

Peripheral glia and neurons jointly regulate activity-induced synaptic remodeling at the *Drosophila* neuromuscular junction

Reviewed Preprint

v2 • October 29, 2025

Revised by authors

Reviewed Preprint

v1 • January 6, 2025

Yen-Ching Chang, Yi-Jheng Peng, Joo Yeun Lee, Annie Wen, Karen T Chang 

Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles, United States • Dept. of Physiology & Neuroscience, Keck School of Medicine, University of Southern California, Los Angeles, United States

 https://en.wikipedia.org/wiki/Open_access
 Copyright information

eLife Assessment

This study presents a **valuable** finding on a new role of glia in activity-dependent synaptic remodeling using the *Drosophila* NMJ as a model system. The evidence supporting the claims of the authors is **convincing**. The authors have addressed most of the reviewers' concerns and help to further clarify the claims. The work will be of interest to neuroscientists working on glia-neuron interaction and synaptic remodeling.

<https://doi.org/10.7554/eLife.104126.2.sa3>

Abstract

In the nervous system, reliable communication depends on the ability of neurons to adaptively remodel their synaptic structure and function in response to changes in neuronal activity. While neurons are the main drivers of synaptic plasticity, glial cells are increasingly recognized for their roles as active modulators. However, the underlying molecular mechanisms remain unclear. Here, using *Drosophila* neuromuscular junction as a model system for a tripartite synapse, we show that peripheral glial cells collaborate with neurons at the NMJ to regulate activity-induced synaptic remodeling, in part through a protein called shriveled (Shv). Shv is an activator of integrin signaling previously shown to be released by neurons during intense stimulation at the fly NMJ to regulate activity-induced synaptic remodeling. We demonstrate that Shv is also present in peripheral glia, and glial Shv is both necessary and sufficient for synaptic remodeling. However, unlike neuronal Shv, glial Shv does not activate integrin signaling at the NMJ. Instead, it regulates synaptic plasticity in two ways: 1) maintaining the extracellular balance of neuronal Shv proteins to regulate integrin signaling, and 2) controlling ambient extracellular glutamate concentration to regulate postsynaptic glutamate receptor abundance. Loss of glial cells showed the same phenotype as loss of Shv in glia. Together, these results reveal that neurons and glial cells homeostatically regulate extracellular Shv protein levels to control activity-induced synaptic remodeling. Additionally, peripheral glia maintains postsynaptic glutamate receptor abundance and

contribute to activity-induced synaptic remodeling by regulating ambient glutamate concentration at the fly NMJ.

Introduction

The nervous system is highly plastic, with the capacity to undergo dynamic alterations in structure and strength in response to changing stimuli and environments (Lamprecht and LeDoux, 2004; Citri and Malenka, 2008). This activity-induced synaptic remodeling process is highly conserved across species and plays crucial roles in circuit formation during development, as well as in stabilizing existing connections post-development. Synaptic remodeling represents a finely orchestrated process, with communications across both the pre- and post-synapses to allow the growth of new synapses, and the stabilization and strengthening of existing ones. While much of the research on synaptic plasticity has concentrated on interactions between presynaptic axon terminals and postsynaptic cells, most synapses are tripartite synapses, with glial cells as the third cell type (Araque and Navarrete, 2010). Beyond simply providing metabolic support, glial cells are increasingly recognized for their roles as active modulators of synaptic plasticity (Sancho et al., 2021). However, the mechanisms by which glial cells and neurons collaborate to coordinate activity-induced synaptic remodeling are not well understood.

The *Drosophila* larval neuromuscular junction (NMJ) is a genetically tractable system and a tripartite glutamatergic synapse that serves as an excellent model system to investigate mechanisms underlying activity-induced synaptic remodeling by glial cells (Banerjee and Bhat, 2008; Freeman, 2015; Kim et al., 2020). The peripheral glial cells at the fly NMJ perform some of the key functions similar to mammalian glia, including controlling neuronal excitability and conduction velocity (Kottmeier et al., 2020; Rey et al., 2023), recycling of neurotransmitters (Rival et al., 2004; Danjo et al., 2011), and engulfing and clearing debris during damage to allow the growth of new boutons (Fuentes-Medel et al., 2009). They also release proteins such as transforming growth factor (TGF- β) to support synaptic growth (Fuentes-Medel et al., 2012), TNF- α (eiger) to influence neuronal survival (Keller et al., 2011), Wingless to regulate GluR clustering (Kerr et al., 2014), and laminin to control animal locomotion (Petley-Ragan et al., 2016). Nevertheless, the role of peripheral glia in regulating activity-induced stabilization and remodeling of the existing synapses at the NMJ remains unknown.

Previous studies on activity-induced synaptic remodeling at the fly NMJ demonstrated that neuronal activity leads to the enlargement of existing boutons, accompanied by increases in postsynaptic GluR abundance (Lee et al., 2017; Chang et al., 2024). Intense neuronal stimulation triggers the release of a protein called Shriveled (Shv) by presynaptic motoneurons, which activates β PS integrin bi-directionally to stimulate synaptic bouton enlargement and elevate GluR levels on the postsynaptic muscles (Lee et al., 2017). Consequently, *shv* mutants display defective post-tetanic potentiation (PTP), a form of functional synaptic plasticity similar to the early phase of long-term potentiation (LTP) seen in mammalian neurons. Here we demonstrate that the Shv protein is also expressed in glial cells and is released extracellularly by peripheral glial cells. Glial Shv not only regulates basal GluR clustering but is also required for activity-induced synaptic remodeling. We further demonstrate that while glial Shv is present extracellularly, it does not respond to neuronal activity nor does it activate integrin signaling, unlike Shv derived from neurons. Instead, glial Shv contributes to synaptic plasticity regulation by modulating the levels of Shv release from neurons and by controlling the levels of ambient glutamate concentration. Restoring ambient glutamate concentration could correct basal GluR abundance and defective synaptic plasticity caused by the loss of glial cells. These results further reveal that regulation of ambient extracellular glutamate concentration by glia is an important mechanism contributing to synaptic plasticity regulation.

Results

Shv is expressed in peripheral glia

To determine the role of Shv in glia, we first monitored its presence in different cell types. To this end, we knocked-in eGFP to the 3'-end of the full-length Shv protein using CRISPR/Cas9-catalyzed homology directed repair (Gratz et al., 2014). Western blots confirmed that the Shv protein is tagged with eGFP (**Fig 1A**), and immunostaining revealed its presence in neurons and glial cells (**Fig 1B**). Glial cells were identified using antibody against reverse polarity (Repo), a transcription factor expressed exclusively in glial cells (Xiong et al., 1994), and neuronal cells were marked either by Elav, a transcription factor expressed in neurons (Robinow and White, 1991), or HRP, which stains the neuronal cell membrane. We found that Shv-eGFP is present in both Repo and Elav positive cells in the larval brain, consistent with our previous report (Lee et al., 2017). Furthermore, Shv-eGFP can be detected at the NMJ, with weak signals in postsynaptic muscles and synaptic boutons, and stronger signals in peripheral glia (**Fig 1C**).

Glial Shv is required for activity-induced synaptic remodeling

Loss of Shv in *shv*¹ mutant was previously shown to be essential for activity-induced synaptic remodeling (Lee et al., 2017). Given that Shv is observed in neurons, glia, and muscle (**Fig 1C**), we determined the tissue-specific requirement for Shv by systematically knocking down *shv* using RNAi and tissue-specific drivers (**Fig 2A**). We found that *shv* knockdown in neurons using the pan-neuronal driver, *elav-GAL4*, generated the same phenotype as *shv*¹ mutant (Lee et al., 2017), namely smaller bouton size, reduced basal GluR intensity, and defective synaptic remodeling in response to neuronal stimulation. However, knockdown of *shv* in glia using the pan-glial specific driver, *repo-GAL4*, resulted in normal bouton size but significantly elevated GluR levels, as well as abolished activity-induced synaptic remodeling. Knockdown of *shv* in glia using another independent RNAi line resulted in the same phenotypes (**Figure 2 – figure supplement 1A**). This increase in GluR is unexpected and further suggests that neuronal and glial Shv acts through different pathways to regulate GluR abundance. Lastly, knockdown of *shv* in post-synaptic muscles using the muscle specific driver *24B-GAL4* did not affect synaptic development or activity-induced synaptic changes. Collectively, these results are consistent with an earlier finding that Shv is predominantly released by neurons to maintain activity-induced synaptic remodeling (Lee et al., 2017), as well as reveal an unexpected requirement for glial Shv in sustaining activity-induced synaptic changes and basal GluR abundance.

Given that reducing Shv in glia altered basal GluR levels during development, we used the GeneSwitch system to determine the temporal requirement for Shv in glia (**Fig 2B**). In the presence of RU486, the *repo-GeneSwitch* driver can undergo a conformational change to activate gene expression in glia (Osterwalder et al., 2001; Roman et al., 2001; Artiushin et al., 2018). First, we confirmed the efficiency of acute *shv* knockdown by performing Western blot analysis of dissected larval brains (**Figure 2 – figure supplement 1B**). Acute glial knockdown using the *repo-GeneSwitch* driver resulted in a 30% reduction in Shv levels (+RU486), similar to the decrease observed with the *repo-GAL4* driver, suggesting that the GeneSwitch driver is functional. Furthermore, knockdown of *shv* by the ubiquitous *tubulin-GAL4* driver completely eliminated Shv protein, indicating that the RNAi construct is effective. We next examined the effect of acute glial *shv* knockdown on synaptic remodeling. As shown in **Figure 2B**, transient glial *shv* knockdown is sufficient to elevate basal GluR levels and abolish activity-induced synaptic remodeling. Taken together, these results suggest that while glial Shv is a minor source, it is acutely required for regulating GluR abundance and synaptic plasticity.

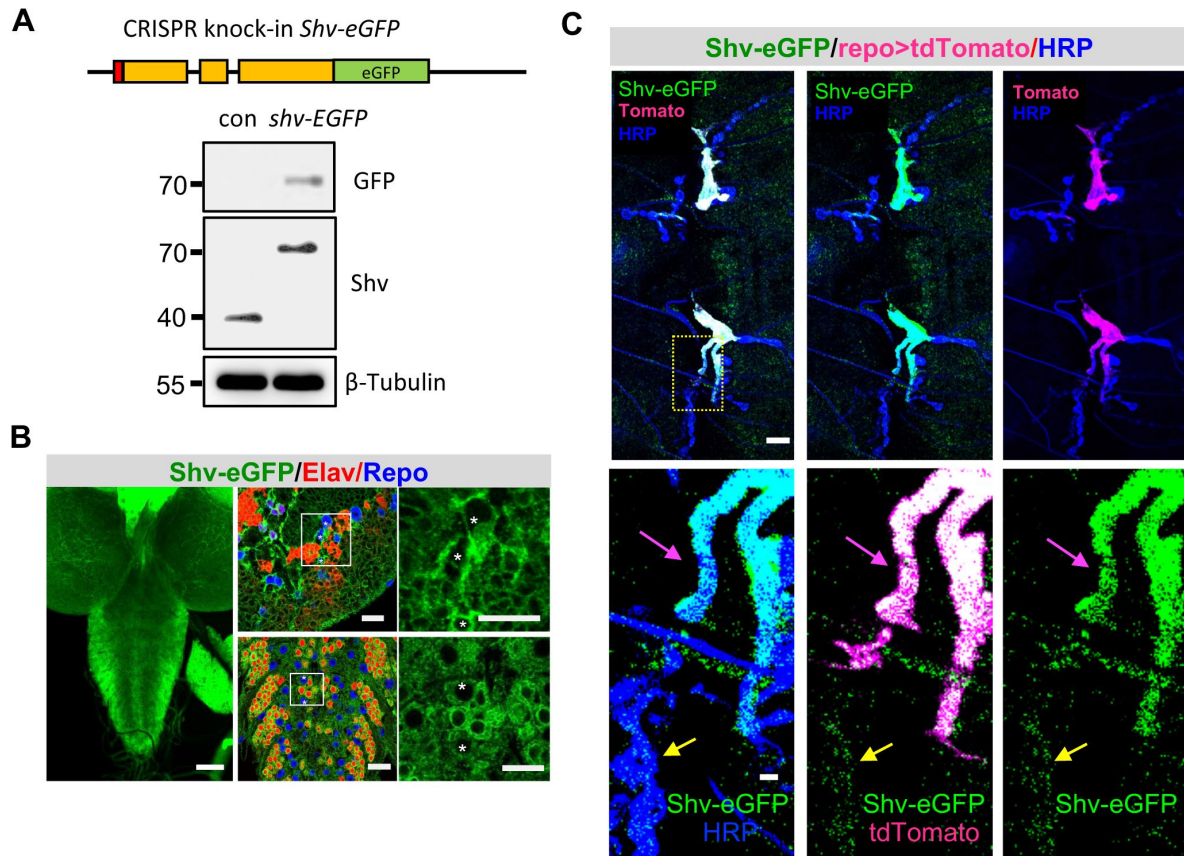


Figure 1.

Endogenous *Shv* tagged with eGFP is detected in glial cells.

(A) Schematic of eGFP knock-in to the *Shv* gene generated using CRISPR/Cas9 system (top). Exons are in orange, signal peptide in red. Western blots using antibodies against GFP and Shv confirms the presence of eGFP in Shv. β-tubulin is used as a loading control. **(B)** Low magnification images of the 3rd instar larval brain showing weak *Shv-eGFP* signal throughout the brain (left). Scale bar = 50 μm. Zoomed in view of the brain hemisphere and ventral nerve cord with neurons and glia marked by *Elav* and *Repo* antibodies, respectively. Asterisks label glial cells with *Shv* expression. Scale bar for brain and VNC are 10 and 15 μm, respectively. **(C)** *Shv-eGFP* can be detected at the NMJ. Glial membrane is marked by membrane targeted *tdTomato* (driven by glial specific *repo-GAL4*) and neuronal membrane labeled by HRP. Zoomed in views show that *Shv-eGFP* colocalizes with glial membrane (magenta arrow) and synaptic boutons (yellow arrow). *Shv-eGFP* also weakly labels the muscle. Note that a single optical slice of the NMJ at muscle 6/7, abdominal segment 2 is shown, which highlights *Shv-eGFP* colocalization with glia and synaptic boutons in this permeabilized prep. The full glial stalk is not visible because it lies in a different focal plane from the branch of interest. Scale bar = 10 μm in the upper panels, and 2 μm in the lower panels.

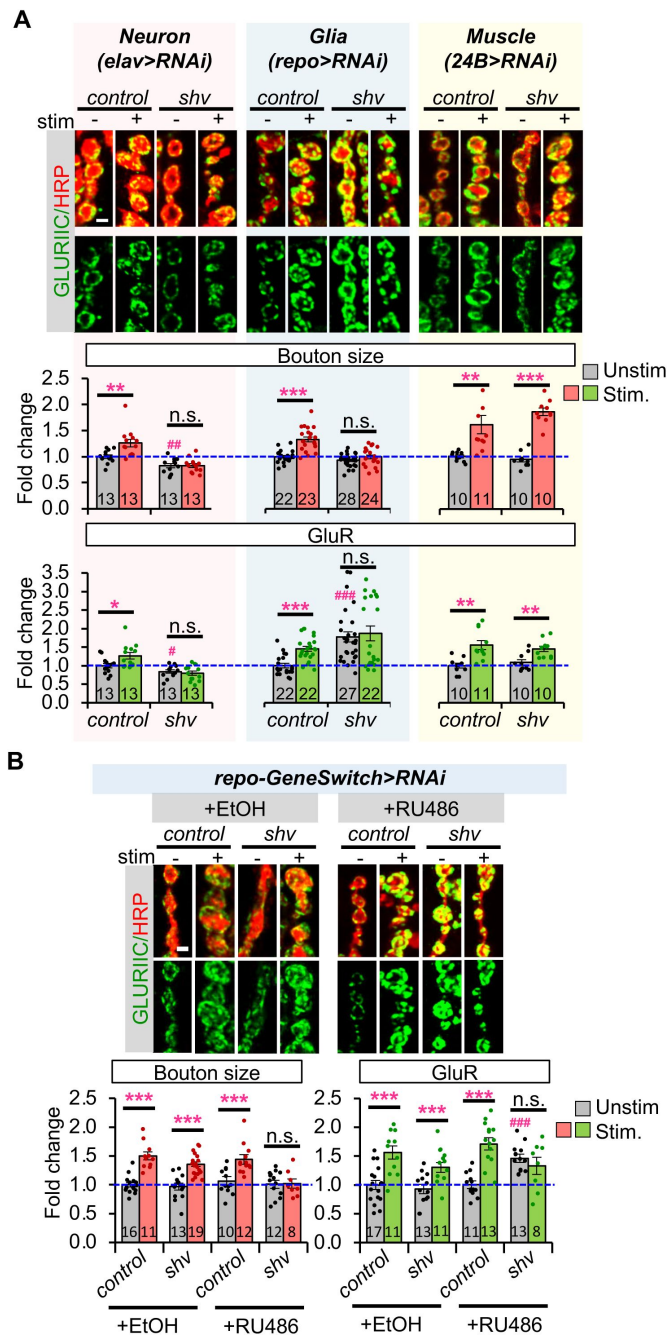


Figure 2.

Shv in glia is necessary for activity-induced synaptic remodeling.

(A) Tissue-specific knockdown of *shv*. Reducing Shv in neurons or glia blocks activity-induced synaptic remodeling, but not when *shv* is knocked down in muscles. Notably, glia-specific knockdown of *shv* increases the levels of GluRIIC, whereas neuronal Shv knockdown decreases basal GluRIIC. (B) Acute knockdown of *shv* in glia using the inducible *repo-GeneSwitch>RNAi* GAL4 driver affects basal GluR intensity and abolishes activity-induced synaptic changes. (A) and (B), Scale bar = 2 μ m. All values are normalized to unstimulated control and presented as mean $\pm$ S.E.M. Control contains TRiP RNAi control vector driven by the indicated driver. Statistics: One-way Anova followed by Tukey's multiple comparison test was used to compare between unstimulated control and unstimulated NMJs across genotypes. Student's t-test was used to compare between unstimulated and stimulated NMJs of the same genotype. # $p \leq 0.05$; ## $p \leq 0.01$; ### $p \leq 0.001$ when comparing unstimulated samples to unstimulated control. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ when comparing stimulated to unstimulated NMJs.

Next, we determined the spatial requirement for Shv in glial cells. The peripheral glia can be divided into three subtypes: wrapping glia (WG) is the innermost layer, wrapping and contacting the peripheral nerve bundle; the subperineurial glial (SPG) covers the WG, establishing the blood-brain barrier; the perineurial glia (PGs) is located on the outermost surface of the nerve that is also part of the blood-brain barrier (Awasaki et al., 2008 [↗](#); Stork et al., 2008 [↗](#); Brink et al., 2012 [↗](#); Freeman, 2015 [↗](#); Fernandes et al., 2024 [↗](#)) (**Fig 3A** [↗](#)). Using GAL4 lines previously shown to drive expression in specific glial subtypes (Stork et al., 2012 [↗](#)), we found that reducing Shv in either SPG or PG was sufficient to block activity-induced synaptic remodeling (**Fig 3B** [↗](#)), phenocopying *shv* knockdown in all glial cells (**Fig 2A** [↗](#)). However, we noticed that knockdown of *shv* in SPG more closely resembles the pan-glial knockdown phenotype, likely because SPG, as the middle glial cell layer in the fly peripheral nervous system, may also influence the PG layer through signaling mechanisms (Lavery et al., 2007 [↗](#)). Conversely, NMJs with *shv* knockdown in wrapping glia (*nrv-Gal4*) exhibited normal activity-induced synaptic remodeling (**Fig 3B** [↗](#)). We also tested whether the astrocyte-like glia located in the central nervous system also contributes by knocking down Shv using *alrm-GAL4* (Doherty et al., 2009 [↗](#)). We observed normal activity-induced synaptic remodeling (**Fig 3** [↗](#)). Together, these results suggest that Shv in PG and SPG glia, both in contact with synaptic boutons, extending into the muscles, and are part of the blood-brain barrier, are crucial for Shv function in glia and for synaptic remodeling.

Glial expression of Shv is sufficient to rescue synaptic plasticity in *shv* mutants

To determine whether Shv from either neuron or glia is sufficient for activity-induced synaptic remodeling, we expressed *shv* (*UAS-Shv*) in either cell type in *shv*¹ mutants, which exhibit defective synaptic remodeling (Lee et al., 2017 [↗](#)). As shown in **Figure 4A** [↗](#), *shv* expression in either neurons or glia was sufficient to restore basal GluR intensity and activity-induced synaptic remodeling; however, glial expression did not normalize bouton size. These data demonstrate that while neuronal Shv is necessary to regulate bouton size during development and modulate synaptic plasticity (**Figure 4A** [↗](#)), Shv derived from glia is also sufficient to maintain basal GluR levels and support activity-induced synaptic modifications.

We have previously shown that neuronal Shv is sufficient to rescue functional plasticity (Lee et al., 2017 [↗](#)), but the role of glial Shv in the process is not known. Electrophysiological recordings demonstrated that *shv*¹ mutants displayed reduced miniature excitatory potential (mEPSP) amplitude but normal evoked EPSP (**Fig 4B** [↗](#)), consistent with a previous report (Lee et al., 2017 [↗](#)). Selective expression of *shv* in glia restored mEPSP amplitude, in line with the rescue in GluR levels. Glial-specific expression of *shv* also rescued post-tetanic potentiation (PTP), an activity-dependent plasticity in *Drosophila* that is functionally similar to the initial stages of long-term potentiation (LTP) (**Fig 4C** [↗](#)). Collectively, these data suggest that glial Shv is sufficient to support functional and structural plasticity.

Glial Shv does not activate integrin signaling

Shv was previously shown to be released by neurons to trigger synaptic remodeling through integrin activation (Lee et al., 2017 [↗](#)); we thus asked whether glial Shv restores synaptic plasticity in *shv*¹ mutants through the same mechanism. **Figure 5A** [↗](#) shows that glia can indeed release Shv, as glial expression of HA-tagged Shv (driven by *repo-GAL4*) can be detected extracellularly when stained using non-permeabilizing conditions. Additionally, Western blot analysis showed that either neuronal or glial expression of Shv-HA resulted in a protein of the same molecular weight (**Figure 5, figure supplement 1A** [↗](#)), suggesting that major differences in protein processing are unlikely. Next, we assessed the effects of glial Shv on integrin signaling by monitoring the levels of phosphorylated focal adhesion kinase (pFAK), as its levels strongly correlate with integrin activation (Mitra et al., 2005 [↗](#); Tsai et al., 2008 [↗](#)). To our surprise, glial expression of *shv* in *shv*¹ mutant did not restore pFAK levels to normal (**Fig 5B** [↗](#)). Furthermore, *shv* knockdown in glial

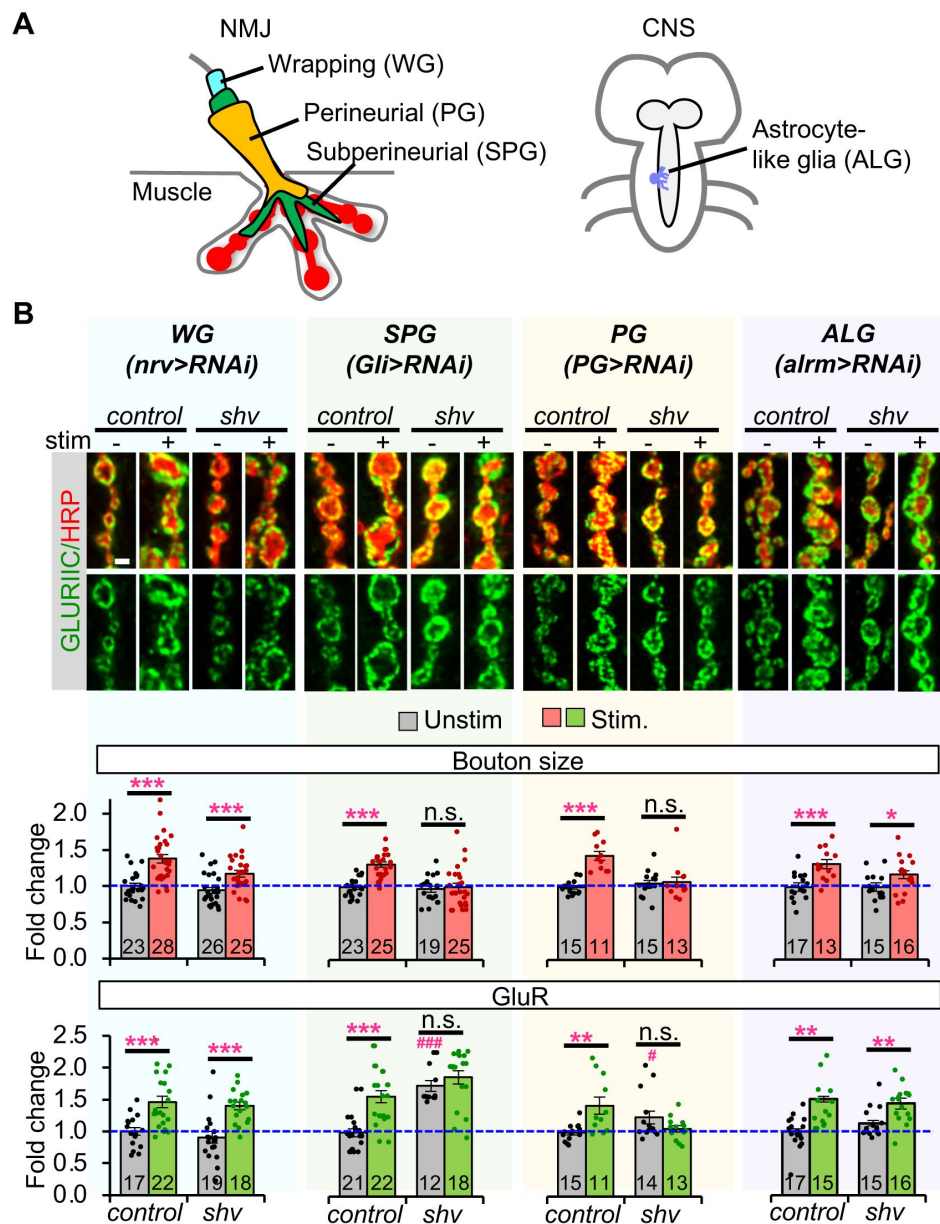


Figure 3.

Expression of *shv* in perineurial and subperineurial glia is required for activity-induced synaptic remodeling.

(A) Diagram of relative membrane position and extension of WG, SPG, and PG at the NMJ. Astrocyte-like glia can be detected in the central brain and ventral nerve cord. **(B)** Representative images and quantification of synaptic changes following knockdown of *shv* in glia subtypes. Knockdown of *shv* in SPG and PG recapitulate the phenotypes of pan-glial knockdown, as well as abolish activity-induced synaptic remodeling. Scale bar = 2 μ m. All values are normalized to unstimulated control and presented as mean $\pm$ S.E.M. Control contains TRiP RNAi control vector driven by the indicated driver. Statistics: One-way Anova followed by Tukey's multiple comparison test was used to compare between unstimulated control and unstimulated NMJs across genotypes. Student's t-test was used to compare between unstimulated and stimulated NMJs of the same genotype. # $p \leq 0.05$; ### $p \leq 0.001$ when comparing unstimulated samples to unstimulated control. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ when comparing stimulated to unstimulated NMJs.

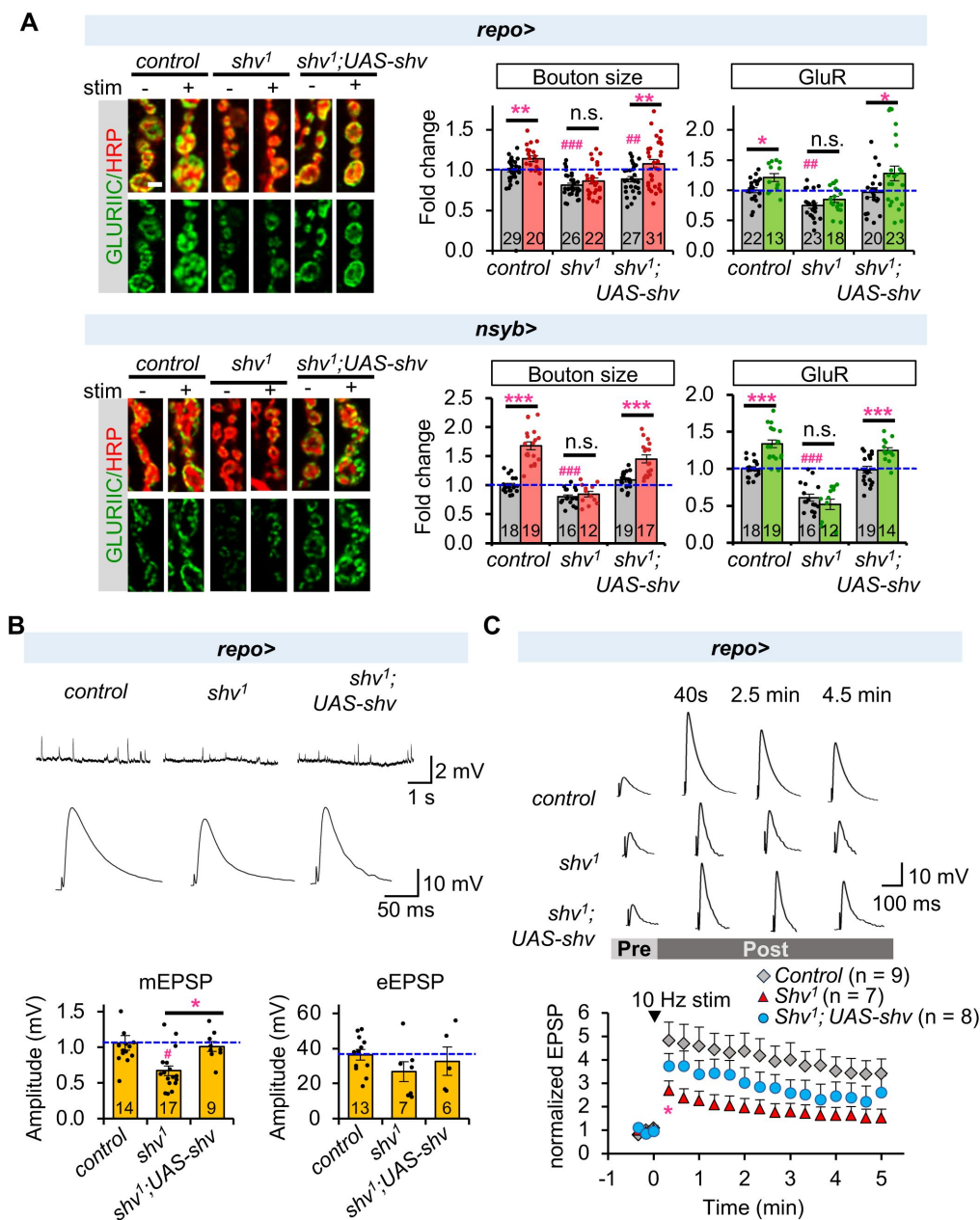


Figure 4.

Glial Shv rescues defective synaptic plasticity observed in *shv*¹ mutant.

(A) Selective expression of *shv* in glia or neurons of *shv*¹ mutants is sufficient to rescue activity-dependent synaptic changes, but glial *shv* expression did not restore basal bouton size. Scale bar = 2 μ m. (B) Representative mEPSP and eEPSP recordings conducted using HL3 solution containing 0.5 mM Ca^{2+} . Average eEPSP amplitude is plotted after nonlinear summation correction. (C) Normalized eEPSP before and following tetanus (10 Hz for 2 min) at the indicated time points. Recordings were done using HL3 solution containing 0.25 mM Ca^{2+} . *shv*¹ showed significantly diminished PTP following stimulation. Expression of *shv* in glial cells of *shv*¹ rescued PTP. The number of NMJs examined is shown in parentheses. Student's t-test was used to compare between control and the indicated genotypes. * shows that *shv*¹ displays PTP that is significantly lower than the control ($p \leq 0.05$), starting from the indicated time and onwards. All values are mean $\pm$ S.E.M. For (A) and (B), one-way Anova followed by Tukey's multiple comparison test was used to compare between unstimulated control and unstimulated NMJs across genotypes. Student's t-test was used to compare between unstimulated and stimulated NMJs of the same genotype. # $p \leq 0.05$; ## $p \leq 0.01$; ### $p \leq 0.001$ when comparing unstimulated samples to unstimulated control. * $p \leq 0.05$; ** $p \leq 0.01$ when comparing stimulated to unstimulated NMJs.

cells exhibited higher pFAK staining compared to the control (**Fig 5C**), a result that is opposite to what was observed with *shv* knockdown in neurons (Lee et al., 2017). *shv* overexpression in glia, although did not change basal pFAK levels, blocked the activity-induced pFAK increases normally seen in control (**Fig 5C**). Collectively, these results suggest that glial Shv does not activate integrin and appears to play an inhibitory role in activity-induced integrin activation.

To elucidate whether glial Shv directly inhibits activity-induced integrin activation, we monitored the amount of Shv released by glia during neuronal activity by expressing Shv-HA in either glia or neurons of *shv*¹ and measured its extracellular levels. **Fig 5D** demonstrates that while stimulation significantly increased extracellular Shv released by neurons, the amount of extracellular Shv released by glial cells was reduced. This activity-induced decrease in glial Shv levels, along with reduced integrin activation (**Fig. 5B**), suggest that glial Shv does not act by directly inhibiting integrin signaling. We further investigated whether the decrease in extracellular glial Shv results from altered release. To address this, we monitored intracellular Shv levels using a permeabilized preparation (we found that detergent treatment stripped away extracellular Shv signal). When combined with non-permeabilized extracellular staining, this approach provides insights into total Shv levels. As shown in **Figure 5D**, there was no intracellular accumulation of Shv and the intracellular levels remained unchanged following stimulation, suggesting that reduced extracellular glial Shv is unlikely due to defects in the release machinery.

Next, we tested the hypothesis that glial Shv instead influences integrin signaling by regulating Shv release from neurons. To this end, we monitored Shv secreted by neurons or glia using the endogenously tagged Shv-eGFP line and tissue-specific knockdown of *shv*. When *shv* was selectively knocked down in glia, an increase in extracellular Shv-eGFP level was observed at the NMJ (**Fig 5E**), which likely originated from neurons. This finding is consistent with a role for glial Shv in suppressing neuronal Shv release and further explains the higher pFAK level observed in case of glial *shv* knockdown (**Fig 5C**). A similar compensatory upregulation from glia was obtained when Shv was knocked down in neurons (**Fig 5E**). To validate that our staining protocol is selective for extracellular proteins, we also stained for cysteine string protein (CSP), an intracellular synaptic vesicle protein predominantly located in the presynaptic terminals (Zinsmaier et al., 1990; Umbach et al., 1994), under the same conditions. CSP was detected only in the permeabilized condition (**Figure 5E**), suggesting that the non-permeabilizing protocol is selective for extracellular proteins. We also monitored the levels of Shv-eGFP in the larval brain, confirming that the *shv*-RNAi approach successfully reduced Shv levels in the selective cell types (**Figure 5 – figure supplement 1B**). Taken together, these results reveal that the amount of Shv released by neurons is homeostatically regulated by Shv produced in glia. Furthermore, unlike neurons, glial Shv release is independent of neuronal activity and does not directly alter integrin signaling.

***Drosophila* peripheral glia and Shv control ambient extracellular glutamate levels to regulate activity-induced synaptic remodeling**

If Shv derived from glia does not activate integrin signaling, how does it restore synaptic plasticity in *shv*¹? One plausible explanation is that glial Shv is required for normal glial growth or survival. We therefore monitored glial morphology when Shv is knocked down in glia by co-expressing membrane targeted *CD4-tdGFP* using the *repo-GAL4* driver. No obvious change in overall glial morphology was observed, with glia continuing to wrap the segmental nerves and extend processes that closely associate with proximal synaptic boutons (**Figure 5, figure supplement 2**). These observations suggest that glial Shv is not essential for maintaining normal glial structure or survival, and is consistent with the idea that glial Shv does not activate integrin, as integrin signaling is required to maintain the integrity of peripheral glial layers (Xie and Auld, 2011; Hunter et al., 2020).

An alternate possibility is that Shv modulates glial function to rescue activity-induced synaptic remodeling. Glial cells have an established role in providing support and maintaining glutamate homeostasis in the nervous system (Augustin et al., 2007 [DOI](#)). The *Drosophila* larval NMJ is a glutamatergic synapse with high ambient glutamate concentration in the hemolymph, with an average in the range of 1-2 mM glutamate (Chen et al., 2009 [DOI](#)). Surprisingly, the larval NMJ does not contain the excitatory amino acid transporter 1 protein (Eaat1), which is essential for removing extracellular glutamate, suggesting that high extracellular glutamate is better tolerated and removed by diffusion through the hemolymph (Rival et al., 2006 [DOI](#); Chen et al., 2009 [DOI](#)). The high ambient glutamate concentration is maintained by the cystine/glutamate antiporter (Cx-T), which imports cystine and exports glutamate into the extracellular milieu (Augustin et al., 2007 [DOI](#); Grosjean et al., 2008 [DOI](#)). The purpose of the high extracellular glutamate is not well understood, but it is thought to control GluR clustering and maintain a reserved pool of GluR intracellularly (Chen et al., 2009 [DOI](#)). Given that we observed significantly upregulated GluR clustering when Shv was reduced in glia, we hypothesized that glial Shv may influence the levels of ambient extracellular glutamate. To detect glutamate level at the synapse, we took advantage of the GAL4/UAS and LexA/LexAop systems to express the glutamate sensor (iGluSnFR) in neurons while simultaneously knocking down or upregulating Shv in glia (Marvin et al., 2013 [DOI](#)) (**Fig 6A** [DOI](#)). Knockdown of Shv in glia significantly reduced iGluSnFR signal, whereas upregulating Shv increased it. As a control, we also expressed Shv in neurons using *Elav-LexA* (**Fig 6A** [DOI](#)). We found neuronal expression of Shv did not alter iGluSnFR signal at the synapse, suggesting that the change in ambient glutamate level is selectively caused by Shv from glia. Additionally, to ascertain that the decrease in iGluSnFR signal reflects a decrease in ambient extracellular glutamate levels rather than glutamate depletion caused by high levels of GluR, we upregulated GluR levels using *mhc-GluRIIA*, which drives GluRIIA expression in muscles (Petersen et al., 1997 [DOI](#)). We found *mhc-GluRIIA* animals exhibited elevated levels of not only GluRIIA but also the obligatory GluRIIC subunit (**Figure 6 – figure supplement 1** [DOI](#)). Despite this increase in GluR expression, we did not observe any change in extracellular glutamate levels, as measured by live imaging using the neuronal iGluSnFR sensor (**Figure 6A** [DOI](#)). Taken together, these results suggest that glial Shv plays a critical role in controlling ambient extracellular glutamate levels.

Next, we set out to determine whether the reduced extracellular ambient glutamate is responsible for the defective synaptic remodeling observed when Shv is depleted in glia. To this end, we incubated the NMJ with HL-3 solution containing 2 mM glutamate for 1 hour before stimulating the NMJ with high KCl. Strikingly, this treatment condition not only corrected basal GluR levels, but also fully rescued the activity-induced synaptic remodeling defect seen in glial knockdown of *shv* (**Fig 6B** [DOI](#)). In contrast, incubating the NMJ with 2 mM glutamate did not correct basal GluR or activity-induced synaptic remodeling defect when Shv is knocked down in neurons (**Figure 6 – figure supplement 2** [DOI](#)). These data are consistent with the iGluSnFR imaging results and further confirm that glial and neuronal Shv acts through distinct pathways to jointly regulate synaptic remodeling.

To further understand the function of glial cells in synaptic plasticity regulation, we ablated glia by inducing *reaper* (*rpr*) expression in early third instar larvae using the inducible *repo-GeneSwitch* driver. This method allows us to examine the acute requirement for glia while avoiding lethality associated with chronic glial ablation. Following RU486 treatment for 24 hours, fragmentation of the glial cell membrane and a reduction in GFP intensity was observed (**Fig 7A** [DOI](#)), indicating degeneration of glial cells. This is consistent with an earlier report demonstrating that Rpr expression is sufficient to induce apoptosis and trigger cell death (White et al., 1996 [DOI](#)). **Fig 7B** [DOI](#) shows that glial ablation caused the same phenotype as *shv* knockdown in glial cells (**Fig 2A** [DOI](#)), with elevated basal GluR levels and blocked activity-induced synaptic remodeling. We hypothesized that if a primary role of glial cells in synaptic plasticity regulation is to maintain ambient extracellular glutamate levels, similar to glial Shv, then correcting extracellular glutamate concentration should be sufficient to restore synaptic plasticity, even in the absence of functional glia. Strikingly, incubating NMJs with 2 mM glutamate not only restored basal GluR levels but also

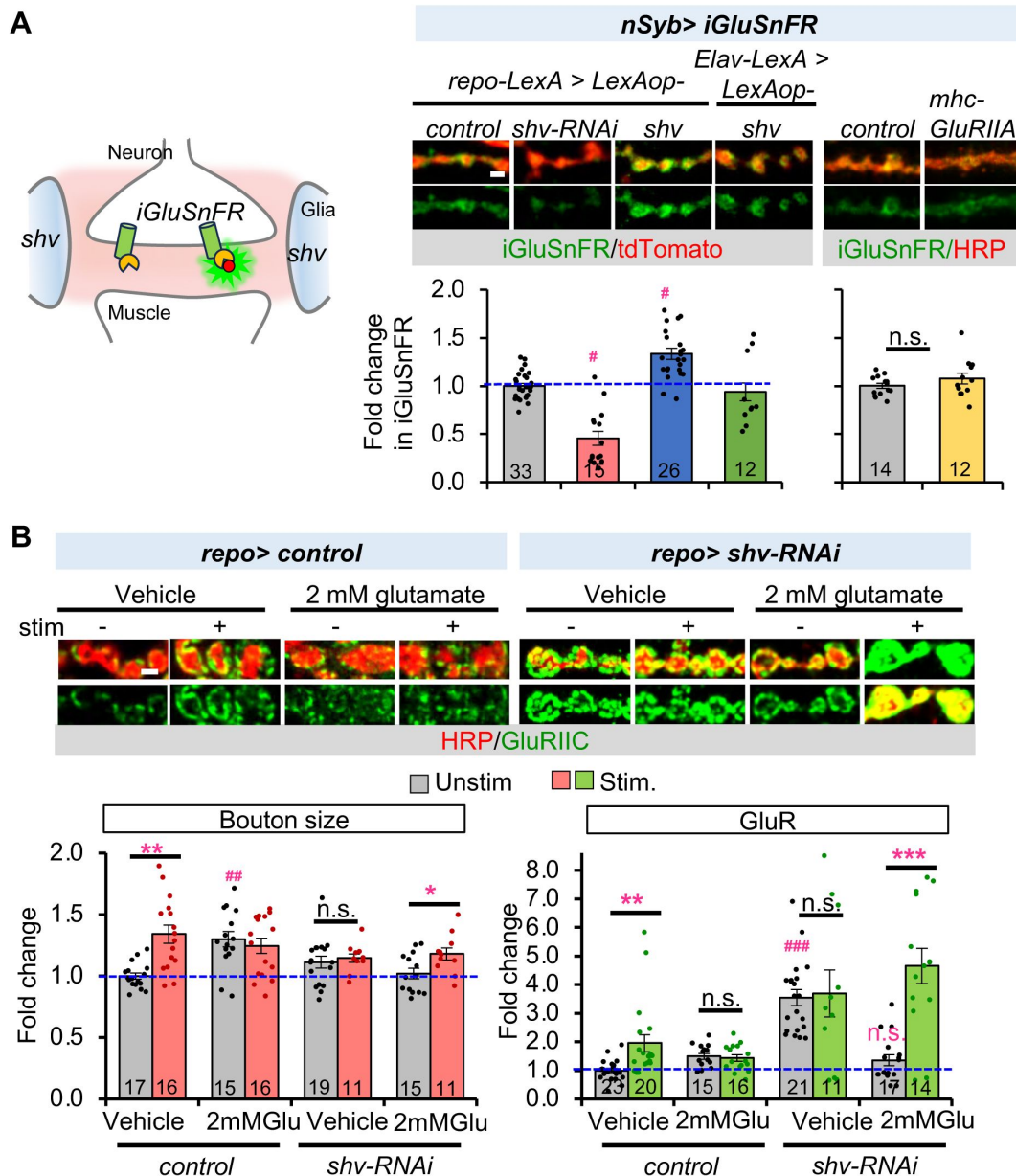


Figure 6.

Glial Shv regulates ambient extracellular glutamate concentration.

(A) Shv expression in glia maintains ambient glutamate concentration at the NMJ. Left panel shows a schematic of the tripartite synapse at the NMJ. iGluSnFR expression in neurons via the GAL4/UAS system can detect extracellular ambient glutamate concentration at the synapse, while glia-specific knockdown or upregulation of Shv is achieved using the LexA/LexAop system. iGluSnFR signals are seen in green and mtdTomato marks neuronal membrane. In addition, overexpression of postsynaptic GluR using *mhc-GluRIIA* doesn't affect iGluSnFR signals. HRP-Cy3 was added after live imaging of iGluSnFR. Lower graph shows quantitation of the relative ambient glutamate concentration at the NMJ. Only Shv from glia affects ambient glutamate concentration, Shv from neurons does not. **(B)** Incubating the NMJ with 2 mM glutamate rescues synaptic remodeling in case of glial Shv knockdown. Vehicle controls represent NMJs dissected in parallel and incubated with HL3 without 2mM glutamate for the same length of time. Scale bar = 2µm. All values are normalized to unstimulated control and presented as mean ± S.E.M. Control contains TRiP RNAi control vector driven by the indicated driver. Statistics: One-way Anova followed by Tukey's multiple comparison test was used to compare between unstimulated control and unstimulated NMJs across genotypes. Student's t-test was used to compare between unstimulated and stimulated NMJs of the same genotype. # $p \leq 0.05$; ### $p \leq 0.001$ when comparing unstimulated samples to unstimulated control. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ when comparing stimulated to unstimulated NMJs.

rescued activity-induced synaptic remodeling caused by glial ablation (**Fig 7B**, **7C**). These results are in line with previous reports that showed low ambient glutamate levels significantly elevated GluR intensity at the *Drosophila* NMJ (Chen et al., 2009), but such increase can be reversed by glutamate supplementation (Augustin et al., 2007; Chen et al., 2009). Together, these data confirm that the ability of peripheral glial cells to maintain high ambient extracellular glutamate concentrations at the NMJ is crucial for synaptic plasticity.

Discussion

In this study, we show that the peripheral glial cells at the *Drosophila* NMJ play an important role in regulating synaptic plasticity. We demonstrate that neurons and glia jointly orchestrate activity-induced synaptic remodeling at the NMJ, with Shv playing a pivotal role. While neurons release Shv in an activity-dependent manner to regulate synaptic remodeling through integrin signaling (Lee et al., 2017), release of Shv by peripheral glial cells does not rely on neuronal activity and does not activate integrin signaling (**Fig 5A-C**). Instead, glial Shv influences activity-induced synaptic remodeling by keeping the levels of Shv released by neurons in check (**Fig 5D-F**) and controlling ambient extracellular glutamate levels (**Fig 7**).

Our data suggest that one mechanism underlying activity-induced synaptic remodeling by glia is through indirect control of Shv release from neurons, thereby maintaining minimal integrin signaling under ambient conditions. We propose that this low baseline integrin signaling enables neurons to respond with high sensitivity to enhanced integrin activation by Shv release from neurons following neuronal activity, leading to rapid synaptic remodeling. Conversely, knocking down Shv in glia removes this suppression, resulting in increased Shv release from neurons, higher basal integrin signaling, GluR levels, and pathway saturation, thereby inhibiting activity-induced synaptic remodeling. Supporting this, overexpression of Shv in neurons elevated basal pFAK and abolished synaptic plasticity (Lee et al., 2017). These findings also raise several intriguing questions, including how neurons and glia distinguish different sources of Shv, and how they sense and communicate this information to regulate extracellular Shv levels from different cells. Based on Western blot analyses of adult heads and larval brains showing that Shv is present as a single band (**Fig. 1A** and **Figure 2 – figure supplement 1B**), the functional differences in neuronal or glial Shv is not likely due to the presence of different isoforms. Consistent with this, FlyBase also suggests that *shv* encodes a single isoform (Ozturk-Colak et al., 2024). However, while we did not detect obvious post-translational modifications when Shv protein was expressed in neurons or glia (**Figure 5 – figure supplement 1A**), we cannot exclude the possibility that different cell types process Shv differently through post-transcriptional or post-translational mechanisms. Notably, *shv* is predicted to undergo A-to-I RNA editing, including an editing site in the coding region, which will result in a single amino acid change (St Laurent et al., 2013). Given that ADAR, the editing enzyme, is enriched in neurons and absent from glia (Jepson et al., 2011), it is possible that such cell-specific editing could contribute to functional differences. It will be interesting to investigate this in the future.

We found that another main function of glial Shv is to regulate ambient extracellular glutamate concentration. We report that overexpression of *shv* elevated ambient glutamate levels at the synapse measured using a genetically encoded glutamate sensor, whereas knockdown of *shv* in glia reduced its level (**Fig 6B**). Furthermore, ablating glia recapitulated the phenotypes of *shv* knockdown in glia, and transiently restoring extracellular ambient glutamate concentration efficiently rescued synaptic plasticity. Based on reports that it has been shown that the *Drosophila* larval NMJ maintains a surprisingly high ambient extracellular glutamate concentration, with an average in the range of 1-2 mM (Augustin et al., 2007). How Shv regulates extracellular glutamate concentration remains to be explored, but a likely mechanism is by affecting the levels or functions of the cystine/glutamate (Cx-T), which imports cystine and exports glutamate into the extracellular matrix. While Shv has been shown to activate integrin, it encodes a homolog of the

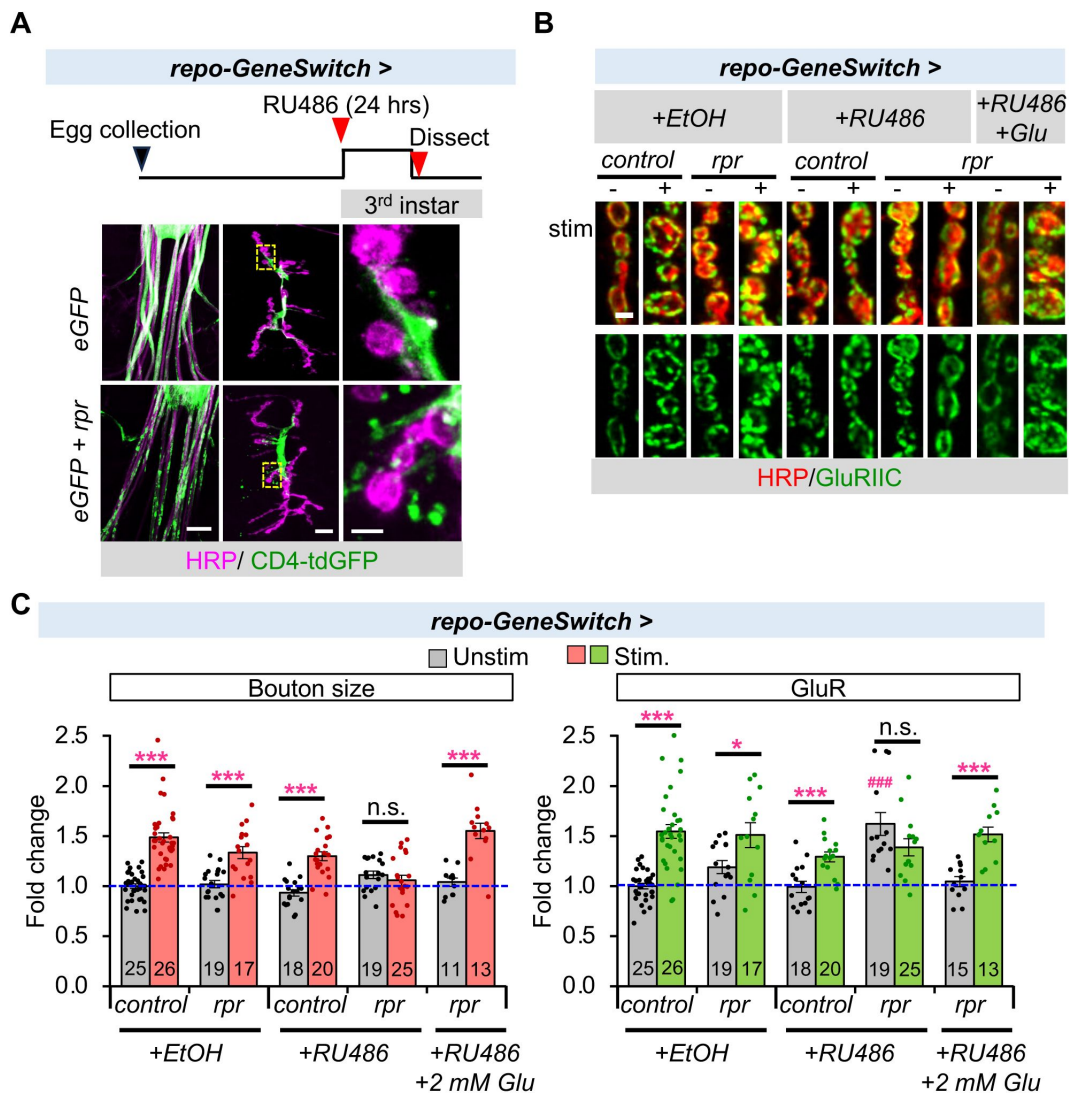


Figure 7.

Acute ablation of glia elevated basal GluR levels and disrupted activity-induced synaptic remodeling, but extracellular glutamate incubation is sufficient to compensate for the loss of glia.

(A) Transient *rpr* expression is sufficient to trigger death of glial cells. Diagram shows the RU486 feeding protocol used to induce glial cell death in 3rd instar larvae. Lower panels show images of the segmental nerves (left) and the NMJ (middle). The zoomed in region of the NMJ (yellow box) is magnified on the right. Glial membrane is marked by CD4-tdGFP, which appears fragmented in the presence of *rpr* expression, indicating glial cell death. Scale bars indicate size in 50, 10 and 2 μ m from left to right. (B) Images and (C) quantitation of synaptic bouton size and GluR abundance. Incubating the NMJ for 10 minutes with 2 mM glutamate is sufficient to overcome loss of glial cells and restore basal GluR level and activity-induced synaptic remodeling, suggesting a main function of peripheral glia in synaptic plasticity regulation is to maintain a high ambient glutamate concentration. Scale bar = 2 μ m in (B). All values are normalized to unstimulated control and presented as mean $\pm$ S.E.M. Control contains TRiP RNAi control vector driven by the indicated driver. Statistics: One-way Anova followed by Tukey's multiple comparison test was used to compare between unstimulated control and unstimulated NMJs across genotypes. Student's t-test was used to compare between unstimulated and stimulated NMJs of the same genotype. ### $p \leq 0.001$ when comparing unstimulated samples to unstimulated control. * $p \leq 0.05$; *** $p \leq 0.001$ when comparing stimulated to unstimulated NMJs.

mammalian DNAJB11 protein (Lee et al., 2016 [↗](#)), which functions as a molecular chaperone vital for proper protein folding in the endoplasmic reticulum (Shen et al., 2002 [↗](#)). Shv thus could potentially be required for the proper folding and the function of the Cx-T, which is located on glial cell membrane at the fly NMJ (Augustin et al., 2007 [↗](#); Grosjean et al., 2008 [↗](#)). Aligned with this, mutations in Cx-T also resulted in elevated basal GluR levels at the fly NMJ (Augustin et al., 2007 [↗](#)). Future studies examining the functional interactions between Cx-T and Shv will shed light on mechanisms for Shv-dependent regulation of ambient extracellular glutamate levels at the NMJ.

How does extracellular glutamate regulate GluR levels and synaptic plasticity? Changes in glutamate levels has been shown to directly impact neurotransmission, glutamate receptor activity, and influence GluR clustering by internalizing the desensitized GluR (Featherstone and Shippey, 2008 [↗](#); Chen et al., 2009 [↗](#); Yao et al., 2018 [↗](#)). We have also shown that basal GluR level is homeostatically regulated by extracellular glutamate concentration (**Fig 7** [↗](#)). It is plausible that a high extracellular glutamate concentration enables the NMJ to maintain a reserved pool of GluRs that can be readily mobilized to the surface upon neuronal activity. Additionally, activation of GluR and downstream signaling pathways could trigger local protein translation machineries to prime the synapses to respond rapidly to changes in neuronal activity. Future studies examining intracellular mechanisms controlling activity-induced GluR increases will lead to better insights on synaptic plasticity regulation.

Materials and Methods

Fly stocks

Flies were maintained at 25°C on a standard fly food consisting of cornmeal, yeast, sugar, and agar, under a 12-hour light/dark cycle unless otherwise specified. The following fly lines were obtained from the Bloomington *Drosophila* Stock Center at Indiana University (BDSC, stock numbers in parentheses), the Vienna *Drosophila* Resource Center and *Drosophila* Genomics Resource Center (VDRC, specified before stock number), or Kyoto stock center (Kyoto, stock number in parentheses): *repo-GAL4* (BDSC, 7415), *24B-GAL4* (BDSC, 1767), *elav-GAL4* (BDSC, 458), *nSyb-GAL4* (BDSC, 51635), *Glutactin-GAL4* (BDSC, 9030), *Nrv-GAL4* (BDSC, 6800), *alrm-GAL4* (BDSC, 67032), *TRiP-RNAi* control (BDSC, 35788), *iGluSnFR* (BDSC, 59611), *UAS-CD4-tdGFP* (BDSC, 35836), *UAS-IVS-myr::tdTomato* (BDSC, 32221), *Elav-LexA* (BDSC, 52676), *UAS-shv-RNAi* (VDRC 108576), *UAS-shv-RNAi*³⁷⁵⁰⁷ (BDSC, 37507), *Mhc.GluRIIA.Myc* (BDSC, 64258), *PG-GAL4* (also known as *NP6293-GAL4*, Kyoto, 105188), and *repo-GeneSwitch-GAL4* (Artiushin et al., 2018 [↗](#)), *repo-LexA* (a gift from Dr. Henry Y. Sun), *UAS-Shv* and *shv*¹ (Lee et al., 2017 [↗](#)).

To visualize the endogenous levels of Shv, Shv with eGFP inserted into the 3'-end of the endogenous Shv gene (Shv-eGFP) was generated by CRISPR/Cas9 genome editing, and homology directed repair (HDR) as described (Gratz et al., 2014 [↗](#)). Briefly, a target Cas9 cleavage site in Shv was selected at the 3' end of Shv without obvious off-target sequence in the *Drosophila* genome using CRISPR optimum Target Finder. The sgRNA target sequence: 5'-GCCGGCGAGCACTTTTATTG was cloned into the pU6-BbsI-chiRNA vector (Addgene, #45946). The vector for HDR contains the 5' homology arm containing Shv genomic region (1000 base pairs at the 3' end of the Shv gene with the stop codon removed) plus eGFP sequence, and the 3' homology arm (1020 base pair downstream of the Cas9 cleavage site in the Shv gene region) was cloned into pHD-DsRed (Addgene, # 51434). Both cloned vectors were injected into fly stocks containing Cas9 (BDSC 55821) and positive genome editing screen by the presence of DsRed by BestGene Inc. Insertion was confirmed by sequencing and Western blots.

LexAop-Shv RNAi and LexAop-Shv fly lines were generated by subcloning Shv-RNAi hairpin sequence based on VDRC Shv-RNAi design or the coding regions of Shv which contains a V5 tag into the pJFRC19-13XLexAop2-IVS-myr::GFP vector (Addgene, 26224). Both lines were inserted into the X chromosome specific position by microinjecting the plasmid into a P{CaryP}attP18 fly line (BDSC, 32107).

RU486 feeding

repo-GeneSwitch embryos were collected in 3 hours windows and early 3rd instar larvae (48 hours after larval hatching), were fed with food containing 10 μ M RU486 (1:1000, 10 mM stock) or 0.1% EtOH (vehicle control) for 24 hours before dissection (Gatto and Broadie, 2008 [↗](#)).

Immunocytochemistry

Dissection and stimulation of the third-instar larvae fillets were done as described (Lee et al., 2017 [↗](#)). Briefly, dissected NMJs were fixed for 3 minutes at room temperature (RT) with Boutin's fixative for GluR staining. For all other antibodies, 4% paraformaldehyde was used (25 minutes at RT). The fixed samples were subsequently washed with either 0.1% Triton X-100 in PBS (PBST) or with PBS alone for a detergent-free condition. The samples were then blocked with 5% normal goat serum in either PBST or PBS, as specified. Primary antibodies used were: Rabbit anti-pFAK, 1:250 (Invitrogen, 44-624G), Rabbit anti-HA, 1:1000 (Sigma, H6908), Rabbit anti-GluRIIC, 1:1000 (Chang et al., 2024 [↗](#)), rat anti-Elav, 1:500 (7E8A10, Developmental Studies Hybridoma Bank at the University of Iowa (DSHB)); mouse anti-Repo, 1:50 (8D12, DSHB); mouse anti-GFP, 1:100 (4C9, DSHB). Cy3/Alexa-647 conjugated anti-HRP, 1:100 for non-permeabilized staining or 1:250 for permeabilized staining (Jackson ImmunoResearch). For iGluSnFR imaging experiments involving *Mhc-GluRIIA*, HRP staining was done after live imaging by incubating with HRP-Cy3 for 5 mins. Secondary antibodies used were Alexa Fluor 488 or 405 conjugated, diluted at 1:250 (Invitrogen). For experiments with glutamate incubation, dissected preps were incubated with 2mM Glutamate solution for 1hr or 10 min before performing stimulation protocol as indicated. Mock controls were treated the same way but without 2mM glutamate.

Imaging and image analysis

Images of synaptic boutons from muscle 6/7, A2 or A3 were taken using Olympus FV3000 confocal microscope at 60X with 1.6 zoom. Images showing expression patterns were taken at lower magnifications with either 10X or 60X objectives. 40x water objectives is used when performing live imaging of iGluSnFR. Mock controls (unstimulated larvae) were always dissected, immunostained, and imaged in parallel with stimulated genotypes for each experiment using the same conditions. Type Ib boutons were analyzed to establish changes in bouton size and GluR levels. Image analyses for GluRIIC, bouton size and extracellular staining were done using the same protocol as described (Lee et al., 2017 [↗](#)).

Electrophysiology

Third-instar larvae were dissected and immersed in a modified HL-3 solution containing NaCl (70 mM), KCl (5 mM), MgCl₂ (10 mM), sucrose (115 mM), HEPES (5 mM), trehalose (5 mM), and NaHCO₃ (10 mM), at pH 7.2, with specified concentrations of CaCl₂ (0.25 or 0.5 mM). Current-clamp recordings were performed on muscle area 6 in abdominal segments A2 or A3, using suction electrodes to stimulate the severed ventral nerves with a 0.3 ms stimulus duration. The recording electrode, filled with 3 M KCl and having a resistance between 15 and 40 M Ω , was used to acquire data. Data with resting potentials more hyperpolarized than -60 mV were analyzed, while datasets were excluded if resting potentials deviated by more than 10 mV during recording or if there was a sudden drop in EPSP amplitude, indicating incomplete nerve function. The experimental setup included an Axopatch 200B Amplifier, a Digidata 1440A for digitization, and pClamp 10.3 software

(Molecular Devices) for control. Data analysis was conducted using MiniAnalysis (Synaptosoft), Clampfit (Molecular Devices), and Microsoft Excel, with nonlinear summation applied to correct the average EPSP.

Western blot

Drosophila larval brain extract was obtained by homogenizing 6-10 larval brains collected on ice in RIPA buffer with EDTA (50 mM Tris-HCl, pH7.5, 1% NP-40, 0.5% NaDoc, 150 mM NaCl, 0.1% SDS, 10 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 250 nM cycloporin A, protease inhibitor cocktail (Roche) using mortar and pestle. Protein homogenate was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Primary antibodies were diluted in blocking solution as following: rabbit anti-Shv, 1:400 (Lee et al., 2017 [DOI](#)); Rabbit Anti-GFP, 1:1000 (NOVUS, NB600-308), Anti- β -tubulin, 1:500 (E7, DSHB), Mouse Anti-Complex V 1:10000 (MitoScience).

Statistical Analysis

All data are shown as mean $\pm$ SEM. Sample sizes, indicated in the graphs or figure legends, represent biological replicates and adhere to established standards in the literature. Comparisons between unstimulated and stimulated samples of the paired genotype were made using Student's t-test. For comparisons involving multiple samples, one-way ANOVA followed by Tukey's multiple comparison test was employed to determine statistical significance. To minimize bias, all samples were randomized during dissection, image collection, and data analysis.

Data Availability

Additional information and requests for reagents used in this study are available upon request.

Acknowledgements

We would like to thank Dr. Amita Seghal and Dr. Henry Y. Sun for the generous gift of *repo-GeneSwitch-GAL4* and *repo-LexA*, respectively. K.T.C. is supported by NIH grants R01NS102260 and R01NS080946.

Additional information

Author contributions

Y-C.C. performed most of the experiments and data analyses. Y-J.P. performed the electrophysiology experiments. A.W. helped with data analyses. J.Y.L. designed and helped to generate the Shv-eGFP line using CRISPR/Cas9, K.T.C. conceived, designed, and supervised all aspects of the project. Y-C.C. and K.T.C. wrote the manuscript with inputs from Y-J.P. All authors reviewed and edited the manuscript.

Funding

NIH/NINDS (R01NS102260)

NIH/NINDS (R01NS080946)

Supplementary Figures

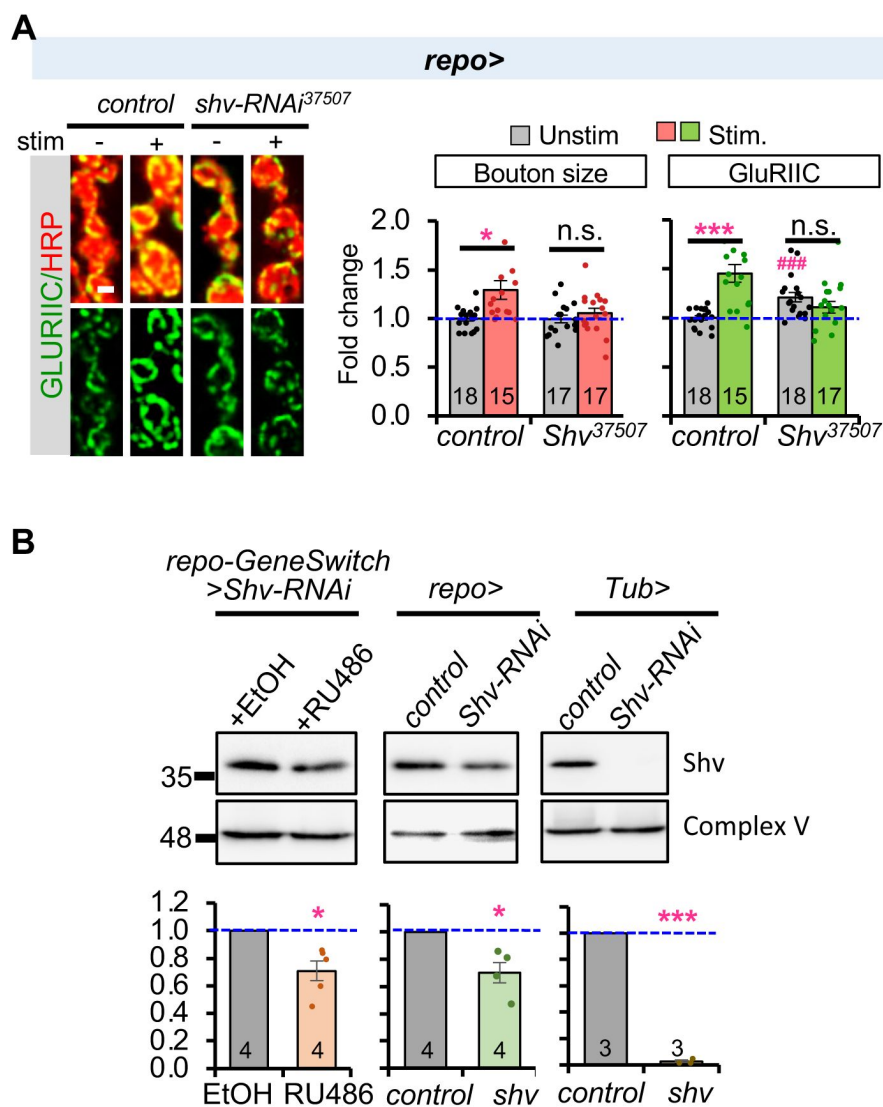


Figure 2 – figure supplement 1.

Independent *Shv-RNAi* line and efficacy of *shv* knockdown by the GeneSwitch system.

(A) Glial specific knockdown of *shv* by *shv-RNAi*³⁷⁵⁰⁷ recapitulates the *shv-RNAi* phenotypes shown in Fig 2. All values are normalized to unstimulated control and presented as mean $\pm$ S.E.M. Statistics: One-way Anova followed by Tukey's multiple comparison test was used to compare between unstimulated control and unstimulated NMJs across genotypes. Student's t-test was used to compare between unstimulated and stimulated NMJs of the same genotype. ### $p \leq 0.001$ when comparing unstimulated samples to unstimulated control. * $p \leq 0.05$; *** $p \leq 0.001$ when comparing stimulated to unstimulated NMJs. (B) Western blot against Shv to show the efficiency of the GeneSwitch system. Complex V is used as internal control. Control contains TriP RNAi control vector driven by the indicated driver. Student's t-test was used to compare control to knockdown. * $p \leq 0.05$; *** $p \leq 0.001$.

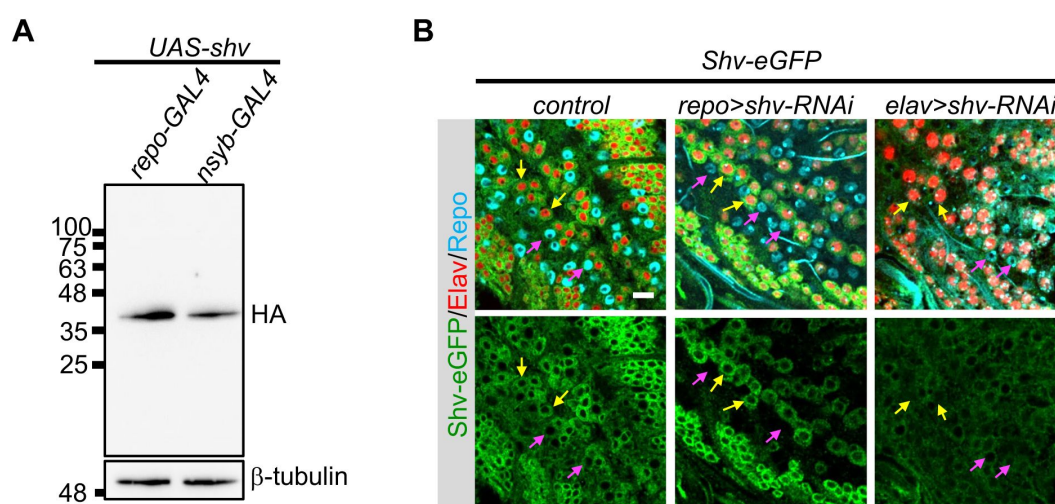


Figure 5 – figure supplement 1.

Neuronal and glial expression of Shv and efficiency of *shv* knockdown by RNAi in neurons and glia.

(A) Western blots using antibodies against HA show no detectable post-translational modification between glial and neuronal OE Shv. β -tubulin is used as a loading control. **(B)** Staining of the larval brain confirms that the RNAi approach effectively reduces Shv-eGFP in the selective cell types. Yellow arrows highlight neurons (Elav positive), magenta arrows point to glial cells (Repo positive). Scale bar = 10 μ m.

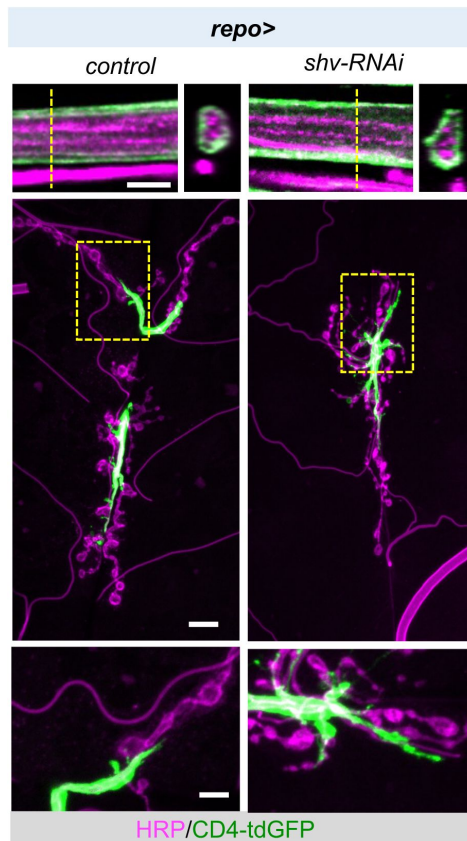


Figure 5 – figure supplement 2.

Integrity of peripheral glial cell membranes.

The top panels show representative images of the segmental nerves in the XY axis (left) and the orthogonal YZ axis view (right). The dashed line indicates the orthogonal projection plane. Knockdown of *shv* in glia doesn't affect the overall glial membrane integrity. Scale bar = 5 μ m. Middle panels show that knockdown of *shv* in glia does not alter the general morphology of peripheral glia at the NMJ. Scale bar = 10 μ m. Lower panels show magnified view of glia closely associated with proximal synaptic boutons (boxed area in the upper panels). Scale bar = 2 μ m.

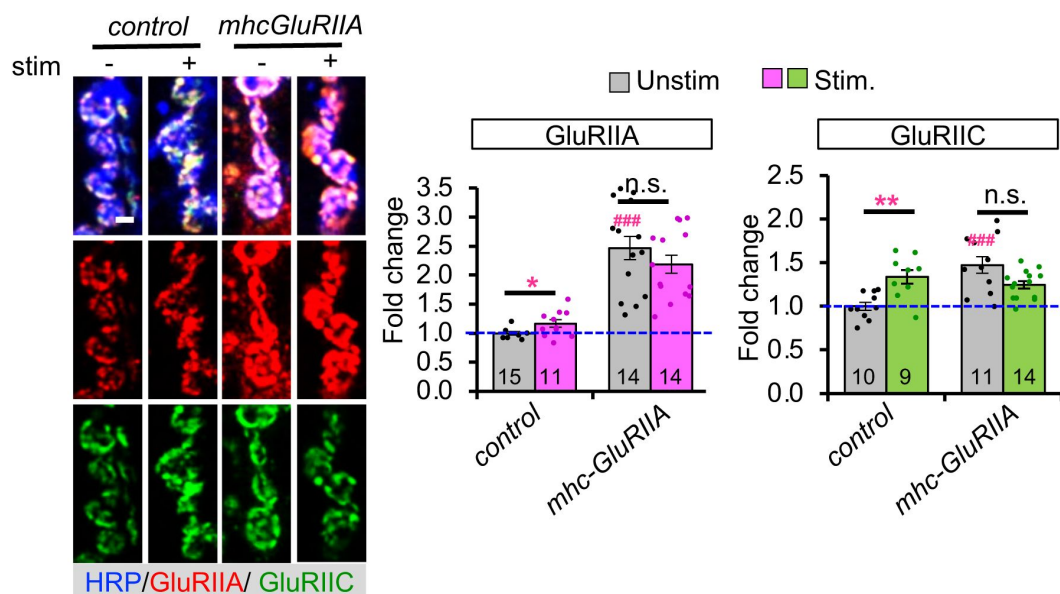


Figure 6 - figure supplement 1.

Validation of GluR intensity in *MhcGluRIIA*.

(A) *MhcGluRIIA* line shows higher basal GluRIIA and GluRIIC levels that failed to further increase upon stimulation. All values are normalized to unstimulated control and presented as mean ± S.E.M. Statistics: One-way Anova followed by Tukey's multiple comparison test was used to compare between unstimulated control and unstimulated NMJs across genotypes. Student's t-test was used to compare between unstimulated and stimulated NMJs of the same genotype. ### p ≤ 0.001 when comparing unstimulated samples to unstimulated control. *p ≤ 0.05; **p ≤ 0.001 when comparing stimulated to unstimulated NMJs.

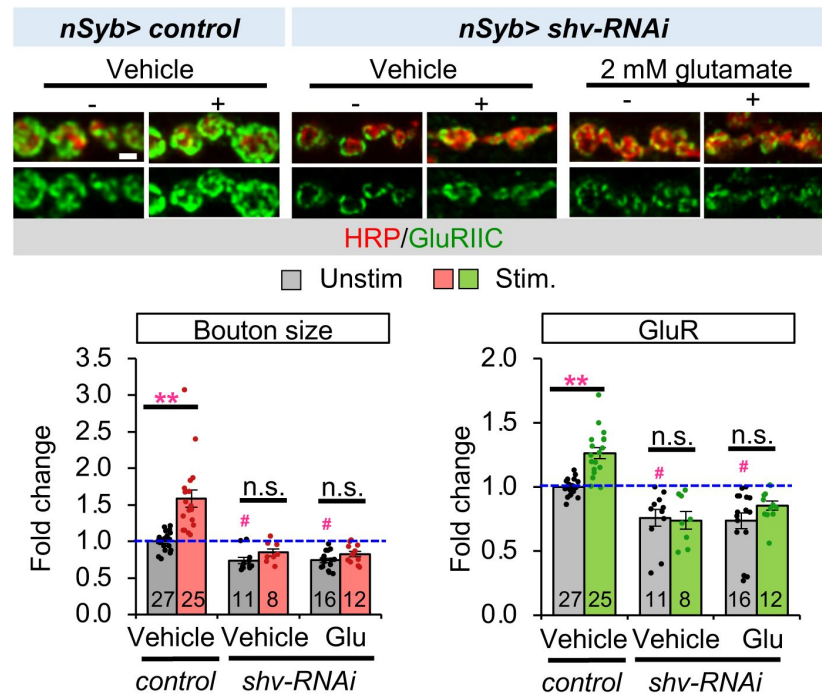


Figure 6 – figure supplement 2.

Defective activity-induced synaptic remodeling caused by the loss of neuronal Shv is not rescued by incubation with 2 mM glutamate.

Vehicle controls represent NMJs dissected in parallel and incubated with HL3 without 2mM glutamate for the same length of time. Scale bar = 2µm. All values are normalized to unstimulated control and presented as mean ± S.E.M. Control contains TRiP RNAi control vector driven by the indicated driver. Statistics: One-way Anova followed by Tukey's multiple comparison test was used to compare between unstimulated control and unstimulated NMJs across genotypes. Student's t-test was used to compare between unstimulated and stimulated NMJs of the same genotype. # $p \leq 0.05$ when comparing unstimulated samples to unstimulated control. * $p \leq 0.05$ when comparing stimulated to unstimulated NMJs.

References

- Araque A, Navarrete M (2010) **Glial cells in neuronal network function** *Philos Trans R Soc Lond B Biol Sci* **365**:2375–2381 [Google Scholar](#)
- Artiushin G, Zhang SL, Tricoire H, Sehgal A (2018) **Endocytosis at the Drosophila blood-brain barrier as a function for sleep** *eLife* **7** [Google Scholar](#)
- Augustin H, Grosjean Y, Chen K, Sheng Q, Featherstone DE (2007) **Nonvesicular release of glutamate by glial xCT transporters suppresses glutamate receptor clustering in vivo** *J Neurosci* **27**:111–123 [Google Scholar](#)
- Awasaki T, Lai SL, Ito K, Lee T (2008) **Organization and postembryonic development of glial cells in the adult central brain of Drosophila** *J Neurosci* **28**:13742–13753 [Google Scholar](#)
- Banerjee S, Bhat MA (2008) **Glial ensheathment of peripheral axons in Drosophila** *J Neurosci Res* **86**:1189–1198 [Google Scholar](#)
- Brink DL, Gilbert M, Xie X, Petley-Ragan L, Auld VJ (2012) **Glial processes at the Drosophila larval neuromuscular junction match synaptic growth** *PloS one* **7**:e37876 [Google Scholar](#)
- Chang YC, Gao Y, Lee JY, Peng YJ, Langen J, Chang KT (2024) **Identification of secretory autophagy as a mechanism modulating activity-induced synaptic remodeling** *Proc Natl Acad Sci U S A* **121**:e2315958121 [Google Scholar](#)
- Chen K, Augustin H, Featherstone DE (2009) **Effect of ambient extracellular glutamate on Drosophila glutamate receptor trafficking and function** *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* **195**:21–29 [Google Scholar](#)
- Citri A, Malenka RC (2008) **Synaptic plasticity: multiple forms, functions, and mechanisms** *Neuropsychopharmacology* **33**:18–41 [Google Scholar](#)
- Danjo R, Kawasaki F, Ordway RW (2011) **A tripartite synapse model in Drosophila** *PloS one* **6**:e17131 [Google Scholar](#)
- Doherty J, Logan MA, Tasdemir OE, Freeman MR (2009) **Ensheathing glia function as phagocytes in the adult Drosophila brain** *J Neurosci* **29**:4768–4781 [Google Scholar](#)
- Featherstone DE, Shippy SA (2008) **Regulation of synaptic transmission by ambient extracellular glutamate** *The Neuroscientist: a review journal bringing neurobiology, neurology and psychiatry* **14**:171–181 [Google Scholar](#)
- Fernandes VM, Auld V, Klambt C (2024) **Glia as Functional Barriers and Signaling Intermediaries** *Cold Spring Harb Perspect Biol* **16** [Google Scholar](#)
- Freeman MR (2015) **Drosophila Central Nervous System Glia** *Cold Spring Harb Perspect Biol* **7** [Google Scholar](#)
- Fuentes-Medel Y, Logan MA, Ashley J, Ataman B, Budnik V, Freeman MR (2009) **Glia and muscle sculpt neuromuscular arbors by engulfing destabilized synaptic boutons and shed presynaptic debris** *PLoS Biol* **7**:e1000184 [Google Scholar](#)

- Fuentes-Medel Y, Ashley J, Barria R, Maloney R, Freeman M, Budnik V (2012) **Integration of a retrograde signal during synapse formation by glia-secreted TGF-beta ligand** *Current biology: CB* **22**:1831–1838 [Google Scholar](#)
- Gatto CL, Broadie K (2008) **Temporal requirements of the fragile X mental retardation protein in the regulation of synaptic structure** *Development* **135**:2637–2648 [Google Scholar](#)
- Gratz SJ, Ukken FP, Rubinstein CD, Thiede G, Donohue LK, Cummings AM, O'Connor-Giles KM (2014) **Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in Drosophila** *Genetics* **196**:961–971 [Google Scholar](#)
- Grosjean Y, Grillet M, Augustin H, Ferveur JF, Featherstone DE (2008) **A glial amino-acid transporter controls synapse strength and courtship in Drosophila** *Nat Neurosci* **11**:54–61 [Google Scholar](#)
- Hunter AC, Petley-Ragan LM, Das M, Auld VJ (2020) **Basigin Associates with Integrin in Order to Regulate Perineurial Glia and Drosophila Nervous System Morphology** *J Neurosci* **40**:3360–3373 [Google Scholar](#)
- Jepson JEC, Savva YA, Yokose C, Sugden AU, Sahin A, Reenan RA (2011) **Engineered alterations in RNA editing modulate complex behavior in Drosophila: regulatory diversity of adenosine deaminase acting on RNA (ADAR) targets** *J Biol Chem* **286**:8325–8337 [Google Scholar](#)
- Keller LC, Cheng L, Locke CJ, Muller M, Fetter RD, Davis GW (2011) **Glial-derived prodegenerative signaling in the Drosophila neuromuscular system** *Neuron* **72**:760–775 [Google Scholar](#)
- Kerr KS, Fuentes-Medel Y, Brewer C, Barria R, Ashley J, Abruzzi KC, Sheehan A, Tasdemir-Yilmaz OE, Freeman MR, Budnik V (2014) **Glial wingless/Wnt regulates glutamate receptor clustering and synaptic physiology at the Drosophila neuromuscular junction** *J Neurosci* **34**:2910–2920 [Google Scholar](#)
- Kim T, Song B, Lee IS (2020) **Drosophila Glia: Models for Human Neurodevelopmental and Neurodegenerative Disorders** *Int J Mol Sci* **21** [Google Scholar](#)
- Kottmeier R, Bittern J, Schoofs A, Scheiwe F, Matzat T, Pankratz M, Klambt C (2020) **Wrapping glia regulates neuronal signaling speed and precision in the peripheral nervous system of Drosophila** *Nat Commun* **11**:4491 [Google Scholar](#)
- Lamprecht R, LeDoux J (2004) **Structural plasticity and memory** *Nat Rev Neurosci* **5**:45–54 [Google Scholar](#)
- Lavery W, Hall V, Yager JC, Rottgers A, Wells MC, Stern M (2007) **Phosphatidylinositol 3-kinase and Akt nonautonomously promote perineurial glial growth in Drosophila peripheral nerves** *J Neurosci* **27**:279–288 [Google Scholar](#)
- Lee JY, Chen JY, Shaw JL, Chang KT (2016) **Maintenance of Stem Cell Niche Integrity by a Novel Activator of Integrin Signaling** *PLoS Genet* **12**:e1006043 [Google Scholar](#)
- Lee JY, Geng J, Lee J, Wang AR, Chang KT (2017) **Activity-Induced Synaptic Structural Modifications by an Activator of Integrin Signaling at the** *J Neurosci* **37**:3246–3263 [Google Scholar](#)

- Marvin JS, Borghuis BG, Tian L, Cichon J, Harnett MT, Akerboom J, Gordus A, Renninger SL, Chen TW, Bargmann CI, Orger MB, Schreiter ER, Demb JB, Gan WB, Hires SA, Looger LL (2013) **An optimized fluorescent probe for visualizing glutamate neurotransmission** *Nat Methods* **10**:162–170 [Google Scholar](#)
- Mitra SK, Hanson DA, Schlaepfer DD (2005) **Focal adhesion kinase: in command and control of cell motility** *Nat Rev Mol Cell Biol* **6**:56–68 [Google Scholar](#)
- Osterwalder T, Yoon KS, White BH, Keshishian H (2001) **A conditional tissue-specific transgene expression system using inducible GAL4** *Proc Natl Acad Sci U S A* **98**:12596–12601 [Google Scholar](#)
- Ozturk-Colak A, Marygold SJ, Antonazzo G, Attrill H, Goutte-Gattat D, Jenkins VK, Matthews BB, Millburn G, Dos Santos G, Tabone CJ, FlyBase C (2024) **FlyBase: updates to the Drosophila genes and genomes database** *Genetics* **227** [Google Scholar](#)
- Petersen SA, Fetter RD, Noordermeer JN, Goodman CS, DiAntonio A (1997) **Genetic analysis of glutamate receptors in Drosophila reveals a retrograde signal regulating presynaptic transmitter release** *Neuron* **19**:1237–1248 [Google Scholar](#)
- Petley-Ragan LM, Ardiel EL, Rankin CH, Auld VJ (2016) **Accumulation of Laminin Monomers in Drosophila Glia Leads to Glial Endoplasmic Reticulum Stress and Disrupted Larval Locomotion** *J Neurosci* **36**:1151–1164 [Google Scholar](#)
- Rey S, Ohm H, Moschref F, Zeuschner D, Praetz M, Klambt C (2023) **Glial-dependent clustering of voltage-gated ion channels in Drosophila precedes myelin formation** *eLife* **12** [Google Scholar](#)
- Rival T, Soustelle L, Strambi C, Besson MT, Iche M, Birman S (2004) **Decreasing glutamate buffering capacity triggers oxidative stress and neurofil degeneration in the Drosophila brain** *Current biology: CB* **14**:599–605 [Google Scholar](#)
- Rival T, Soustelle L, Cattaert D, Strambi C, Iche M, Birman S (2006) **Physiological requirement for the glutamate transporter dEAAT1 at the adult Drosophila neuromuscular junction** *J Neurobiol* **66**:1061–1074 [Google Scholar](#)
- Robinow S, White K (1991) **Characterization and spatial distribution of the ELAV protein during Drosophila melanogaster development** *J Neurobiol* **22**:443–461 [Google Scholar](#)
- Roman G, Endo K, Zong L, Davis RL (2001) **P[Switch], a system for spatial and temporal control of gene expression in Drosophila melanogaster** *Proc Natl Acad Sci U S A* **98**:12602–12607 [Google Scholar](#)
- Sancho L, Contreras M, Allen NJ (2021) **Glia as sculptors of synaptic plasticity** *Neurosci Res* **167**:17–29 [Google Scholar](#)
- Shen Y, Meunier L, Hendershot LM (2002) **Identification and characterization of a novel endoplasmic reticulum (ER) Dnaj homologue, which stimulates ATPase activity of BiP in vitro and is induced by ER stress** *J Biol Chem* **277**:15947–15956 [Google Scholar](#)
- St Laurent G, Tackett MR, Nechkin S, Shtokalo D, Antonets D, Savva YA, Maloney R, Kapranov P, Lawrence CE, Reenan RA (2013) **Genome-wide analysis of A-to-I RNA editing by single-molecule sequencing in Drosophila** *Nat Struct Mol Biol* **20**:1333–1339 [Google Scholar](#)

- Stork T, Bernardos R, Freeman MR (2012) **Analysis of glial cell development and function in *Drosophila*** *Cold Spring Harb Protoc* **2012**:1–17 [Google Scholar](#)
- Stork T, Engelen D, Krudewig A, Silies M, Bainton RJ, Klambt C (2008) **Organization and function of the blood-brain barrier in *Drosophila*** *J Neurosci* **28**:587–597 [Google Scholar](#)
- Tsai PI, Kao HH, Grabbe C, Lee YT, Ghose A, Lai TT, Peng KP, Van Vactor D, Palmer RH, Chen RH, Yeh SR, Chien CT (2008) **Fak56 functions downstream of integrin alphaPS3betanu and suppresses MAPK activation in neuromuscular junction growth** *Neural Dev* **3**:26 [Google Scholar](#)
- Umbach JA, Zinsmaier KE, Eberle KK, Buchner E, Benzer S, Gunderson CB (1994) **Presynaptic dysfunction in *Drosophila* csp mutants** *Neuron* **13**:899–907 [Google Scholar](#)
- White K, Tahaoglu E, Steller H (1996) **Cell killing by the *Drosophila* gene reaper** *Science* **271**:805–807 [Google Scholar](#)
- Xie X, Auld VJ (2011) **Integrins are necessary for the development and maintenance of the glial layers in the *Drosophila* peripheral nerve** *Development* **138**:3813–3822 [Google Scholar](#)
- Xiong WC, Okano H, Patel NH, Blendy JA, Montell C (1994) **repo encodes a glial-specific homeo domain protein required in the *Drosophila* nervous system** *Genes Dev* **8**:981–994 [Google Scholar](#)
- Yao L, Grand T, Hanson JE, Paoletti P, Zhou Q (2018) **Higher ambient synaptic glutamate at inhibitory versus excitatory neurons differentially impacts NMDA receptor activity** *Nat Commun* **9**:4000 [Google Scholar](#)
- Zinsmaier KE, Hofbauer A, Heimbeck G, Pflugfelder GO, Buchner S, Buchner E (1990) **A cysteine-string protein is expressed in retina and brain of *Drosophila*** *J Neurogenet* **7**:15–29 [Google Scholar](#)

Author information

Yen-Ching Chang

Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles, United States

Yi-Jheng Peng

Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles, United States

Joo Yeun Lee[#]

Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles, United States

[#]Present address: Department of Ophthalmology, University of California, San Francisco, San Francisco, United States

Annie Wen

Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles, United States

Karen T Chang

Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles, United States, Dept. of Physiology & Neuroscience, Keck School of Medicine, University of Southern California, Los Angeles, United States

For correspondence: changkt@usc.edu

Editors

Reviewing Editor

Margaret Ho

National Yang Ming Chiao Tung University, Taipei, Taiwan

Senior Editor

Lu Chen

Stanford University, Stanford, United States of America

Reviewer #2 (Public review):

In this paper Chang et al follow up on their lab's previous findings about the secreted protein Shv and its role in activity-induced synaptic remodeling at the fly NMJ. Previously they reported that shv mutants have impaired synaptic plasticity. Normally a high stimulation paradigm should increase bouton size and GluR expression at synapses but this does not happen in shv mutants. The phenotypes relating to activity-dependent plasticity were completely recapitulated when Shv was knocked down only in neurons and could be completely rescued by incubation in exogenously applied Shv protein. The authors also showed that Shv activation of integrin signaling on both the pre- and post-synapse was the molecular mechanism underlying its function in plasticity. Here they extend their study to consider a role of Shv derived from glia in modulating synaptic features at baseline and remodeling conditions. The authors show evidence that Shv is expressed in both neurons and glia. Despite the fact that neuron-specific RNAi knockdown of Shv recapitulated the plasticity phenotypes seen in whole animal mutants, the authors asked whether glial-specific knockdown would have any effects. Surprisingly, knockdown of Shv only in glia also blocked plasticity, just like neuron-specific knockdown, and supporting an important role for glial-derived Shv in plasticity. Unlike neuronal knockdown, though, glial knockdown also caused abnormally high baseline GluR expression. Restoring Shv in ONLY glia in mutant animals is sufficient to completely rescue the plasticity phenotypes and baseline GluR expression, but glial-Shv does not appear to activate integrin signaling which was shown to be the mechanism for neuronally derived Shv to control plasticity. This suggests a different or indirect mechanism of action for glial-derived Shv. This led the authors to hypothesize that glial Shv might work via controlling the levels of neuronal Shv and/or extracellular glutamate. To test these hypotheses, they provide evidence that in the absence of glial Shv, synaptic levels of Shv go up overall, suggesting that glial Shv could somehow have a suppressive effect on release of neuronal Shv. This would indirectly modulate integrin signaling to control plasticity. Using an extracellular glutamate sensor in presynaptic boutons, they also observe decreased signal (extracellular glutamate) from the sensor in glial Shv KD animals, and increased signal in glial Shv overexpression animals, supporting the hypothesis that glial Shv can regulate glutamate levels somehow. These results establish glia as an important source of Shv in these processes and identify some mechanisms for how this might

be accomplished. Several outstanding questions remain-most importantly: how/why do glial-derived and neuronal-derived Shv have different effects when in the same space? No obvious isoform or size differences were found, and the same rescue construct expressed either in neurons or glia could have different effects on integrin activation or glutamate levels. Answering these questions using modified rescue constructs will be an important future direction to understand Shv function specifically and how neurons and glia work together in this context--and potentially many other contexts.

Comments on revisions:

The authors addressed my and the other reviewers' concerns from the original review adequately and this has strengthened the paper substantially.

One small omission to correct: In Figures 4 and 6, the graphs in the figures do not have a legend for the colored bars.

<https://doi.org/10.7554/eLife.104126.2.sa2>

Reviewer #3 (Public review):

Summary:

The manuscript by Chang and colleagues provides compelling evidence that glia-derived Shriveled (Shv) modulates activity-dependent synaptic plasticity at the *Drosophila* neuromuscular junction (NMJ). This mechanism differs from the previously reported function of neuronally released Shv, which activates integrin signaling. They further show that this requirement of Shv is acute and that glial Shv supports synaptic plasticity by modulating neuronal Shv release and the ambient glutamate levels. However, there are a number of conceptual and technical issues that need to be addressed.

Major comments

- (1) From the images provided for Fig 2B +RU486, the bouton size appears to be bigger in shv RNAi + stimulation, especially judging from the outline of GluR clusters.
- (2) The shv result needs to be replicated with a separate RNAi.
- (3) The phenotype of shv mutant resembles that of neuronal shv RNAi - no increased GluR baseline. Any insights why that is the case?
- (4) In Fig 3B, SPG shv RNAi has elevated GluR baseline, while PG shv RNAi has a lower baseline. In both cases, there is no activity induced GluR increase. What could explain the different phenotypes?
- (5) In Fig 4C, the rescue of PTP is only partial. Does that suggest neuronal shv is also needed to fully rescue the deficit of PTP in shv mutants?
- (6) The observation in Fig 5D is interesting. While there is a reduction in Shv release from glia after stimulation, it is unclear what the mechanism could be. Is there a change in glial shv transcription, translation or the releasing machinery? It will be helpful to look at the full shv pool vs the released ones.
- (7) In Fig 5E, what will happen after stimulation? Will the elevated glial Shv after neuronal shv RNAi be retained in the glia?
- (8) It would be interesting to see if the localization of shv differs based on if it is released by neuron or glia, which might be able to explain the difference in GluR baseline. For example,

by using glia-Gal4>UAS-shv-HA and neuronal-QF>QUAS-shv-FLAG. It seems important to determine if they mix together after release? It is unclear if the two shv pools are processed differently.

(9) Alternatively, do neurons and glia express and release different Shv isoforms, which would bind different receptors?

(10) It is claimed that Sup Fig 2 shows no observable change in gross glial morphology, further bolstering support that glial Shv does not activate integrin. This seems quite an overinterpretation. There is only one image for each condition without quantification. It is hard to judge if glia, which is labeled by GFP (presumably by UAS-eGFP?), is altered or not.

(11) The hypothesis that glutamate regulates GluR level as a homeostatic mechanism makes sense. What is the explanation of the increased bouton size in the control after glutamate application in Fig 6?

(12) What could be a mechanism that prevents elevated glial released Shv to activate integrin signaling after neuronal shv RNAi, as seen in Fig 5E?

(13) Any speculation on how the released Shv pool is sensed?

Comments on revisions:

The authors have addressed most of my previous comments and questions in their revision.

<https://doi.org/10.7554/eLife.104126.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public review):

In this manuscript, Chang et al. investigated the cell type-specific role of the integrin activator Shv in activity-dependent synaptic remodeling. Using the Drosophila larval neuromuscular junction as a model, they show that glial-secreted Shv modulates synaptic plasticity by maintaining the extracellular balance of neuronal Shv proteins and regulating ambient extracellular glutamate concentrations, which in turn affects postsynaptic glutamate receptor abundance. Furthermore, they report that genetic perturbation of glial morphogenesis phenocopies the defects observed with the loss of glial Shv. Altogether, their findings propose a role for glia in activity-induced synaptic remodeling through Shv secretion. While the conclusions are intriguing, several issues related to experimental design and data interpretation merit further discussion.

We appreciate the insightful and constructive comments. We have added new data and modified the text to address your concerns. In doing so, the manuscript has been substantially strengthened. Please see our detailed point-by-point response below.

Reviewer #2 (Public review):

In this paper Chang et al follow up on their lab's previous findings about the secreted protein Shv and its role in activity-induced synaptic remodeling at the fly NMJ. Previously they reported that shv mutants have impaired synaptic plasticity. Normally a high stimulation paradigm should increase bouton size and GluR expression at synapses but this does not happen in shv mutants. The phenotypes relating to activity dependent plasticity were completely recapitulated when Shv was knocked down only in neurons

and could be completely rescued by incubation in exogenously applied Shv protein. The authors also showed that Shv activation of integrin signaling on both the pre- and post-synapse was the molecular mechanism underlying its function. Here they extend their study to consider the role of Shv derived from glia in modulating synaptic features at baseline and remodeling conditions. This study is important to understand if and how glia contribute to these processes. Using cell-type specific knockdown of Shv only in glia causes abnormally high baseline GluR expression and prevents activity-dependent increases in bouton size or GluR expression post-stimulation. This does not appear to be a developmental defect as the authors show that knocking down Shv in glia after basic development has the same effects as lifelong knockdown, so Shv is acting in real time. Restoring Shv in ONLY glia in mutant animals is sufficient to completely rescue the plasticity phenotypes and baseline GluR expression, but glial-Shv does not appear to activate integrin signaling which was shown to be the mechanism for neuronally derived Shv to control plasticity. This led the authors to hypothesize that glial Shv works by controlling the levels of neuronal Shv and extracellular glutamate. They provide evidence that in the absence of glial Shv, synaptic levels of Shv go up overall, presumably indicating that neurons secrete more Shv. In this context which could then work via integrin signaling as described to control plasticity. They use a glutamate sensor and observe decreased signal (extracellular glutamate) from the sensor in glial Shv KD animals, however, this background has extremely high GluR levels at the synapse which may account for some or all of the decreases in sensor signal in this background. Additional controls to test if increased GluR density alone affects sensor readouts and/or independently modulating GluR levels in the glial KD background would help strengthen this data. In fact, glial-specific shv KD animals have baseline levels of GluR that are potentially high enough to have hit a ceiling of expression or detection that accounts for the inability for these levels to modulate any higher after strong stimulation and such a ceiling effect should be considered when interpreting the data and conclusions of this paper. Several outstanding questions remain-why can't glial derived Shv activate integrin pathways but exogenously applied recombinant Shv protein can? The effects of neuronal specific rescue of shv in a shv mutant are not provided vis-à-vis GluR levels and bouton size to compare to the glial only rescue. Inclusion of this data might provide more insight to outstanding questions of how and why the source of Shv seems to matter for some aspects of the phenotypes but not others despite the fact that exogenous Shv can rescue and in some experimental paradigms but not others.

We appreciate your insightful comments. We have added new data and modified the text to address your concerns. In doing so, the manuscript has been substantially strengthened. Please also see the enclosed point-by-point response.

To address the question of whether altered GluR density alone affects sensor readouts, we expressed GluR using a mhc promoter-driven GluRIIA fusion line, which increases total GluRIIA expression in muscle independently of the Gal4/UAS system. As shown in Figure 6 – figure supplement 1, mhc-GluRIIA animals exhibited elevated levels of not only GluRIIA but also the obligatory GluRIIC subunit. Despite this increase in GluR expression, we did not observe any change in extracellular glutamate levels, as measured by live imaging using the neuronal iGluSnFR sensor (updated Figure 6A). These results suggest that elevated GluR density alone does not alter iGluSnFR sensors dynamics and further support our conclusions.

In regard to the question about ceiling effect, we do not think that the lack of GluR enhancement in *repo>shv-RNAi* is due to a saturated postsynaptic state. This is based on results in Figure 6, which shows that GluR levels can increase up to fourfold upon stimulation in the presence of glutamate, whereas *repo>shv-RNAi* results in only a ~2-fold increase in baseline GluR concentration. These results suggest that the synapse retains the capacity for further upregulation.

To address the question of why exogenously applied Shv activates integrin while glial derived Shv does not, we tested whether glia and neurons could differentially modify Shv. Based on Western blot analyses of adult heads and larval brains showing that Shv is present as a single band (Fig. 1A and Figure 2 – figure supplement 1B), the functional differences in neuronal or glial Shv is not likely due to the presence of different isoforms. Consistent with this, FlyBase also suggests that shv encodes a single isoform. However, while we did not detect obvious posttranslational modifications when Shv protein was expressed in neurons or glia (Figure 5 – figure supplement 1A), we cannot exclude the possibility that different cell types process Shv differently through post-transcriptional or post-translational mechanisms. Notably, shv is predicted to undergo A-to-I RNA editing, including an editing site in the coding region, which will result in a single amino acid change (St Laurent et al., 2013). Given that ADAR, the editing enzyme, is enriched in neurons and absent from glia (Jepson et al., 2011), such cell-specific editing could contribute to functional differences. It will be interesting to investigate this in the future. We have now included this in the Discussion section.

Additionally, we have now included new data on neuronal Shv rescue of shv¹ mutants as suggested in the updated Figure 4. Consistent with previous findings that neuronal Shv rescues integrin signaling and electrophysiological phenotypes (Lee et al., 2017), we found that it also restores bouton size, GluR levels, and activity-induced synaptic remodeling. These results support the functional contribution of neuronal Shv.

Reviewer #3 (Public review):

Summary:

The manuscript by Chang and colleagues provides compelling evidence that glia-derived Shriveled (Shv) modulates activity-dependent synaptic plasticity at the Drosophila neuromuscular junction (NMJ). This mechanism differs from the previously reported function of neuronally released Shv, which activates integrin signaling. They further show that this requirement of Shv is acute and that glial Shv supports synaptic plasticity by modulating neuronal Shv release and the ambient glutamate levels. However, there are a number of conceptual and technical issues that need to be addressed.

We appreciate the insightful and constructive comments. We have added new data and modified the text to address your concerns. In doing so, the manuscript has been substantially strengthened. Please see our detailed point-by-point response below.

Major comments:

(1) From the images provided for Fig 2B +RU486, the bouton size appears to be bigger in shv RNAi + stimulation, especially judging from the outline of GluR clusters.

Thank you for pointing this out. We have selected another image to better represent the data.

(2) The shv result needs to be replicated with a separate RNAi.

We have used another independent RNAi line targeting shv to confirm our findings (BDSC 37507). This shv-RNAi³⁷⁵⁰⁷ line also showed the same phenotype, including increased GluR levels and impaired activity-induced synaptic remodeling line (new Figure 2 – figure supplement 1A).

(3) The phenotype of shv mutant resembles that of neuronal shv RNAi - no increased GluR baseline. Any insights why that is the case?

This is an interesting question. We speculate that neuronal Shv normally has a dominant role in maintaining GluR levels during development, mainly through its ability to activate integrin signaling. Consistent with this, we have shown that mutations in integrin leads to a drastic reduction in GluR levels at the NMJ (Lee et al., 2017). While we have shown that neuronal knockdown of shv elevates Shv from glia (Fig. 5E), glial Shv cannot activate integrin signaling (Fig. 5B, 5C). Additionally, high levels of glial Shv will elevate ambient glutamate concentrations (Figure 6A), which will likely reduce GluR abundance and impair synaptic remodeling (Augustin et al. 2007, Chen et al., 2009, and Figure 6B). Therefore, neuronal knockdown of Shv resulted in the same phenotype as *shv*¹ mutant.

(4) In Fig 3B, SPG shv RNAi has elevated GluR baseline, while PG shv RNAi has a lower baseline. In both cases, there is no activity induced GluR increase. What could explain the different phenotypes?

SPG is the middle glial cell layer in the fly peripheral nervous system and may also influence the PG layer through signaling mechanisms (Lavery et al., 2007), therefore having a stronger effect. We have now mentioned this in the text.

(5) In Fig 4C, the rescue of PTP is only partial. Does that suggest neuronal shv is also needed to fully rescue the deficit of PTP in shv mutants?

This is indeed a possibility. We have shown that neuronal and glial Shv each contribute to activity-induced synaptic remodeling through different mechanisms. It will be interesting test this in the future.

(6) The observation in Fig 5D is interesting. While there is a reduction in Shv release from glia after stimulation, it is unclear what the mechanism could be. Is there a change in glial shv transcription, translation or the releasing machinery? It will be helpful to look at the full shv pool vs the released ones.

Thank you for the suggestion. To address this, we monitored the levels of intracellular Shv using a permeabilized preparation (we found that the addition of detergent to permeabilize the sample strips away extracellular Shv). Combined with the extracellular staining results, we can get an idea about the total amount of Shv. As shown in the updated Figure 5D, intracellular Shv levels (permeabilized) remained unchanged following stimulation, indicating that there is no intracellular accumulation and that the observed decrease in extracellular Shv is unlikely due to impaired release machinery.

(7) In Fig 5E, what will happen after stimulation? Will the elevated glial Shv after neuronal shv RNAi be retained in the glia?

Thank you for the interesting question. We agree that examining Shv distribution following neuronal activity would be highly informative. While we plan to perform time-lapse experiments in future studies to address this, we feel that such analyses are beyond the scope of the current manuscript.

(8) It would be interesting to see if the localization of shv differs based on if it is released by neuron or glia, which might be able to explain the difference in GluR baseline. For example, by using glia-Gal4>UAS-shv-HA and neuronal-QF>QUAS-shv-FLAG. It seems important to determine if they mix together after release? It is unclear if the two shv pools are processed differently.

We agree that investigating whether neuronal and glial shv pools colocalize or are differentially processed is an important future direction. We hope to examine how each pool responds to stimulation in the shv¹ mutant background using LexA and Gal4 systems in the future

(9) Alternatively, do neurons and glia express and release different Shv isoforms, which would bind different receptors?

Thank you for the questions. We have now addressed this in the discussion and also enclosed below:

Based on Western blot analyses of adult heads and larval brains showing that Shv is present as a single band (Fig. 1A and Figure 2 – figure supplement 1B), the functional differences in neuronal or glial Shv is not likely due to the presence of different isoforms. Consistent with this, FlyBase also suggests that shv encodes a single isoform (Ozturk-Colak et al., 2024). However, while we did not detect obvious post-translational modifications when Shv protein was expressed in neurons or glia (Figure 5 – figure supplement 1A), we cannot exclude the possibility that different cell types process Shv differently through posttranscriptional or post-translational mechanisms. Notably, shv is predicted to undergo A-to-I RNA editing, including an editing site in the coding region, which could result in a single amino acid change (St Laurent et al., 2013). Given that ADAR, the editing enzyme, is enriched in neurons and absent from glia (Jepson et al., 2011), such cell-specific editing could contribute to functional differences. It will be interesting to investigate this in the future.

(10) It is claimed that Sup Fig 2 shows no observable change in gross glial morphology, further bolstering support that glial Shv does not activate integrin. This seems quite an overinterpretation. There is only one image for each condition without quantification. It is hard to judge if glia, which is labeled by GFP (presumably by UAS-eGFP?), is altered or not.

Thank you for raising this concern. To strengthen our claim, we now include additional images (Figure 5, figure supplement 2). No obvious change in overall glial morphology was observed, with glia continuing to wrap the segmental nerves and extend processes that closely associate with proximal synaptic boutons (Figure 5, figure supplement 2). These observations suggest that glial Shv is not essential for maintaining normal glial structure or survival, and is consistent with the idea that glial Shv does not activate integrin, as integrin signaling is required to maintain the integrity of peripheral glial layers.

(11) The hypothesis that glutamate regulates GluR level as a homeostatic mechanism makes sense. What is the explanation of the increased bouton size in the control after glutamate application in Fig 6?

We speculate that it could be due to a retrograde signaling mechanism activated by elevated extracellular glutamate, allowing neurons to modulate bouton morphology in response to synaptic demand. It will be interesting to investigate this possibility in the future.

(12) What could be a mechanism that prevents elevated glial released Shv to activate integrin signaling after neuronal shv RNAi, as seen in Fig 5E?

One potential mechanism is post-translational or post-transcriptional processing of Shv. Although our Western blots did not reveal differences in the molecular weight of glial vs. neuronal Shv, we cannot exclude the possibility that modifications not readily detectable by this method are responsible. Additionally, as mentioned in the Discussion section, post-

transcriptional processing such as A-to-I RNA editing could introduce changes in the Shv protein, potentially altering its ability to interact with or activate integrin.

(13) Any speculation on how the released Shv pool is sensed?

The same RNA editing modification mentioned earlier or post-translational modifications in Shv may also influence how it is sensed by target cells.

Reviewer #1 (Recommendations for the authors):

Issues Regarding Cell Type-Specific Secretion and the Role of Shv:

Extracellular Secretion of Shv:

(1) The data in Figure 1 suggest that Shv is not secreted under resting conditions, challenging the proposed extracellular role of Shv. It remains unclear whether Shv secretion can be confirmed using Shv-eGFP (knock-in) following high K⁺ stimulation.

We apologize for not being clear. In Figure 1, Shv signals we've shown are from permeabilized preparation, which preferentially labels intracellular Shv. We do observe secreted Shv-eGFP following stimulation (Figure 5E), consistent with our hypothesis. However, endogenous extracellular Shv-eGFP signal is very weak, and was therefore detected using the GFP antibody and amplified with a fluorescent secondary antibody. We have now also included additional controls in Figure 5E to demonstrate the specificity of the staining.

(2) In Figure 5D, total Shv staining should be included to evaluate potential presynaptic accumulation of intracellular Shv, which may lead to extracellular secretion upon stimulation. Additionally, the representative images of glial rescue do not seem to align with the quantification data; more extracellular Shv signals were observed after stimulation.

Thank you for the comments. We monitored the levels of intracellular Shv using a permeabilized preparation (detergent treatment stripped away extracellular Shv signal). When combined with non-permeabilized extracellular staining, this approach provides insights into total Shv levels. We found no intracellular accumulation of Shv and the intracellular levels remained unchanged following stimulation (updated Figure 5D), suggesting that reduced extracellular Shv is not likely due to impaired release. Additionally, we have selected another image for glial rescue by avoiding the trachea region, which better represent the quantification data.

(3) In Figure 5E, "extracellular" Shv staining in *repo>shv-RNAi* samples appears localized within synaptic boutons. This raises concerns about the staining protocol potentially labeling intracellular proteins. Control experiments using presynaptic cytosolic markers are needed to confirm staining specificity.

Thank you for the thoughtful suggestion. To validate that our staining protocol is selective for extracellular proteins, we also stained for cysteine string protein (CSP), an intracellular synaptic vesicle protein predominantly located in the presynaptic terminals (Zinsmaier et al., 1990; Umbach et al., 1994), under the same conditions. CSP was detected only in the permeabilized condition (updated Figure 5E), suggesting that the non-permeabilizing protocol is selective for extracellular proteins.

(4) The study does not clarify why Shv knockdown in either perineurial glia or subperineurial glia abolishes stimulus-dependent synaptic remodeling. Does Shv secretion occur from PG, SPG, or both toward the synaptic bouton?

Thank you for raising this point. SPG is the middle glial cell layer in the fly peripheral nervous system and may also influence the PG layer through signaling mechanisms (Lavery et al., 2007). Consistent with this, we observed a stronger effect on GluR levels when SPG was disrupted compared to PG. It will be interesting to distinguish whether Shv is released by PG or SPG in the future.

(5) The possibility of an inter-glial role for Shv via integrin signaling in regulating glial morphogenesis is underexplored. The rough morphological characterization in Supplemental Figure 2 requires more detailed quantification and the use of sub-glial typespecific GAL4 drivers.

We now include additional images (Figure 5, figure supplement 2) to examine the overall glial morphology. There was no obvious change in gross glial morphology, with glia continuing to wrap the segmental nerves and extend processes that closely associate with proximal synaptic boutons when shv is knocked down in glia (Figure 5, figure supplement 2). These observations suggest that glial Shv is not essential for maintaining normal glial structure or survival, and is consistent with the idea that glial Shv does not activate integrin, as integrin signaling is required to maintain the integrity of peripheral glial layers (Xie and Auld, 2011; Hunter et al., 2020).

(6) While repo>shv rescues stimulus-dependent bouton size and GluR increases in the shv mutant (Figure 5), the interaction between neuronal and glial Shv remains unclear. Does neuronal Shv influence the expression or distribution of glial Shv?

We agree that investigating whether neuronal and glial shv pools influence each other's expression or distribution is an important future direction. We hope to investigate this in more detail in the future using LexA-LexOp and GAL4/UAS dual expression systems.

Issues Regarding the Regulation of GluR and Perisynaptic Glutamate by Glial Shv:

(7) The methodology for iGluSnFR measurement (Figure 6A) is inadequately described. If anti-HRP staining was used to normalize signals, it suggests the experiment may have involved fixed tissue. However, iGluSnFR typically measures glutamate levels in live cells, raising concerns about the validity of this approach in fixed samples.

We apologize for not being clear about the method used to measure iGluSnFR. The original figure was generated from imaging iGluSnFR signals immediately following fixation. To address the reviewer's concern and validate these results, we have now performed live imaging experiments using a water dipping objective to measure iGluSnFR intensity in unfixed preparations (new Figure 6A). To label synaptic boutons, we co-expressed mtdTomato using the neuronal driver, nSybGAL4. The results from the live imaging experiments confirmed our original observations that glial Shv required to control ambient extracellular glutamate levels (see updated Fig. 6A and text). Additionally, to ascertain that the decrease in iGluSnFR signal reflects a decrease in ambient extracellular glutamate levels rather than glutamate depletion caused by high levels of GluR, we upregulated GluR levels using mhc-GluRIIA, which drives GluRIIA expression in muscles (Petersen et al., 1997). We found mhc-GluRIIA animals exhibited elevated levels of not only GluRIIA but also the obligatory GluRIIC subunit. However, iGluSnFR signals at the synapse remained unchanged (Figure 6A), suggesting that elevated GluR density alone does not reduce signals. Taken together, these results suggest that glial Shv plays a critical role in controlling ambient extracellular glutamate levels.

(8) As shown in Figure 2, repo>shv-RNAi increases GluR levels before high K⁺ stimulation, potentially saturating postsynaptic GluR expression and precluding further increases

upon stimulation.

Our data in Figure 6 show that GluR levels can increase up to four-fold upon stimulation in the presence of glutamate, whereas *repo>shv-RNAi* results in only a ~2-fold increase in baseline GluR concentration. These results suggest that the synapse retains the capacity for further upregulation. Thus, we do not think that the lack of GluR enhancement in *repo>shv-RNAi* is due to a saturated postsynaptic state, but rather reflects a requirement for glial Shv in activity-dependent modulation.

(9) Despite glial shv knockdown lowering extracellular glutamate levels, GluR levels unexpectedly increase (Figure 6B). This contradicts the known requirement for high ambient glutamate concentrations to promote GluR clustering and membrane expression (Chen et al., 2009). Furthermore, adding 2 mM glutamate reverses these increases, suggesting additional complexity in the regulation of Shv synaptic remodeling.

Thank you for the comment and the opportunity to clarify this point. While it may seem counterintuitive at first glance, our observations are in line with previous reports that showed low ambient glutamate levels significantly elevated GluR intensity at the *Drosophila* NMJ (Chen et al., 2009), but such increase can be reversed by glutamate supplementation (Augustin et al., 2007; Chen et al., 2009). We have revised the text to more clearly reflect this connection.

*(10) If glial Shv promotes GluR expression, why does the increased extracellular Shv from neuronal shv knockdown (*elav>shv-RNAi*, Figure 5E) fail to elicit stimulus-dependent GluR elevation?*

We speculate that this is because glial Shv does not activate integrin signaling (Figure 5B, C), and elevated glial Shv increases ambient glutamate concentration (Figure 6A), thereby reducing GluR expression (Augustin et al., 2007; Chen et al., 2009). This is indeed what we observed when *shv* is knocked down in neurons.

Additional Issues:

(11) The type of bouton used for quantification (e.g., Ib or Is boutons) is not specified, which is critical for interpreting the results.

We apologize for not being clear. We analyzed type Ib boutons as done previously (Lee et al., 2017 and Chang et al., 2024), and have now included this information in the Methods section.

*(12) The extent of Shv protein depletion in the *repo*-GeneSwitch system needs validation to confirm the efficacy of the knockdown.*

Thank you for the suggestion. We confirmed the efficiency of acute *shv* knockdown by the *repo*-GeneSwitch system by performing Western blot analysis of dissected larval brains (Figure 2 – figure supplement 1B). Acute glial knockdown using the *repo*-GeneSwitch driver resulted in a 30% reduction in Shv levels, similar to the decrease observed with the *repo*-GAL4 driver, suggesting that the GeneSwitch driver is functional. Furthermore, knockdown of *shv* by the ubiquitous tubulin-GAL4 driver completely eliminated Shv protein, indicating that the RNAi construct is effective.

Reviewer #2 (Recommendations for the authors):

(1) General comment on statistics/data presentation: The authors employ an unusual method of using both one-way ANOVA and multiple t-test stats for the same data. Would a 2-way ANOVA be the more appropriate solution to this problem (to analyze across

genotype and stimulation condition)? Also a chart in the supplementals showing all comparisons rather than just the fraction explicitly reported in the graphs would be helpful (it is not clear if no indication on significance indicates no difference or just not reported between some of the baseline levels, especially since everything is presented as ratios and in some cases this could help with data interpretation of which baseline levels are different and how they compare to other baselines and other post-stim levels). Further, there are no sample sizes given for any experiment, nor are any values of means, SD, etc ever explicitly given.

We appreciate the thoughtful suggestion. While a two-way ANOVA could be used to examine interaction effects between genotype and stimulation condition, our analysis was designed to address a specific biological question: whether each genotype, independent of baseline levels, is capable of undergoing activity-dependent synaptic remodeling. To this end, we used t-tests to directly compare unstimulated vs. stimulated conditions within each genotype, allowing us to determine whether stimulation produces a significant effect in an all-or-none manner. In parallel, we applied one-way ANOVA with post hoc tests to analyze differences among baseline (unstimulated) conditions across genotypes. This approach is justified by the fact that stimulation was applied acutely and separately, and therefore the baseline values should not be influenced by the stimulated condition. Because we were not aiming to compare the extent of synaptic remodeling between genotypes, we did not use a two-way ANOVA to analyze interaction effects across all conditions.

In response to the reviewer's suggestion, we have now added the sample number in the graphs. Additionally, in the Methods section, we include information that each sample represents biological repeats, and that data are presented as fold-change relative to unstimulated controls from the same experimental batch. This normalization is necessary, as absolute GluR intensities can vary depending on microscope settings and staining conditions.

(2) To clarify distinct roles of Shv coming from neurons vs glia it would help if the authors could include more data on the rescue of shv mutants with UAS-Shv in neurons alone. This data is never shown in the manuscript and data on what effect this rescue has on the pertinent phenotypes in this paper (bouton size and GluR staining) is not reported in the referred to 2017 paper. What this does and does not do for these phenotypes has important implications for how to interpret the glia-only rescue findings.

Thank you for the suggestion. We have now included new data on neuronal Shv rescue in shv¹ mutants as suggested (updated Figure 4A). Consistent with previous findings that neuronal Shv rescues integrin signaling and electrophysiological phenotypes (Lee et al., 2017), we found that it also restores bouton size, GluR levels, and activity-induced synaptic remodeling. These results support the functional contribution of neuronal Shv.

(3) Figure 1C: Where are the images in the periphery taken? The morphology of the glia is odd in that "blobs" of glial membrane seemingly unattached to anything else are floating about? Perhaps these are a thin stalk projection and so the connection to the main glia "stalks" are just cut off? Could a specific individual synapse be shown? Also consider HRP shown on its own so that where the actual boutons are could be more clear. It seems like both the Tomato and HRP channels are really overexposed making visualizing the morphology quite confusing. Also why not use the antibody against Shv to directly visualize expression which is more direct than a knock-in tagged version?

Figure 1C shows a single optical slice of the NMJ at muscle segment 2, selected to clearly highlight Shv-eGFP localization at a branch in close contact with the glial membrane. The glial stalk is not visible in this image because it lies in a different focal plane from the branch of interest. We have now specified this information in the figure legend. In the original figure,

the HRP signal (405 channel) was oversaturated, which interfered with visual clarity. In the updated Figure 1C, we reduced the intensity of overexposed channels to better reveal the weak Shv-eGFP signal and fine glial processes. While we have generated an antibody against Shv, the amount is extremely limited, and hence the Shv-eGFP fusion serves as a valuable tool for visualizing subcellular localization.

(4) Do glutamate levels really rise in glia Shv KD? Although iGluSnFR signal changes could it be the high level of GluR at the synapse acting as sponges to sequester glutamate so that it can't stimulate the sensor as well? One way to test this would be to overexpress or KD GluRs in muscle in wildtype (or in the repo>Shv RNAi background) to see if that alone can modulate iGluSnFR signals?

Thank you for suggesting this important control. To address the question of whether high level GluR density alone could influence neuronal iGluSnFR sensor readouts, we expressed GluR using a mhc promoter-driven GluRIIA fusion line, which increases total GluRIIA expression in muscle independently of the Gal4/UAS system. As shown in Figure 6 – figure supplement 1, mhc-GluRIIA animals exhibited elevated levels of not only GluRIIA but also the obligatory GluRIIC subunit. Despite this increase in GluR expression, we did not observe any change in extracellular glutamate levels, as measured by live imaging using the neuronal iGluSnFR sensor (updated Figure 6A). These results suggest that elevated GluR density alone does not alter iGluSnFR sensors dynamics and further support our conclusions.

(5) The authors have some Shv constructs that can't be secreted or can't bind to integrins. Performing cell type specific rescues with these constructs might also help distinguish how source matters for each proposed sub-function of Shv though this may be outside the scope of this study.

Thank you for noticing the Shv constructs we have. We hope to further test subfunctions of Shv in the future.

(6) At one point the authors discuss experiments that measure how much Shv is released by glia during neuronal stimulation. Then state that "These data indicate that glial Shv does not directly inhibit integrin signaling." But how this experiment relates to integrin signaling is not explained and unclear.

We apologize for the confusion. We have now updated the text to better explain our logic: “This activity-induced decrease in glial Shv levels, along with reduced integrin activation (Fig. 5B), suggest that glial Shv does not act by directly inhibiting integrin signaling.”

Reviewer #3 (Recommendations for the authors):

Minor comments

(1) Readers are left wondering what causes the increased baseline of GluR after glial shv RNAi at Fig 1, which is addressed much later. It would be helpful to preemptively mention this.

Thank you for the suggestion. To maintain a logical flow, we chose to first present the phenotypic data in Figures 1 and 2 and then return to the mechanistic explanation once we introduced ambient glutamate measurements.

(2) Be consistent with eGFP vs EGFP.

Thank you, we have corrected the inconsistencies.

(3) Scale bar for Fig 1B is missing in the low-magnification panel.

Thank you for pointing out. We've put in the scale bar for Figure 1B.

(4) Fig 1C, it would be helpful to elaborate on the anatomy. For example, what NMJ/abdominal segment is this? Why only some axons are surrounded by glia?

Figure 1C presents a single optical slice of the NMJ at muscle segment 2, chosen to highlight Shv-eGFP localization at a branch closely juxtaposed to the glial membrane. The glial stalk is not shown in this image because it resides in a different focal plane than the branch being visualized. We have now included this information in the figure legend.

(5) For Fig 3B, while it is stated that "we observed normal synaptic remodeling using *alrmGAL4*," the effect size is smaller. There seems to be a decrease in the amount of synaptic remodeling occurring?

Thank you for pointing this out. Our primary goal was to determine whether each genotype, regardless of baseline GluR levels, is capable of undergoing activity-dependent synaptic remodeling in response to stimulation. For this reason, we focused on detecting the presence or absence of remodeling rather than comparing the extent of remodeling across genotypes. While a smaller effect on activity-induced bouton size was observed with *alrm-GAL4*, the change was still statistically significant, indicating that remodeling does occur in this genotype. Currently, we do not have a clear biological interpretation for differences in the magnitude of remodeling, and therefore chose not to emphasize cross-genotype comparisons.

<https://doi.org/10.7554/eLife.104126.2.sa0>