanding how neurons respond to perturbations in adjacent neurons will provide insight into nervous system plasticity in both healthy and disease states.

Received June 11, 2020; revised Nov. 28, 2020; accepted Dec. 17, 2020.


Stocks obtained from the Bloomington Drosophila Stock Center (National Institutes of Health [NIH] Grant P40-OD-018537) were used in this study. The monoclonal antibodies 3C10, 4F3, and 8B4D2 were deposited to the Developmental Studies Hybridoma Bank (DSHB) by C. Goodman, while the ncl2 antibody was deposited by E. Buchner and were obtained from the DSHB, created by the Eunice Kennedy Shriver National Institute of Child Health and Development of the NIH and maintained at The University of Iowa, Department of Biology (Iowa City, IA). This work was supported by NIH Grants R01-NS-102342 (to R.A.C.) and T32-GM-007183 (to M. L.-R.), funding from the University of Chicago Biological Sciences Division (BSO) International Student Fellowship (to Y.W.), and funding from the BSD Office of Diversity and Inclusion (to R.A.C.) and the Grossman Institute for Neuroscience, Quantitative Biology and Human Behavior (to R.A.C.). We thank Edwin “Chip” Ferguson, Ellie Heckscher, Kai Zinn, Kai Wang, Hong Liu, Richard Fehon, David Pincus, and members of the Carrillo laboratory for helpful discussions and comments. We also thank Vyta Bindokas for helpful guidance for Imaris analysis. The authors declare no competing financial interests.

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https://doi.org/10.1523/JNEUROSCI.1492-20.2020

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Introduction

The nervous system is characterized by complex wiring patterns that include different neurons converging onto the same postsynaptic cell. This wiring paradigm is found in pyramidal neurons that receive input from excitatory and inhibitory contacts (Megías et al., 2001), and in esophageal striated muscles that receive enteric and vagal nerve inputs (Kallmünzer et al., 2008; Neuhuber and Wörl, 2016). While dynamic regulation of individual synapses has been examined (Berry and Nedivi, 2017; Kruijssen and Wierenga, 2019), interplay between convergent neurons has been predominantly studied by monitoring postsynaptic spine changes (Oh et al., 2015; Hedrick et al., 2016; Jungenitz et al., 2018; Chistiakova et al., 2019). Understanding how convergent neurons respond to perturbations will shed light on the etiologies of neurodegenerative disorders, such as amyotrophic lateral sclerosis, which display progressive neuronal cell death and devastating functional consequences (Cluskey and Ramsden, 2001).

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Figure 1. 1b and 1s MNs differentially contribute to the total EPSPs. A, Schematic of the innervation pattern of a subset of 1s MNs (vCE: dark green; dCE: light green) and 1b MNs (m6-1b: rust; m12-1b: orange; m4-1b: peach). Muscles analyzed in this study are marked by the red boxes. B, Representative frames showing the baseline fluorescence (top), 1b + 1s firing event (middle), and 1b alone firing event (bottom) of m6 in MHK-CDB:eGFP::Shi larvae (1b: red; 1s: blue; also see Movie 1). C–E, Representative EPSP traces of 1b + 1s and 1b alone on m6 (C), m12 (D), and m4 (E). Traces and graphs are color coded as indicated in the color key. F–H, Paired EPSP amplitudes of m6 (F, t_{14}=18.60, p < 0.0001, paired t test), m12 (G, t_{14}=15.73, p < 0.0001, paired t test), and m4 (H, t_{14}=7.43, p < 0.0001, paired t test). I, Calculated EPSP ratios of 1b/1b + 1s of m6, m12, and m4 (t_{14}=26.03, p < 0.0001, one-way ANOVA, Tukey post hoc test). Error bars indicate ± SEM **** p < 0.0001. n values (NMJs/larva) are 15/12, 15/12, and 16/15, respectively.
including facilitation and depression (Hallermann et al., 2010). As each muscle is coinervated and individual synaptic activities can be distinguished, this system provides an ideal platform to investigate structural and physiological plasticity changes that enable one MN to compensate for perturbations of a convergent MN. A recent study found that ablating the 1s MN on muscle 1 (m1) elevated evoked neurotransmission of the corresponding 1b NMJ (Aponte-Santiago et al., 2020). However, each muscle is innervated by a unique 1b–1s MN pair, so it is unclear whether this plasticity is consistent across all muscles.

### Materials and Methods

**Fly and antibody reagents**

Fly lines and antibodies used in this study are listed in [Tables 2 and 3](#).

**Dissection and immunocytochemistry**

Embryonic dissections were performed as previously described (Lee et al., 2009). Egg-laying chambers were set up with adult flies (15–20 females and 10–15 males) and capped with grape juice plates (3% agar, 1.3% sucrose, 25% grape juice in water). After 6 h laying periods, grape...
plates covered in embryos were collected. Embryos were staged on dou-
ble-sided tape using the autofluorescence and shape of the gut (Bownes,
1975; Hartenstein et al., 1987) under a Zeiss V20 stereoscope, and then
dechorionated with a sharpened metal probe and placed on grape juice
agar. Embryos were transferred to double-sided tape on a Superfrost
Plus slides (catalog #22–037-246, Thermo Fisher Scientific) with the dis-
ssecting area outlined by a PAP pen (catalog #195506, Research Products
International), and then covered with 0.22 μm filtered PBS (0.01 M so-
dium phosphate, 150 mM sodium chloride). Embryos were opened
with an electrolytically sharpened tungsten wire, removed from the

Figure 2. A8-GAL4 drives expression in 1s MNs and can be used to ablate 1s MNs. A, B, Representative third instar larval VNC z-sections showing ventral (A) and dorsal (B) cell bodies labeled with
GFP (green) and Eve (magenta; labels nuclei of dCE and other neurons) in A8–>GFP. Arrows indicate GFP-positive vCE and dCE cell bodies. Carets indicate other cells that express A8. Asterisks indicate two
axons exiting the VNC. C, D, 3D representations of A8-expressing neurons in the VNC viewed from lateral (C) and dorsal (D) sides (same VNC as A and B). Arrow in C shows a ventral cell (left, green) that
projects an axon to the dorsal midline. Arrowhead in D shows an ipsilateral projection from a dorsal cell. Heat map colors are as follows: red denotes the dorsal-most region; and blue denotes the ventral
most region. Asterisks indicate axons exiting the VNC similar to B. E, F, Representative third instar larval abdominal hemisegment labeled with GFP (green) and the postsynaptic marker DLG (magenta) in
A8–>GFP. Ventral muscles (m6, m7, m13, and m12) innervated by vCE (E) and dorsal muscles (m4, m3, m2 and m1) innervated by dCE (F). Arrows indicate 1s NMJs and arrowheads indicate 1b NMJs.
G, H, Representative third instar larval VNCs lacking vCE (G) and dCE (H) labeled with GFP (green) and Eve (magenta) in A8–>GFP;hid,rpr. Dashed circles mark the absence of vCE and dCE. Note that both
vCE and dCE cell bodies are ablated by the third instar stage. Asterisk marks a GFP-positive cell that remained. I, J, Representative third instar larval abdominal hemisegment showing ventral muscles (I)
and dorsal muscles (J), labeled with GFP (green) and DLG (magenta) in A8–>GFP;hid,rpr. Note that all NMJs from vCE and dCE are absent (no GFP). Arrowheads indicate 1b NMJs.
vitellin membrane, and then adhered to the charged slide. Dissected embryos were washed once with PBS, and then fixed for 30 min at room temperature using 4% paraformaldehyde (Electron Microscopy Sciences). Samples were then washed three times in 0.05% PBST (PBS with 0.05% Triton X-100), and then blocked for 1 h in 5% normal goat serum (5% goat serum diluted in 0.05% PBST). Samples were incubated in primary antibody solutions overnight at 4°C and washed three times in PBST. Samples were then incubated with secondary antibody solutions at room temperature for 2 h and washed three times with PBST. Finally, samples were mounted in Vectashield (Vector Laboratories), and the coverslip was sealed with nail polish.

First and third instar larval dissections were performed as previously described (Ashley et al., 2019). Wandering third instar larvae were dissected in PBS on Sylgard-184 (Dow) dishes and pinned down using sharpened 0.1 mm insect pins (catalog #26002–10, Fine Science Tools). For first instar larvae, electrolytically sharpened tungsten pins were used to accommodate the size of smaller larvae. Samples were then fixed for 30 min using 4% paraformaldehyde or 5 min in Bouin’s solution for glutamate receptor IIA (GluRIIA) and then transferred to 0.5 ml Eppendorf tubes. Samples were blocked and treated with primary and secondary antibodies as the embryo samples described above. All larval washes and antibody incubations were performed with mild agitation on a nutator.

Image acquisition
All imaging was acquired on a Zeiss LSM800 confocal microscope with either a 20× plan-apo 0.8 numerical aperture (NA) objective, a 40× plan-neofluor 1.3 NA objective, or a 63× plan-apo 1.4 NA objective. Laser

**Figure 3.** *A8>*hid,rpr* -induced cell death occurs after 1s innervation. **A–L,** Representative images depicting the presence or absence of vCE and dCE cell bodies from embryonic stage 15 (**A–D**), stage 17 (**E–H**), and early first instar (**I–L**) larval VNCs labeled with GFP (green), Eve (magenta), and Fasciclin 2 (blue) in control (**A8>*GFP**), and 1s-ablated (**A8>*GFP,hid,rpr**). Arrows and asterisks indicate the cells expressing or not expressing A8, respectively. **A, B,** A8 expression begins at embryonic stage 15 in **A8>*GFP,hid,rpr,** vCEs, and dCEs undergo apoptosis starting at embryonic stage 17 (**G, H**) noted by the loss of Eve staining in dCEs and GFP-positive debris (indicated by arrowhead), and are completely absent in early first instar larvae (**K, L**). **M, N,** Representative 1s NMJs labeled with GFP (green) and a muscle marker, phalloidin (magenta), in control (**M**) and 1s-ablated early first instar larvae (**N**). Note that 1s NMJs are labeled by GFP in control animals, and some 1s NMJs are still present in **A8>*GFP,hid,rpr** animals, suggesting that *A8>*hid,rpr- induced cell death happens after 1s NMJ formation. **O,** Innervation frequency of 1s MNs in control and 1s-ablated late first instar larvae. Three muscles (m6, m12, and m4) were pooled and analyzed together. All 1s NMJs were eliminated in 1s-ablated animals by this stage. n values (NMJs/larva) are 76/5 and 86/5 for **O**.

**Figure 3.** *A8>*hid,rpr* -induced cell death occurs after 1s innervation. **A–L,** Representative images depicting the presence or absence of vCE and dCE cell bodies from embryonic stage 15 (**A–D**), stage 17 (**E–H**), and early first instar (**I–L**) larval VNCs labeled with GFP (green), Eve (magenta), and Fasciclin 2 (blue) in control (**A8>*GFP**), and 1s-ablated (**A8>*GFP,hid,rpr**). Arrows and asterisks indicate the cells expressing or not expressing A8, respectively. **A, B,** A8 expression begins at embryonic stage 15 in **A8>*GFP,hid,rpr,** vCEs, and dCEs undergo apoptosis starting at embryonic stage 17 (**G, H**) noted by the loss of Eve staining in dCEs and GFP-positive debris (indicated by arrowhead), and are completely absent in early first instar larvae (**K, L**). **M, N,** Representative 1s NMJs labeled with GFP (green) and a muscle marker, phalloidin (magenta), in control (**M**) and 1s-ablated early first instar larvae (**N**). Note that 1s NMJs are labeled by GFP in control animals, and some 1s NMJs are still present in **A8>*GFP,hid,rpr** animals, suggesting that *A8>*hid,rpr- induced cell death happens after 1s NMJ formation. **O,** Innervation frequency of 1s MNs in control and 1s-ablated late first instar larvae. Three muscles (m6, m12, and m4) were pooled and analyzed together. All 1s NMJs were eliminated in 1s-ablated animals by this stage. n values (NMJs/larva) are 76/5 and 86/5 for **O**.
intensity, pinhole, and gain were adjusted to increase the signal but avoid overexposure. All samples from the same experiment were imaged under identical conditions. Representative images are the maximum projection of the corresponding confocal z-stack (Image).

**Image analysis**

Dorsal common exciters and ventral common exciters identification. The existence of ventral common exciters (vCE) and dorsal common exciters (dCE) MNs (two 1s type MNs) was confirmed in embryos and first instars. dCEs were identified by the expression of GFP, the transcription factor Even-skipped (Eve), and their positions in the ventral nerve cord (VNC). vCEs were identified by the expression of GFP and their positions in the VNC. Final confirmation was done by identifying the muscle innervation patterns in larval abdominal hemisegments.

1b bouton counting. 1b bouton counting was performed in third instar samples. Boutons were examined using HRP and scored as 1s or 1b based on the Discs large (DLG) signal, as 1s boutons have a smaller and dimmer DLG signal than 1b boutons (Guan et al., 1996). Satellite boutons were identified as small bud-like structures emerging from “parent” boutons (Lee and Wu, 2010).

**Bruchpilot and GluRIIA quantification.** Bruchpilot (BRP) and GluRIIA signals at 1b NMJs were quantified in a manner similar to that described in the study by Han et al. (2020). Briefly, BRP and GluRIIA in 1s NMJs were excluded in controls by using the GFP signal (1b>GFP) to create a surface boundary (Imaris, Oxford Instruments) and setting the BRP and GluRIIA signal intensities inside 1s NMJs to zero. Next, a 1b NMJ surface was created based on the masked BRP, or GluRIIA, channel, and the total intensities of horseradish peroxidase (HRP), DLG, and BRP or GluRIIA were collected. For normalization, 1b>A8>hid,prr and control samples were normalized to corresponding mean control values. For BRP/HRP and GluRIIA/DLG ratios, each sample was normalized to the sum intensity of either HRP or DLG, respectively.

**Electrophysiology and analysis**

Current-clamp recordings were performed as previously described (Meng et al., 2020). Third instar larvae were dissected in modified HL3 saline (70 mM NaCl, 5 mM KCl, 10 mM MgCl2, 10 mM NaHCO3, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES) with 0.3 mM calcium. Segmental nerves were cut near the ventral nerve cord to remove VNC input and then the larval fillet was perfused with modified HL3 saline containing 0.5 mM calcium. Body-wall muscle 6, 12, or 4 in abdominal segment 3 were impaled with a 15–30 MΩ sharp electrode filled with 3 M KCl and recorded for miniature EPSPs (mEPSPs) for 2 minutes. All mEPSP amplitudes from the same genotype were pooled together and binned with at 0.01 mV increments to calculate the cumulative probability.

Nerves were drawn into a suction electrode and stimulated to elicit EPSPs. Specifically, for muscle 6 and 12 EPSP recordings, the whole segmental nerve bundle was stimulated, whereas for muscle 4 EPSP recording, the intersegmental nerve (ISN) above muscle 5 was stimulated. For each muscle, 24 EPSPs were elicited at 0.2 Hz and the largest 12 EPSPs were averaged to indicate the mean EPSP (while measuring EPSPs in A8>A8>hid,prr, we sometimes observed smaller EPSPs, similar to those reported by Lenuicka and Keshishian (2000)). All resting potentials are reported in Table 4.

Because of nonlinear summation of quantal units of large EPSPs, we calculated corrected EPSPs using the equation defined by Martin (1955) and elaborated by Feeney et al. (1998). Quantal content (QC) was then calculated by dividing the corrected mean EPSP amplitude by the mean mEPSP amplitude for each muscle. Specifically, in genotypes where 1b and 1s NMJs were both present, the mean of the smaller two-thirds of the mEPSP amplitudes was used to represent the 1b-derived mEPSP. This assumption was based on the published spontaneous frequencies of 1b and 1s NMJs (Newman et al., 2017). Then the 1b-derived EPSP was divided by the 1b-derived mEPSP amplitude to estimate the 1b-derived QC. Similarly, the 1b + 1s EPSP was divided by the overall mEPSP amplitude to represent 1b + 1s QC. Finally, the 1b-derived QC was normalized to the 1b + 1s QC to indicate the estimated 1b baseline QC (1b QC/1b + 1s QC).

Paired-pulse recordings were performed under two-electrode voltage-clamp configuration (Li et al., 2018) in modified HL3 saline with 0.5 mM calcium. A second sharp electrode with 10–15 MΩ resistance was used to inject current. Muscle 6 was clamped at −70 mV, and the nerve was stimulated by two pulses 20 ms apart at 0.1 Hz. Only muscles with a leak current <10 nA were subjected to analysis. Paired-pulse ratios (EPSC2/EPSC1) were calculated by dividing the second EPSC by the first EPSC.

Signals were amplified using a MultiClamp 700B amplifier ( Molecular Devices) for EPSP and mEPSP recordings or a Geneclamp reporter by Lenuicka and Keshishian (2000)). All resting potentials are reported in Table 4. **Figure 4.** 1b NMJs expand upon ablation of 1s MNs. A–F, Representative NMJ arbors (1b arbors and 1s arbors, arrowheads and arrows, respectively) of m6 (A, D), m12 (B, E), and m4 (C, F) labeled with DLG (green) and HRP (magenta) in control (1b>GFP) and 1s-ablated (1b>GFP,hid,prr) third instar larvae. Insets are 5× zoomed images of corresponding dashed regions in D–F. Satellite boutons are indicated by asterisks. Note that 1s NMJs are absent in 1s-ablated animals. Images and graphs are color coded as indicated in the color key. G, Quantification of 1b bouton number of m6 (m6>1s QC, p = 0.02, unpaired t test), m12 (m12>1s QC, p = 0.001, unpaired t test), and m4 (m4>1s QC, p = 0.001, unpaired t test) in control and 1s-ablated animals (satellite 1b boutons were not included). H, Quantification of satellite boutons of m6 (m6>1s QC, p = 0.025, unpaired t test), m12 (m12>1s QC, p = 0.0269, unpaired t test with Welch’s correction), and m4 (m4>1s QC, p = 0.0019, unpaired t test with Welch’s correction) in control and 1s-ablated animals. Error bars indicate ±SEM. *p < 0.05, **p < 0.01, ***p < 0.001. n values (NMJs/larva) are 16/8, 16/8, 15/8, 15/8, and 15/8, respectively.
Loss of 1s MNs decreases overall mEPSP amplitudes and increases 1b mEPSP frequencies. Figure 5. Representative mEPSP recordings of m6 (A), m12 (B), and m4 (C) in control (A8>GFP) and 1s-ablated (A8>GFP;hid, rpr) animals. Traces and graphs are color coded as indicated in the color key. D–F, Pooled cumulative probability distributions of m6 (D; p < 0.0001, K-S test), m12 (E; p < 0.0001, K-S test), and m4 (F; p < 0.0001, K-S test). G, Quantification of mEPSP amplitude of m6 (t (22) = 2.630, p = 0.0175, unpaired t test), m12 (t (22) = 2.294, p = 0.0317, unpaired t test), and m4 (t (22) = 3.700, p = 0.0009, unpaired t test) in control and 1s-ablated animals. Each data point represents the average mEPSP amplitude from one sample. H, Quantification of mEPSP frequencies of m6 (t (22) = 1.224, p = 0.2339, unpaired t test), m12 (t (22) = 2.331, p = 0.0291, unpaired t test), and m4 (t (22) = 0.8369, p = 0.4095, unpaired t test) in control and 1s-ablated animals. Each data point represents the average mEPSP frequency from one sample. Error bars indicate ± SEM. n = 8 per genotype, n = 10 for controls, males were also selected. All statistical analyses were performed using Prism 8 software (GraphPad). Average and SEM are reported. Outliers are determined by Q-test and excluded from the sample pools. For each data point, at least eight animals per genotype were dissected and at least two biological replicates were examined. All data were assumed to follow a Gaussian distribution. As we were making comparisons within specific target cells, and not between targets, most comparisons were performed by Student’s t test (Welch’s correction was used in cases of unequal variance) or Kolmogorov–Smirnov (K-S) test. Comparisons between target cells was performed with one-way ANOVA followed by Tukey’s test.

Results

1b and 1s inputs contribute to postsynaptic activity in a target-specific manner

Converging inputs contribute to the overall postsynaptic response. In this study, we examined to what extent one input can influence the structure and function of a convergent input. To address this, we first determined the activity contribution of each MN on the postsynaptic muscle target in a wild-type condition. We chose m6, m12, and m4 because (1) prior studies showed that each 1b contributes a unique percentage of the total postsynaptic activity, (2) these muscles have been frequently analyzed in NMJ studies (Nose, 2012; Menon et al., 2013), and (3) the 1b and 1s innervation patterns on these muscles enabled the identification of common and muscle-specific principles (all muscles have unique 1b MNs, but m6 and m12 are innervated by the same 1s MN).

We measured the 1b MN contribution to the total EPSP on a muscle by muscle basis. 1b and 1s axons fasciculate into nerve bundles as they exit the VNC, which impeded us from physically stimulating each neuron independently without patching each cell body (Choi et al., 2004). To circumvent this, we combined NMJ electrophysiology with a postsynaptically targeted genetically encoded calcium indicator, SynapGCaMP6f (Menon et al., 2017). Some of the earliest 1b alone firing events and 1b + 1s firing events (Movie 1). If a stimulation only triggered a GCaMP firing event at 1b NMJs but not 1s NMJs, the corresponding EPSP was counted as a 1b alone EPSP. If a stimulation triggered GCaMP fluorescence changes at both 1b and 1s NMJs, the corresponding EPSP was counted as a 1b + 1s EPSP. For each sample, 1b alone EPSPs and 1b + 1s EPSPs were averaged respectively. 1b alone/1b + 1s was calculated by dividing the mean 1b alone EPSP by the mean 1b + 1s EPSP of each sample.

Experimental design and statistical analysis

In all experiments, we included A8>GFP to ensure that 1s NMJs were present in control animals (Ashley et al., 2019; Aponte-Santiago et al., 2020). For experiments with DIP-α null, A8>GFP larva, males were selected as DIP-α on the X chromosome. For A8>GFP/+ controls, males were also selected. All statistical analyses were performed using Prism 8 software (GraphPad). Average and SEM are reported. Outliers are determined by Q-test and excluded from the sample pools. For each data point, at least eight animals per genotype were dissected and at least two biological replicates were examined. All data were assumed to follow a Gaussian distribution. As we were making comparisons within specific target cells, and not between targets, most comparisons were performed by Student’s t test (Welch’s correction was used in cases of unequal variance) or Kolmogorov–Smirnov (K-S) test. Comparisons between target cells was performed with one-way ANOVA followed by Tukey’s test.
evidence for the existence of two distinct MN populations was uncovered >40 years ago (Jan and Jan, 1976) through observations that different voltage injections elicited two populations of muscle EPSPs. It was later found that lower voltage injection generated action potentials in 1b MNs (Lnenicka and Keshishian, 2000); therefore, we varied the stimulus protocol to independently elicit and record EPSPs from 1b alone and 1b + 1s together (see Materials and Methods). SynapGCaMP6f fluorescence changes at 1b and 1s NMJs confirmed whether the recorded EPSPs were because of 1b alone or 1b + 1s activity (Fig. 1B, Movie 1). Using this procedure, the average total EPSP amplitude (1b + 1s) in m6 was 31.55 mV and the average 1b-derived EPSP was 17.17 mV (Fig. 1C,F). Thus, the m6-1b MN contributes 54% of the total m6 EPSP (Fig. 1I). Interestingly, at m12, the 1b MN contributes 31% of the total EPSP, and at m4 the 1b MN represents 62% (Fig. 1D,E,G–I). Thus, we determined the contribution of each MN (1b and 1s) to the postsynaptic muscle activity in wild-type larvae and found that the relative strength of each MN differed between muscles, with the m4-1b MN contributing the most and the m12-1b MN the least. Notably, m6-1b MN and m12-1b MN contributions are different although they are innervated by the same 1s MN. These data established a model in which to introduce perturbations and examine synaptic plasticity.

Cell-specific genetic ablation of 1s MNs by ectopic hid,rpr expression

To begin to examine whether one input can respond to perturbations in an adjacent input, we needed to disrupt one MN and monitor the impact on the convergent MN. Additionally, being able to disrupt one input before or after innervation can shed light on whether initial innervation is required for synaptic plasticity. We needed to identify drivers that are expressed specifically within subsets of the convergent neurons. In a previous study, we showed that DIP-α is expressed in subsets of neurons in the larval VNC, including interneurons and two 1s MNs, and that the removal of DIP-α impeded 1s innervation of m4 (Ashley et al., 2019). To gain genetic access specifically to the 1s MNs, we examined a GAL4 driver derived from the DIP-α promoter. In the adult neuromuscular circuit, this driver [hereafter referred to as A8-GAL4 or (A8)] was found to be expressed in a small subset of MNs (Venkatasubramanian et al., 2019). We used A8-GAL4 to drive UAS-GFP and found expression in only two pairs of segmentally repeated neurons in the third instar VNC (Fig. 2A,B, arrows). The labeled neurons located ventrally in the VNC have axons that project medially and dorsally toward the neuropil (Fig. 2C, arrow). The other A8-expressing neurons are located in the dorsal region of the VNC and showed an ipsilateral projection with a large dendritic arbor (Fig. 2D, arrowhead).

Examination of nerves exiting the VNC showed Figure 6. 1b NMJs elevate evoked neurotransmission in a target-specific manner in the absence of 1s inputs. A–C, Representative EPSP traces of m6 (A), m12 (B), and m4 (C) in control (A8>GFP) and 1s-ablated (A8>GFP, hid,rpr) animals. Traces and graphs are color coded as indicated in the color key. D–F, Quantification of EPSP amplitudes in m6 (D, t(22) = 8.306, p < 0.0001, unpaired t test), m12 (E, t(22) = 13.82, p < 0.0001, unpaired t test), and m4 (F; t(22) = 3.057, p = 0.0046, unpaired t test) in control and 1s-ablated animals. G–I, Normalized EPSPs of m6 (G, t(22) = 2.301, p = 0.0300, unpaired t test), m12 (H, t(22) = 1.552, p = 0.1332, unpaired t test), and m4 (I; t(22) = 4.605, p < 0.0001, unpaired t test) in control and 1s-ablated third instar larvae. Normalized EPSPs in A8>GFP,hid,rpr are compared with the EPSP ratio of 1b+1s calculated from corresponding muscles in Figure 1I, indicated by gray dashed line. Note m6-1b slightly compensates, m12-1b does not compensate and m4 largely compensates the loss of 1s MNs. J–L, Normalized QC of m6 (J, t(22) = 1.239, p = 0.2268, unpaired t test), m12 (K; t(22) = 2.119, p = 0.0442, unpaired t test), and m4 (L; t(22) = 4.639, p < 0.0001, unpaired t test) in control and 1s-ablated larvae. Normalized QCs were compared with 1b baseline QC (see Materials and Methods). Note m4-1b shows an increased QC, while m12-1b shows a decrease. Error bars indicate ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ns - not significant. n values (NMJs/larva) are 12/9, 12/11, 12/9, 12/12, 14/11, and 17/14, respectively.
two axons in each bundle (Fig. 2BD asterisks), suggesting that these A8-positive neurons are MNs. The dorsal neurons were colabeled with the transcription factor Eve, which labels three medial neurons, aCC, pCC, and MNISN-1s (Do et al., 1988; Broadus et al., 1995), and thus these neurons were confirmed as MNISN-1s (also called the dCE) based on their location (Fig. 2B). Drosophila larval MNs make connections with their muscle targets with very high fidelity, allowing us to unequivocally determine the identity of the MNs based on their innervation pattern. In A8>GFP third instar larvae, one axon innervates the ventral muscles including muscles 6, 7, 12, and 13, similar to the connectivity pattern of MNISNb-d-1s (also called the vCE). The other axon innervates the dorsal muscles, corresponding to dCE (Figs. 1A, 2E,F). To distinguish 1b and 1s NMJs, we also stained for DLG as 1b NMJs are surrounded by significantly more DLG (Guan et al., 1996; Fig. 2E,F). A8 is not expressed in the third 1s MN that innervates lateral transverse muscles. Importantly, A8 does not label any other MNs but only a few additional cells in one segment of the VNC (Fig. 2A, carets). In summary, A8 labels two 1s MNs (the vCE and dCE) within each hemisegment.

We used A8-GAL4 to drive head involution defective (hid) and reaper (rpr) in vCE and dCE. hid and rpr have important functions in programmed cell death, and ectopic expression of both genes more reliably induces neuronal death than either gene alone (Zhou et al., 1997; Pauls et al., 2015). As shown in Figure 2A–F, A8>GFP robustly labels 1s MNs but in third instar larvae that ectopically express hid,rpr (A8>GFP,hid,rpr), all vCE or dCE cell bodies and NMJs are absent (Fig. 2G–J). Thus, the expression of hid,rpr is sufficient to genetically ablate 1s MNs.

To determine whether cell death occurred before or after innervation, we examined earlier developmental stages and visualized GFP in 1s MN cell bodies and NMJs in A8>GFP and A8>GFP,hid,rpr. Neuromuscular innervation is established at embryonic stage 16 (Halpern et al., 1991; Broadie and Bate, 1993; Yoshiihara et al., 1997), so we focused on stage 15 and later stages including stage 17 and first instar larvae. In stage 15 embryo controls (A8>GFP), only a subset of 1s MNs are detected since not all dCE cell bodies (Eve positive) are costained with GFP and no vCE cell bodies are observed (Fig. 3A,B). Age-matched A8>GFP,hid,rpr embryos showed GFP expression patterns similar to those of controls (Fig. 3CD); thus, no cell death occurred before neuromuscular innervation. By embryonic stage 17, more vCE and dCE cell bodies expressed A8 but the lack of GFP in some cells suggests that A8 expression has not reached maximal levels (Fig. 3EF). In stage 17 A8>GFP,hid,rpr embryos, cells were undergoing apoptosis, as revealed by significant decreases in GFP and by the loss of Eve staining (suggesting nuclear degradation; Fig. 3GH). Finally, in A8>GFP early first instar larvae, all vCE and dCE cell bodies were labeled (Fig. 3IJ) and NMJs were present (Fig. 3M). Age-matched A8>GFP,hid,rpr larvae completely lacked vCE and dCE cell bodies (Fig. 3KL), although some 1s NMJs were still observed (Fig. 3MN), suggesting that 1s NMJs were established before ablation in this genetic background. By late first instar stage, A8>GFP,hid,rpr larval show no traces of 1s NMJs on m6, m12, and m4 (Fig. 3O). Together, A8-GAL4 is specifically expressed in vCE and dCE, and driving ectopic expression of cell death genes with A8 triggers apoptosis in 1s MNs after synaptic contacts are established.

1b NMJs expand upon loss of adjacent 1s MNs
NMJ structural plasticity can be induced by the perturbation of synaptic function (Budnik et al., 1990; Jarecki and Keshishian, 1995; Sigrist et al., 2003; Goel et al., 2019a; Perry et al., 2020). Here, we examined whether the loss of 1s MNs can induce structural changes at adjacent 1b NMJs. The size of each NMJ is well characterized (represented by the number of boutons), and this allows us to observe structural changes because of perturbations. We genetically ablated 1s MNs (vCE and dCE) and counted the number of boutons on m6, m12, and m4 in wandering third instar larvae (NMJ expansion is complete; Li et al., 2002). We observed an increase in the number of 1b boutons on all three muscles (Fig. 4A–G) when comparing them to A8>GFP controls, suggesting that 1b NMJs expanded when adjacent 1s MNs were ablated. Because m4-1s innervation frequency is ~80% in wild type (Ashley et al., 2019; Aponte-Santiago et al., 2020), we used A8>GFP to confirm the presence of m4-1s in controls and excluded muscles lacking 1s innervation. Interestingly, we also found an increase in small budding boutons, called satellite boutons, emanating from 1b boutons (Fig. 4DF, insets, H). These structures represent immature but functional boutons as they contain all synaptic machinery and postsynaptic receptors (Torroja et al., 1999; Dickman et al., 2006; O’Connor-Giles et al., 2008; O’Connor-Giles and Ganetzky, 2008; Lee and Wu, 2010; Carrillo et al., 2015). Thus, all 1b NMJs initiate structural plasticity mechanisms to respond to the loss of adjacent 1s MNs.

1b NMJs elevate their rate of spontaneous release upon loss of 1s MNs
To determine to what extent a synaptic input can influence the functional synaptic plasticity of a converging input, we first examined 1b spontaneous neurotransmitter release at muscles where 1s MNs were ablated after innervation. 1b and 1s MNs have unique spontaneous release properties. For example, 1b-derived spontaneous events (stimulus-independent release of neurotransmitter vesicles; also referred to as mEPSPs) have smaller amplitudes compared with 1s-derived mEPSPs (Nguyen and Stewart, 2016; Newman et al., 2017). Therefore, the ablation of 1s inputs should shift the mean mEPSP amplitude toward the smaller 1b-like amplitude if there is no compensation. We performed current-clamp recordings from m6, m12, and m4. Indeed, A8>GFP,hid,rpr revealed decreased mEPSP amplitudes compared with A8>GFP controls, and a significant shift in cumulative amplitude probability distribution (Fig. 5AG). Because of the inability of standard NMJ electrophysiology experiments to distinguish between 1b and 1s mEPSPs in controls, we are unable to unambiguously determine whether the 1b mEPSP
amplitudes are affected by the loss of 1s inputs. Nonetheless, we can conclude that 1b NMJs cannot fully restore the average mEPSP amplitudes to wild-type levels.

Another measure of stimulus-independent activity is the rate of spontaneous neurotransmitter release. In prior studies, mEPSP frequencies were found to be higher at 1b NMJs than 1s NMJs (e.g., 2.3 Hz at m4-1b NMJ and 1 Hz at m4-1s NMJ; Newman et al., 2017), thus, if the elimination of 1s MNs does not affect the rate of 1b spontaneous release, overall mEPSP frequencies should decrease by about one-third. However, we did not observe any reduction of mEPSP frequencies on m6, m12, and m4 when comparing m4 with and without convergent 1s MNs. Unlike spontaneous neurotransmitter release, EPSPs require stimulation to depolarize the presynaptic neuron above threshold. This suprathreshold stimulation triggers an action potential to induce neurotransmitter release and elicit a postsynaptic response. To examine whether 1b MNs can compensate for the loss of 1s synaptic drive, we recorded EPSPs in both A8>GFP and A8>GFPhid,rpr animals, and displayed the smallest 1b contribution. Meanwhile, m12-1b did not compensate for the loss of 1s inputs, we normalized the EPSPs (A8>GFP,hid,rpr EPSP/A8>GFP EPSP) and compared these values to the calculated 1b/1b + 1s ratio in Figure 1I. This analysis allows for the comparison of 1b-derived EPSPs with and without convergent 1s MNs.

We observed target-specific changes in 1b-derived EPSPs (Fig. 6G–I). At m4, we observed a significant increase in 1b-derived EPSPs compared with the control 1b/1b + 1s ratio (Fig. 6I). By repeating the analysis at other muscles, we found a mild increase in 1b-derived EPSPs at m6 (Fig. 6G) and, surprisingly, no change at m12 (Fig. 6H). When comparing the degree of EPSP compensations (Fig. 6G–I) to the wild-type 1b contribution to total EPSP at each muscle (Fig. 1I), a pattern arises whereby the m4-1b has both the most compensation and the largest 1b contribution. Meanwhile, m12-1b did not compensate and displayed the smallest 1b contribution.

Next, we examined QC, a calculation of the approximate amount of neurotransmitter released per stimulation. We found that the normalized QC of m4-1b in A8>GFP,hid,rpr was significantly increased compared with the m4-1b baseline QC (see Materials and Methods), whereas there was no change at m6-1b, and even a decrease at m12-1b (Fig. 6J–L). To test whether the loss of 1s MNs perturbed short-term 1b NMJ plasticity, we...
examined paired-pulse facilitation (PPF) as it is a measure of evoked release probability \( (P_r) \). We measured m6 PPF in \( A8>GFP, hid, rpr \) and found an enhancement compared with \( A8>GFP \) controls (Fig. 7). Although these data suggest an increase in 1b paired-pulse ratio (PPR), this can also be explained by the loss of 1s-specific synaptic depression (Newman et al., 2017) as both MNs are stimulated in controls. Furthermore, we were unable to definitively isolate 1b-specific PPF in controls as the second EPSC could be either 1b derived or 1b + 1s derived. Overall, when ablating 1s MNs after NMJ formation, 1b MNs that innervate the same muscle upregulated their rate of spontaneous release, and, importantly, the m4-1b MN significantly compensated the total EPSP by increasing neurotransmitter release.

Active zone and glutamate receptor levels are unaltered by ablation of 1s MNs
One way to increase both spontaneous and evoked release is by the addition of more AZs. We stained for BRP, an AZ scaffolding protein (Kittel et al., 2006), to visualize 1b AZs in the presence or absence of convergent 1s MNs. Despite elevated 1b synaptic drive, we observed no change in overall 1b AZ intensity (Fig. 8A–G), although the larger NMJ sizes (Fig. 4G) led to reduced AZ densities (BRP/HRP; Fig. 8H). These data suggest that the number of AZs remains constant although NMJ size increases, similar to previous studies (Goel et al., 2019a, b; Aponte-Santiago et al., 2020; Goel et al., 2020).

Changes in the postsynaptic glutamate receptor density or localization can contribute to functional responses. To test whether the functional synaptic plasticity we observed was because of changes in glutamate receptor levels, we visualized the GluRIIA (Petersen et al., 1997). m6-1b NMJs showed a significant increase in GluRIIA intensity, whereas m12-1b and m4-1b did not show a significant increase (Fig. 9A–G). Interestingly, 1b NMJ GluRIIA density (Fig. 9H) was unaffected, suggesting that receptor abundance may not contribute to 1b synaptic plasticity. Overall, these data indicate that presynaptic BRP or postsynaptic GluRIIA levels are not an integral component of 1b synaptic plasticity induced by the ablation of 1s MNs.

Loss of the 1s-derived EPSP triggers 1b synaptic plasticity
Ablation is a crude perturbation that completely removes all 1s MN synaptic activity and signaling pathways. Here, we focus on 1s evoked neurotransmission to examine whether its removal mimics the ablation of 1s MNs. Tetanus toxin light chain (TNT) is a clostridial toxin that cleaves the vesicle SNARE synaptobre- vin, which is required for calcium-dependent vesicle release (Sweeney et al., 1995). Ectopic expression of TNT blocks evoked neurotransmission without affecting spontaneous release and synaptic morphology (Sweeney et al., 1995; Aponte-Santiago et al., 2020). Therefore, we expressed TNT with \( A8-GAL4 \) to specifically block 1s-derived EPSPs and measured 1b responses. We focused on m4-1b NMJs as these showed robust EPSP compensation, and we observed similar 1b synaptic plasticity when compared with ablation of 1s MNs. Structurally, the m4-1b NMJ was larger (Fig. 10A–C) and displayed more satellite boutons (Fig. 10D). Functionally, we observed an increase in overall mEPSP frequency (Fig. 10E,F) and 1b-derived EPSP amplitude (when
Comparing the normalized EPSP to the 1b/1b + 1s ratio (Fig. 10G,H), the compensatory 1b-derived EPSP, however, was less than that observed with 1s ablation (Fig. 6i). QC in A8>GFP, TNT was calculated as before (see Materials and Methods; 1b baseline QC), then normalized and compared with 1b baseline QC. Importantly, we observed a similar increase in QC (Fig. 10f). These results suggest that the loss of 1s-derived EPSPs was sufficient to trigger 1b synaptic plasticity, although other 1s-derived signals likely contribute since the ablation of 1s induces a more robust 1b response.

Robust 1b synaptic plasticity requires initial 1s innervation

Above, we examined the structural and functional synaptic plasticity of one neuron when a converging neuron is ablated after innervation (A8>GFP,hid,rpr). To probe whether initial coinervation is required for plasticity, we explored another context where m4-1b synaptic plasticity is not because of the genetic manipulation.

Next, we examined 1b structural and functional plasticity in another context where m4 is naturally missing 1s innervation. In wild-type larvae, ~20% of m4s lack 1s NMJs (Fig. 11c, control), which could underlie why m4-1b MNs are able to compensate for 1s MN perturbation. However, comparing wild-type m4s that naturally lack 1s innervation with those that have 1s innervation, we observed no change in 1b bouton numbers (Fig. 12a, b). Similarly, 1b-derived mEPSP, EPSP and QC were unaffected (Fig. 12c-f). This confirms that lack of synaptic plasticity in DIP-αnull larvae is not because of the genetic manipulation. Further, these data show that m4-1b MNs that naturally lack 1s MNs are unable to compensate, suggesting that robust m4-1b synaptic plasticity is not because of the variable 1s innervation observed on m4, but instead, induced when adjacent neurons are perturbed (for our model, see Discussion).

Discussion

The major gap that this article addresses is to what extent one synaptic input can influence the structural and functional...
plasticity of a converging input. Here, we examine the *Drosophila* neuromuscular circuit and demonstrate 1b synaptic plasticity induced by the loss of convergent 1s MNs. The muscles examined in this study, m6, m12, and m4, are coinnervated by unique 1b MNs. Furthermore, some 1b MNs elevated evoked neurotransmission and uncovered that 1b MNs contribute a unique percentage of the total AZs or QCs. Genetic ablation of 1s MNs (vCE and dCE) after innervation led to the expansion of 1b MN pairs. First, we established an activity baseline in wild type larvae for control and mutant animals (1s calculated from m4 in Fig. 6). Thus, even without an increase in AZs or QCs the replasticity of a converging input.
AZ properties to respond to the loss of 1s inputs. Overall, our data suggest that silent AZs may become activated to increase the pools of spontaneous and evoked AZs as the ablation of 1s MNs led to enhanced 1b spontaneous release rates, target-specific compensation of EPSPs, and increased QC. Additionally, spontaneous and evoked activities may be independently regulated. Furthermore, the readily releasable pool size is under dynamic control during synaptic plasticity (Weyhersmüller et al., 2011; Müller et al., 2012) and could modulate m4-1b-evoked neurotransmitter release. Detailed examination of AZs will significantly bolster our understanding of the mechanism underlying 1b NMJ plasticity.

Prior studies reported that spontaneous neurotransmitter release regulates synaptic development in both mammals and Drosophila (Joseph et al., 2012; Choi et al., 2014; Andreae and Burrone, 2015, 2018; Cho et al., 2015; Kavalali, 2015). Thus, the expanded size of all 1b NMJs following 1s ablation may be caused by the elevated spontaneous activity. These data also suggest that all 1b MNs can detect and respond to the loss of adjacent 1s inputs. However, the ability to differentially compensate the spontaneous and evoked activity is likely because of independent mechanisms since only some 1b MNs elevate their EPSPs.

**Correlation between synaptic weight and target-specific plasticity**

In complex neural circuits, dissecting contributions of individual inputs to the total postsynaptic plasticity, also referred to as synaptic weight (Magee and Cook, 2000; Bhalla, 2008), remains difficult because of thousands of converging inputs on a single cell. The larval NMJ facilitates the partitioning of synaptic inputs as each muscle is innervated by few MNs. In this study, we combined electrophysiology with calcium imaging and found that 1b synaptic weights differ on m6, m12, and m4. Together with the degree of EPSP compensation after the ablation of 1s MNs, there was a direct correlation with the level of target-specific synaptic weight. Thus, robust 1b MNs that carry more synaptic drive may be endowed with certain synaptic plasticity mechanisms that respond to the loss of adjacent inputs. However, we cannot rule out regulatory roles for type II and type III MNs (Gorczyca et al., 1993; Chang and Keshishian, 1996; Koon et al., 2011) that are present on some muscles.

Interestingly, a similar correlation exists in Hebbian plasticity, where stronger synapses are more likely strengthened than weaker ones (Song et al., 2000; Babadi and Abbott, 2010). This correlation is also reflected in PHP. Two studies examined input-specific PHP on different muscles. On m4, PHP can be induced only at 1b NMJs (Newman et al., 2017); however, on m6, PHP can be induced on both 1b and 1s NMJs (Genc and Davis, 2019). This correlates with our observation that the m4-1b has more synaptic weight than m4-1s, whereas m6-1b and m6-1s have similar synaptic weights. Together, homeostatic plasticity varies in target-specific and input-specific manners, suggesting heterogeneous mechanisms.

**Establishing an EPSP set point for 1b MN synaptic plasticity**

Models of synaptic homeostasis rely on an activity set point to stabilize neurons when confronted with perturbations (LeMasson et al., 1993; Liu et al., 1998; Turrigiano, 2007; Davis, 2013; O’Leary et al., 2014). Each target neuron must account for all presynaptic inputs to produce a defined output (i.e., the set point). The structural and functional properties of each input thus determine not only its contribution to the postsynaptic activity but also its ability to respond to perturbations in synaptic function. For example, transcription factors not only regulate the expression of ion channels that shape neuronal excitability, but also homeostatic mechanisms (Turrigiano, 2007; Davis, 2013; Parrish et al., 2014; Engelmann and Haenold, 2016; Diering et al., 2017; Schaukowitch et al., 2017). Like many activity-dependent processes (Ataman et al., 2008; Carrillo et al., 2010; Berke et al., 2013; Vonhoff and Keshishian, 2017a), the optimal set point may be established during a narrow time window of development. This hypothesis was tested in a Drosophila seizure mutant by inhibiting activity during embryonic development and observing the suppression of seizures in postembryonic stages (Giachello and Baines, 2015). Thus, manipulating activity during an embryonic critical period may alter the activity set point.
In this study, one intriguing hypothesis is that 1b + 1s coinervasion determines the EPSP set point during embryogenesis and is referenced by some 1b NMJs to compensate for the loss of 1s MNs. We propose a model to describe how 1b NMJs increase their sizes and spontaneous and evoked neurotransmission because of the loss of convergent 1s MNs. The neuromuscular innervation map is formed during late embryonic development (Prokop et al., 1996; Yoshihara et al., 1997; Vonhoff and Keshishian, 2017b). Since the maximum EPSP amplitude is established at the first instar stage and maintained throughout larval development (Davis and Goodman, 1998; Li et al., 2002), the set point is likely determined by the initial 1b + 1s coinervasion. Blocking the initial formation of 1s NMJs would create a set point that is devoid of 1s influence; and thus, the corresponding 1b NMJs would not compensate toward the wild-type EPSP but instead to this alternate EPSP set point. If the 1s MN is ablated after synaptogenesis, the correct set point is established and the 1b responds accordingly. Removing 1s innervation at various time points during larval development will provide a deeper understanding of the temporal dynamics of 1b plasticity and whether the EPSP set point is maintained by 1b + 1s activities.

**Synaptic plasticity in other convergent neural circuits**

The *Drosophila* neuromuscular circuit is a reductionist system with polynervated muscles. The loss of an input triggering a compensatory increase in synaptic growth or function of a convergent input could be viewed as synaptic competition. Synaptic competition is well characterized at the vertebrate neuromuscular junction, where neonatal muscles are innervated by several motor axons, but only one survives to adulthood (Sanes and Lichtman, 1999; Tapia et al., 2012). Neuromuscular activity is critical for this pruning process but the signaling pathway is not completely understood (Schuldiner and Yaron, 2015; Lee, 2020). Studying convergent synaptic plasticity at the *Drosophila* NMJ may shed light on this process.

Mechanisms uncovered at the NMJ can act in more complex circuits in vertebrates and invertebrates. For example, in the fly VNC, each MN receives sensory information from many interneurons to regulate motor behaviors (Hecksher et al., 2015; Schneider-Mizell et al., 2016; Kohsaka et al., 2019). The convergence of inputs on one MN may establish a homeostatic set point in the MN, similar to the muscle. The complex interneuron–MN connectivity more closely resembles that of vertebrate CNS neurons with polysynaptic dendrites (Kim et al., 2009; Hecksher et al., 2015; Kohsaka et al., 2019; Zarin et al., 2019). Additionally, age-related (Bergado and Almaguer, 2002; Mattson and Magnus, 2006; Mostany et al., 2013; Griffith et al., 2014; Petralia et al., 2014) and disease-related (Gorman, 2008; Milnerwood and Raymond, 2010; Lepeta et al., 2016; Salvadoros et al., 2017; Smith-Dijak et al., 2019) changes in synaptic function and cell survival have been observed in CNS and neuromuscular circuits. In patients and animal models of Alzheimer’s disease, while some neurons are depressed in amyloid-β plaque-enriched regions, other neurons show a compensatory hyperactivation (Busche et al., 2012; Merlo et al., 2019). Thus, future studies at the NMJ and other circuits will elucidate the mechanisms governing how and when the activity set point is defined in a target-specific manner and how neurons respond to dysfunctional neighbors.

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