

Supplementary Materials and Methods

Drosophila crosses and stocks

Ubiquitous and neuron-specific expression was achieved with the GAL4-UAS system [GAL4-dependant upstream activator sequence] (Brand and Perrimon, 1993). *UAS-miRNA_{Myo}* and *Myo-GAL4* lines (Awasaki et al., 2011) were a gift from T. Awasaki from Tzumin Lee lab at Janelia Farm; Bloomington Stock Center lines: *Mef2-GAL4* (#27390), *repo-GAL4* (#7415), *24B-GAL4* (#1767) and *C164-GAL4* (#33807); *UASmyoRNAi* (#33132) was received from Vienna Drosophila Resource Center; *UASmyoglianin* (2nd chr.), *UAS-myoglianin* (3rd chr.), *UAS-Smad2*↑ and *UAS-MAD*↑ were a kind gift from M. O'Connor, University of Minnesota. The *UAS-Smad2*↑ and *UASMAD*↑ lines have the two of the serines in SSVS motif at the C-terminal tail changed to aspartate, rendering Smad2 and MAD constitutively active. *Mef2-GAL4/UASmiRNA_{Myo}/MAD*↑ and *Mef2-GAL4/UAS-miRNA_{Myo}/Smad2*↑ were created using standard *Drosophila* crossing schemes. The *slit-GAL4* line was a kind gift from Iris Salecker, The Frances Crick Institute; *fb-GAL4* line was received from I. Bjedov, UCL; *np1-GAL4* is the Drosophila Genetic Resource Center (Kyoto) stock #112001; *UAS-mCD8-GFP* line was a gift from Y. Grosjean, CNRS/Université de Bourgogne. *wDah* was the “wild-type” strain used in all experiments. The white Dahomey (*wDah*) stock was derived by incorporation of the *w1118* mutation into the outbred Dahomey background by back-crossing.

NMJ and GFS electrophysiology

NMJ recordings were performed using pClamp 10, an Axoclamp 900A amplifier and Digidata 1440A (Molecular Devices, USA) in hemolymph-like solution 3 (HL-3). Recording electrodes (10–30 MΩ) were filled with 3 M KCl. mEJCs were recorded in the presence of 0.5 μM

tetrodotoxin (Tocris, UK). All synaptic responses were recorded from muscles with input resistances ≥ 4 M Ω and resting potentials more negative than -60 mV at 25°C as differences in recording temperature cause changes in glutamate receptor kinetics and amplitudes (Postlethwaite et al., 2007). Holding potentials were -60 mV. The extracellular HL-3 contained (in mM): 70 NaCl, 5 KCl, 20 MgCl₂, 10 NaHCO₃, 115 sucrose, 5 trehalose, 5 HEPES and 0.5–3 CaCl₂ (as specified). Average single eEJC amplitudes (stimulus: 0.1 ms, 1–5 V) are based on the mean peak eEJC amplitude in response to ten presynaptic stimuli (recorded at 0.2 Hz). Nerve stimulation was performed with an isolated stimulator (DS2A, Digitimer). All data were digitized at 10 kHz and for miniature recordings, 200 s recordings we analyzed to obtain mean mEJC amplitudes, decay and frequency values. mEJC and eEJC recordings were off-line low-pass filtered at 500 Hz and 1 kHz, respectively. Materials were purchased from Sigma-Aldrich (UK) unless otherwise stated. Quantal content was estimated for each recording by calculating the ratio of eEJC amplitude/average mEJC amplitude followed by averaging recordings across all NMJs for a given genotype. For GFS recordings, individual adult flies were anaesthetized by cooling on ice and secured in dental wax placed inside a small Petri dish, ventral side down, with the wings held outwards in the wax to expose lateral and dorsal surfaces of the thorax. A tungsten earth wire (ground electrode) was placed in the abdominal cavity. Extracellular stimulation of the Giant Fiber interneurons was achieved by placing two electrolytically (NaOH) sharpened tungsten electrodes through the eyes and into the brain to deliver a 40 V pulse for 0.03 ms using an npi electronic ISO-STIM 01D stimulator. For the thoracic stimulation, the stimulating electrodes were moved from the brain and carefully placed through the cuticle at the anterior end of the thorax and into the fused thoracic ganglia in the ventral part of the thorax. Recordings were made using glass microelectrodes (resistance 40–60 M Ω) filled with 3 M KCl and inserted into the TTM and a contralateral DLM through the thoracic cuticle. Responses were amplified using Axoclamp 900A microelectrode amplifier (Molecular Devices, USA) and the data digitized using an analog-digital Digidata 1440A digitizer (Molecular Devices, USA) and Axoscope 10.5 software (Axon Instruments, USA).

Immunocytochemistry and Western blots

Dissections were performed using a modified *Drosophila* saline (HL3) with physiological levels of glutamate (in mM; 135 NaCl, 5 KCl, 4 MgCl₂, 1.8 CaCl₂, 5 N-

Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 72 sucrose, and 2 L-glutamate). For glutamate receptor (GluRIIA) and Brp staining, dissected 3rd instar larval preparations were fixed for 30 min in Bouin's fixative. Mouse monoclonal anti-GluRIIA (8B4D2) and anti-Brp (nc82) antibodies were obtained from the University of Iowa Developmental Studies Hybridoma Bank (Iowa City, USA) and used at 1:100 and 1:20, respectively. AlexaFluor-conjugated goat anti-mouse secondary antibodies were used at 1:200. TRITC-labeled anti-horseradish peroxidase (HRP) antibodies (staining neuronal membranes) were used at 1:100. To visualize larval muscles, phalloidin was added to fresh larval preparations fixed for 30 min with 4% paraformaldehyde. DAPI was added prior to mounting to stain myofiber nuclei. Quantitative image analysis was performed with ImageJ (NIH, Bethesda, USA) on maximum intensity projection Z-stacks. We first drew a circle around individual boutons delineated by the HRP (horseradish peroxidase) signal and quantified the mean postsynaptic immunofluorescence in the second (GluRIIA/B) channel. The same bouton outline was then moved to a nearby region of the same muscle and fluorescence measured in the same way as for the bouton. The value for the first measurement was divided by the second number representing muscle fluorescence. The fluorescence intensity ("signal intensity") was quantified using underexposed images as "integrated density" (area x mean fluorescence) and normalized to the control genotype (e.g. *+/Mef2-GAL4*). Individual Brp puncta were counted manually in 1b boutons across the NMJ. The pre-synaptic NMJ area was measured by delineating the area labelled by HRP. For Western blots, samples were homogenized in Laemmli buffer containing 0.5% β -Mercaptoethanol and boiled at 95°C for 5 min for gel electrophoresis. Gels were run at 150 V and transferred to nitrocellulose membrane via semi-dry transfer methods using BioRad. All westerns blots were normalised against actin staining (Abcam #ab8224 Ms-anti-actin [1:10000] in 5% milk/TBS-T). 10% SDS-PAGE gels with 3.75% stacking gel were run for p-AKT quantification (Cell Signalling #4060) which was also normalised against total-AKT (Cell Signaling #9272). p-GSK (Cell Signaling #9331) was also run on 10% gel whereas p-S6K (Cell Signaling #9209) was run on 8% SDS-PAGE gels. The *n* in the figure legend denotes the number of biological replicates.

RNA extractions

Larval preps (6 preps per sample, 3-5 samples per genotype per experiment) were dissected in cold HL3 medium and transferred straight into 1 ml of ice cold Trizol. The samples were

ribolysed for 20 s and then incubated at room temperature for 5 min. 0.2 ml chloroform was added, samples were vortexed for 15 s then incubated at room temp for 2 mins before centrifugation at 12,000 rpm for 15 min at 4°C. The clear aqueous layer was transferred (about 60% of total Trizol volume) to a fresh tube. 0.5 ml room temperature isopropanol was added, mixed and incubated at room temperature for 15 min. Samples were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was removed and RNA pellets were washed (x4 with 70% ethanol/DEPC water (~ 400 µl). Supernatants were removed and pellets were dried at room temp before re-suspending in 15 µl of RNase free (DEPC) water. RNA concentration was determined using a NanoDrop.

cDNA synthesis using superscript system for RT-PCR

As per protocol (Invitrogen), cDNA was synthesized from 5 µg of RNA in a total volume of 5 µl (volumes adjusted using sterile DEPC-treated water). cDNA was synthesized as per SOP. Briefly, 10x DNase buffer and DNase was added to the RNA and incubated at 37°C for 15 min. 24 mM EDTA (pH 8, RNase free) was added and samples heated to 75°C for 5 min then chilled on ice. 0.5 µg/µl Oligo dT and 10 mM dNTP mix was added, heated to 65°C for 5 min then chilled on ice. 5x RT buffer, DEPC-treated water, 0.1 M DTT and RNaseOut Recombinant RNase Inhibitor was added, incubated at 42°C for 2 min then 1 µl of SuperScript II RT was added and the samples were incubated at 42°C for 50 min before terminating the reaction at 70°C for 15 min then chilling the cDNA on ice. cDNA was stored on ice until PCR reaction, or at -20°C. All values are the average of four replicates, and standardized to 4 housekeeping genes: *rp49*, *β-actin*, *α-tubulin* and *tbp* (sequences for *myoglianin* primers are: 5'-CGCAGAAACCTGGATGAAGT-3' and 5'ATTTCACCAGCTTTGGATGG-3'; product size is 196 bp).

Larval microinjections

2nd instar larvae were secured onto adhesive tape and injected posterior-laterally using a pedal operated microinjector (PicoSpritzer II, Parker) and needles pulled from glass capillaries (0.58/1 mm ID/OD; #30-0019 Harvard Apparatus). Myostatin (recombinant human; PeproTech #120-00) was injected from a 1 µg/mL solution in Ringers buffer (10 mM Tris-HCl pH 7.2, 182 mM KCl, 46 mM NaCl, 3 mM CaCl₂•2H₂O) supplemented with 0.1% w/v BSA and 0.25% w/v blue food dye (FD&C Blue No.1) to visualize successful delivery (Cocheme et al., 2012). The injected amount per larva equates to 50 pg of Myostatin (±4 SEM, n=6). The injection volume was quantified by homogenizing groups of 5 injected larvae

and measuring the blue dye content from the A629 against a serial dilution of the injection solution (Wong et al., 2009).

Time to pupariation and weight measurements

Pupariation and wet weight measurements were done on larvae reared under the same conditions (food composition, humidity and temperature) as the larvae used for other experiments. Measuring the time to pupariation was done essentially as described recently (Johnson et al., 2013). Eggs were collected for 1-2 h and collected. 24 h later, first instar larvae were placed onto Petri dishes containing regular fly food (SYA). Larvae (~10 groups of 10 larvae per genotype) were inspected daily; following the beginning of the pupariation period animals were scored every 2-3 h and the number of pupariated larvae was counted. The blue segment of bars in Figure S1E denotes period between the first and last pupariation event. Third instar larvae were weighed on a microbalance (Denver Instrument SI-64) in groups of 3; the mean of the 3 measurement was counted as a single data point.

Crawling speed

Larval motility was measured using camera recordings followed by video analysis using fly tracking software (Scott Pletcher, University of Michigan). Videos were recorded at 2 frames per second and converted to AVI file format, which was analyzed using VideoFly software. The distance travelled by individual larvae during the first minute following their placement onto a large Petri dish was used to calculate their crawling speed.

Cell culture experiments

Reagents and antibodies

Antibodies used for immunofluorescence analysis in this study were mouse monoclonal anti-PSD95 (Pierce, MA1-045); mouse monoclonal anti-Gephyrin (referred to as 'GPHN') (Synaptic Systems, 3B11); chicken polyclonal anti-MAP2 (Abcam, Ab5392); rabbit polyclonal anti-VGAT (Synaptic Systems, 131003); Guinea pig anti-vGLUT1 (Synaptic Systems, 135304); Alexa Fluor 488-, 555-, 633 conjugated goat anti-mouse, anti-rabbit, anti-chicken, anti-Guineapig antibodies were from Life Technologies. Reagents concentrations shown, with the exception of DMSO, were determined to be the minimum effective concentrations after serial dilution, as assessed by their measured deviation from control according to the criteria analysed. DMSO referred to as 'Control' (1:1000, Sigma); TGF β 1 (R&D Systems) was used at 5 ng/ml (within physiological levels (Ramesh et al., 1990)); BMP2 (R&D Systems) was used at supraphysiological levels of 10 ng/ml to induce acute Smad 2 signalling; GDF8, also known as myostatin (R&D Systems) was used at 10 ng/ml (within physiological levels (Lakshman et al., 2009)); GDF11 (R&D Systems) was used at 10 ng/ml (within physiological levels (Schafer et al., 2016), Alantolactone referred to as 'TGF β bypass' (400 nM, Sigma SML0415) and A8301, referred to as 'TGF β inhib' (400 nM, Tocris 2939).

Neuronal cell culture and treatments

All experimental procedures were carried out in accordance with institutional animal welfare guidelines and the UK Animals (Scientific Procedures) Act 1986. Rat cortical neuron cultures were prepared from E18 Sprague-Dawley rat embryos as described previously (Arancibia-Carcamo et al., 2009) with the following modifications: Glass bottomed 96 well culture plates were coated with poly-D-lysine (0.05 mg/ml, Sigma) in PBS over night at 37°C, 5% CO₂, after which, a second coating of laminin (0.01 mg/ml, Sigma) in PBS was applied for 2 h at 37°C, 5% CO₂. Approximately 4×10^4 isolated cortical neuronal cells were seeded per well and allowed to attach in neuronal attachment media (Minimum Essential Medium Eagle's with Earl's BSS (Sigma), 10% (w/v) FBS (ThermoFisher), 1 mM sodium pyruvate (Sigma), 20% (w/v) glucose (Sigma), 2 mM glutamax (ThermoFisher), antibiotic-antimycotic (Sigma)) and maintained at 37°C, 5% CO₂ for 12 h after which the media was exchanged to neuronal maintenance media (Neurobasal (ThermoFisher), 2% (w/v) B27 (ThermoFisher), 2 mM glutamax (ThermoFisher), antibiotic-antimycotic (Sigma) and cultures maintained at 37°C, 5% CO₂. Neuronal maintenance media was exchanged every three days for the first 6

days *in vitro* (DIV) after which treatments were added to the neuronal culture in fresh neuronal maintenance media and maintained for a further 5 DIV prior to fixation. Optimum treatment concentrations for each condition were determined by serial dilution using a 5 times dilution between concentration points between well rows.

Fixation and fluorescence labelling for microscopy

Treated cells were fixed in 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature, followed by two washes with PBS and then incubated for 1 h in 50 mM NH₄Cl in PBS to quench the residual PFA. Cells were then permeabilized with 0.1% Triton-PBS and immunolabeled in the presence of horse serum (Life Technologies) at an antibody dilution of 1:5000 for anti-MAP2, 1:500 for anti-vGLUT1, 1:200 for antiPSD95, 1:500 for anti-Gephyrin and 1:200 for anti-VGAT primary antibodies, 1:1000 for all secondary antibodies and 1:10,000 for DAPI staining of DNA. Fluorescently labelled samples were analysed and images captured with a 20x air objective using a widefield high-content analysis system (ImageXpress Micro XLS, Molecular Devices).

Image processing and quantitation

Image analysis was performed using a protocol established in CellProfiler image analysis software (Kamentsky et al., 2011) and is a variation on a protocol established previously (Nieland et al., 2014). A set of image analysis algorithms or 'pipeline' was constructed to measure the properties of interest within the cortical neuron culture labelled with either DAPI, anti-MAP2, anti-PSD95 and anti-vGLUT1 or with DAPI, antiMAP2, anti-Gephyrin and anti-VGAT. Each image-set, corresponding to one field of view or site and comprising four fluorescently labelled channels, were analysed independently using this pipeline. 9 sites per well were analysed and repeated in triplicate experiments. In brief, an illumination correction function was calculated for each channel using a median filter (200x200 pixels) to correct for illumination variations across each 96-well plate. Each image set was then processed in an imaging pipeline as follows. The four channels' raw images were divided by their respective plate/channel illumination function. Firstly, segmentation of the nuclei of each cell in the field of view was identified corresponding to an arbitrary fluorescence intensity, median size (20 to 60 pixels) and shape (circular) according their DAPI DNA labelling. Next, neurites were identified extending from the nuclei using an arbitrary fluorescence intensity corresponding to anti-MAP2 labelling and were enhanced using the neurite enhance module within CellProfiler by the 'Tubeness' method after which, a mask

within all channels according to the identified neurite network, minus that of the nuclei, was created. Only PSD95, vGLUT1, Gephyrin and VGAT punctae within the masked region, with a typical diameter range of range of 8 to 20 pixels and having an arbitrary threshold of fluorescence intensity associated with their corresponding antibody labelling were identified as 'primary punctae objects' for analysis. Excitatory and inhibitory synapses were subsequently identified and designated as 'synaptic objects' if two 'primary punctae objects' were determined to co-localise in the pre and post synaptic marker channels of either PSD95 and vGLUT1 (excitatory) or Gephyrin and VGAT (inhibitory). The cortical neuron cell number per image was measured by counting the number of nuclei from which there were neurite extensions. The neurite network area per image, identified as described above, was measured as the pixel area occupied per image. The 'synaptic object' size for the image pair size overlap was measured as pixel area overlap. In addition, the count of both 'synaptic objects' and non-synaptic 'primary punctae objects' per unit area of the neurite network were measured. Data per well were determined by first aggregating the data of images taken within the same well for all sites and then over replicate wells and experiments. A dilution series of per well of the reagents used in each condition was undertaken to determine their minimum effective concentration as assessed by their deviation from control conditions. Results shown were calculated from the data derived from each condition at its minimum effective concentration and are determined from the median measurements per image per condition, normalised to neuron cell number per well and represent the average of triplicate experiments expressed as a percentage of control conditions. The average number of neuron cells per condition per experiment were >200. The median numbers of pre and post excitatory or inhibitory marker punctae per condition per experiment measured were >1200 and >1000, or >1100 and >800, respectively. The median numbers of excitatory and inhibitory synapses per condition per experiment measured were >400 and >600, respectively.

Supplementary References

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Wong, R., Piper, M. D., Wertheim, B. and Partridge, L. (2009). Quantification of food intake in *Drosophila*. *PLoS one* **4**, e6063.

Supplementary Figures

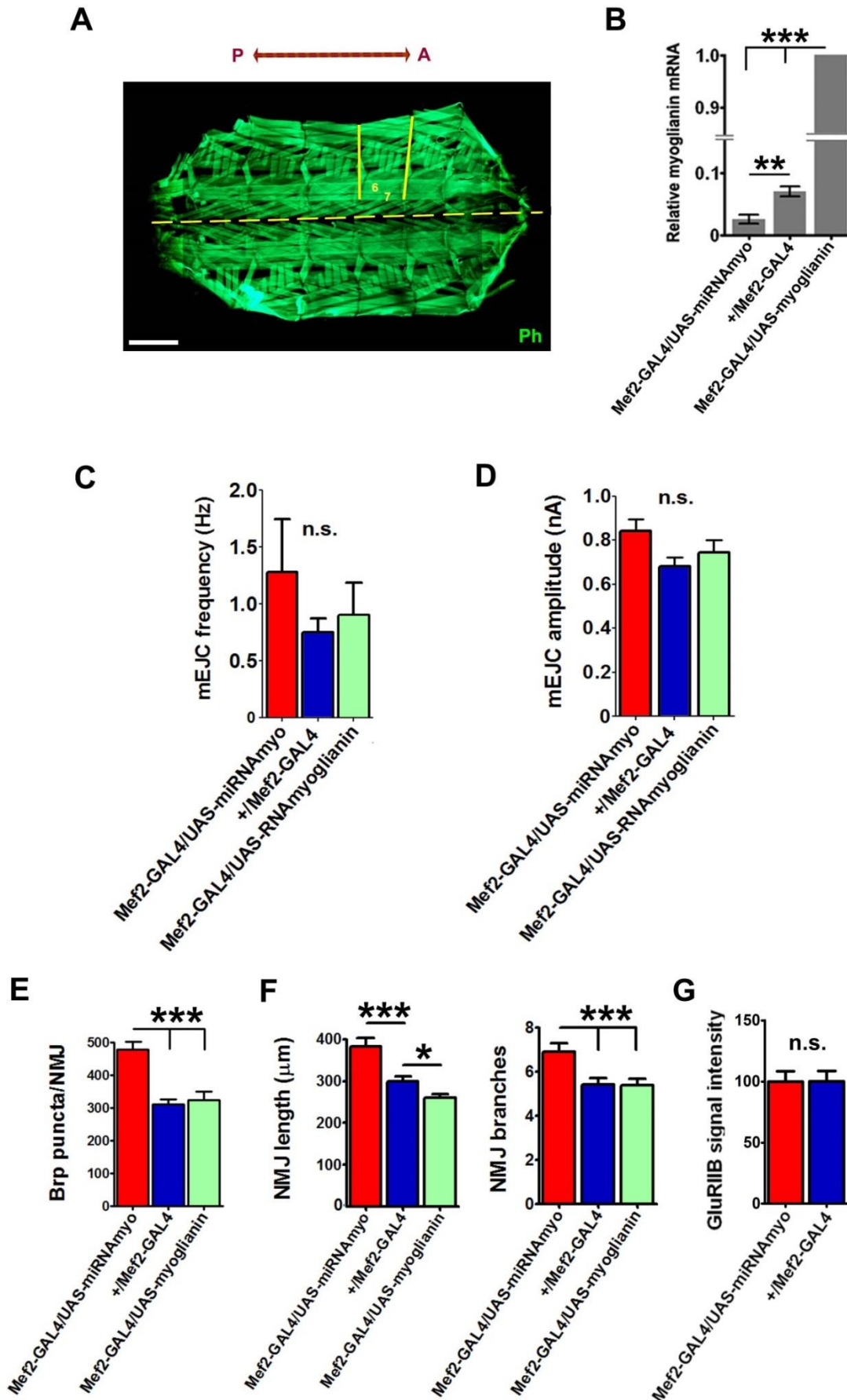


Figure S1

Figure S1. (A) Larval body muscles in third instar larvae stained with phalloidin. A third hemisegment containing muscles 6 and 7 is marked with yellow bars. Dashed line marks the midline. The posterior (P) and anterior (A) directions are denoted with the double-headed arrow above the image. Scale bar: 500 μm . (B) Relative levels of the *myo* transcript in 3rd instar larval muscle preparations ($n = 4$ per genotype). (C) Expression of *myoglianin* constructs in body-wall muscles did not affect the frequency of miniature excitatory junctional currents (mEJCs) at the larval NMJ ($n = 6-16$), or the mean amplitude of miniature excitatory responses ($n = 7-16$) (D). (E) Total number of Brp puncta at the 6/7 NMJ ($n = 10-15$). (F) GluRIIA signal intensity at the NMJ ($n = 6-10$). (G) NMJ length (*left*), and number of 6/7 NMJ branches (*right*) ($n = 11-21$). (H) Synaptic GluRIIB remained unchanged upon motoneuronal *myo* manipulation ($n = 5-7$). All panels: error bars indicate SEM (ANOVA + Tukey's post-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not significant).

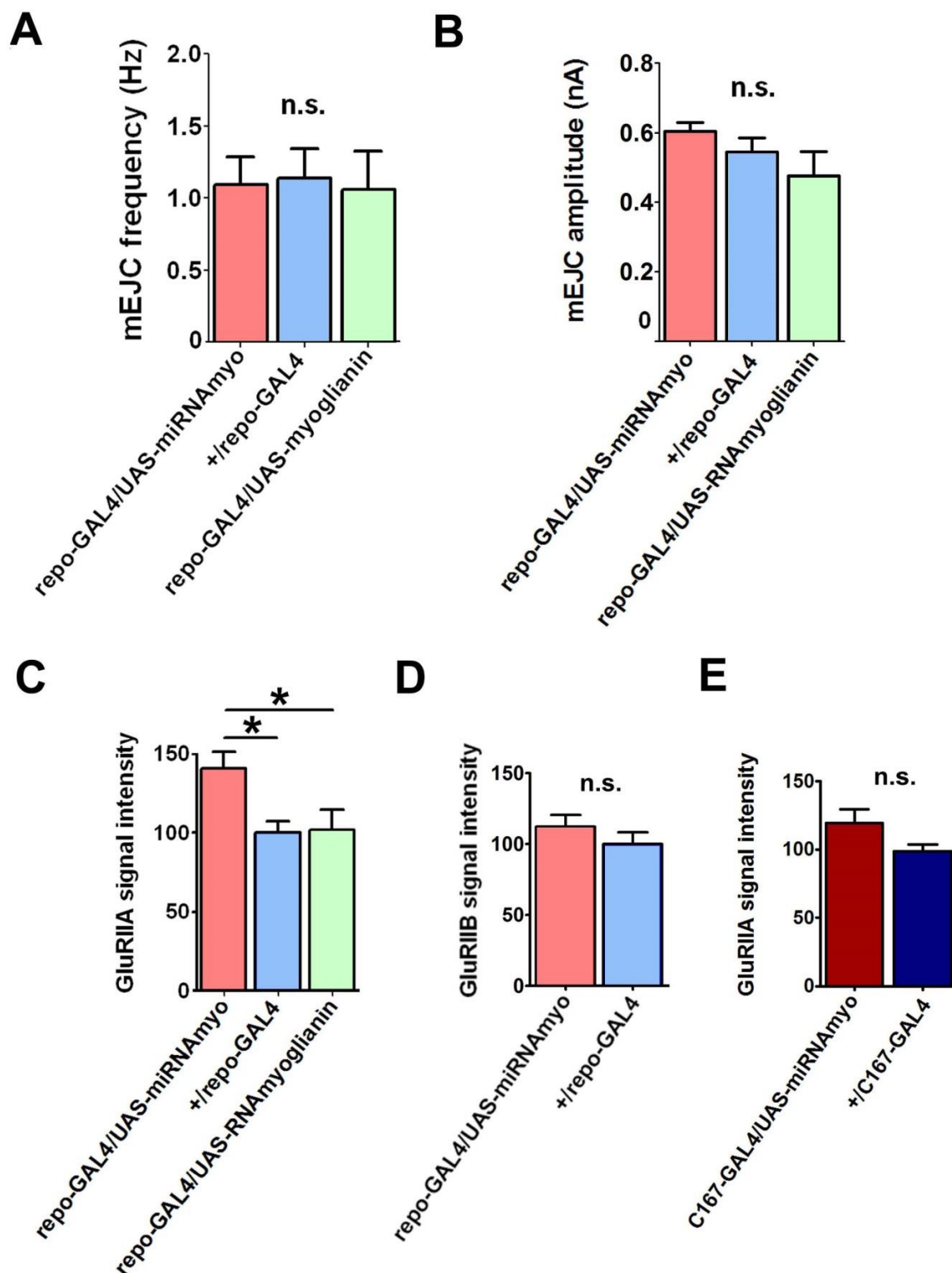


Figure S2.

Figure S2. (A and B) No change in the frequency of mEJCs (A) or mean ‘mini amplitude’ (B) upon *myo* manipulation in glial cells ($n = 6-12$). (C) Quantification of GluRIIA signal intensities in larvae expressing various *myo* constructs in glial cells ($n = 7-10$). (D) Glial expression levels of *myo* did not affect the NMJ density of GluRIIB receptors ($n = 6-7$). (E) Synaptic GluRIIA remained unchanged upon motoneuronal *myo* silencing ($n = 7$). All panels: Error bars indicate SEM (ANOVA + Tukey’s post-test or unpaired t-test (for comparison between 2 genotypes): * $p < 0.05$, n.s.= not significant).

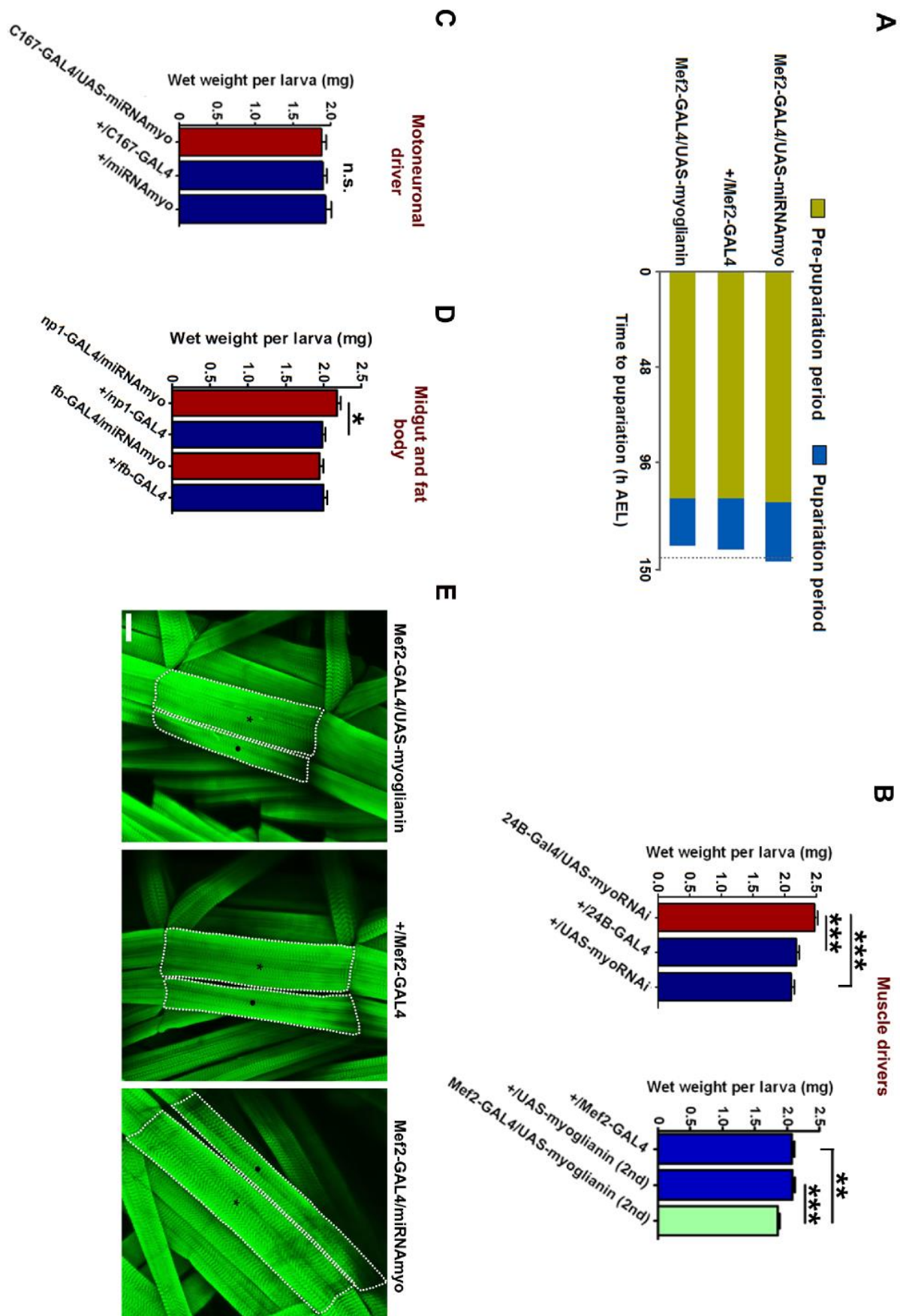


Figure S3.

Figure S3. (A) Developmental progression in flies expressing variable levels of *myo* ($n = \sim 100$ per genotype). Blue marks the period of time during which the larvae pupariated. (B) Larval wet weight is increased when a different muscle driver (*24B-GAL4*) is combined with a *UAS-myoRNAi* transgenic construct (*left*), and decreased when an alternative *UAS-myoglianin* (2nd chromosome) construct is used (*right*). (C) Wet weight in the larvae with *myo* levels manipulated in motoneurons (*C167-GAL4* driver), or (D) fat bodies (*fb-GAL4* driver) and midgut (*np1-GAL4* driver) ($n = 922$ for weight measurements). All panels: error bars indicate SEM (ANOVA + Tukey's post-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and n.s. = not significant). (E) Representative images of the muscles 6 (asterisk) and 7 (circle) examined for fiber area in Figure 3D. Scale bar: 40 μm .

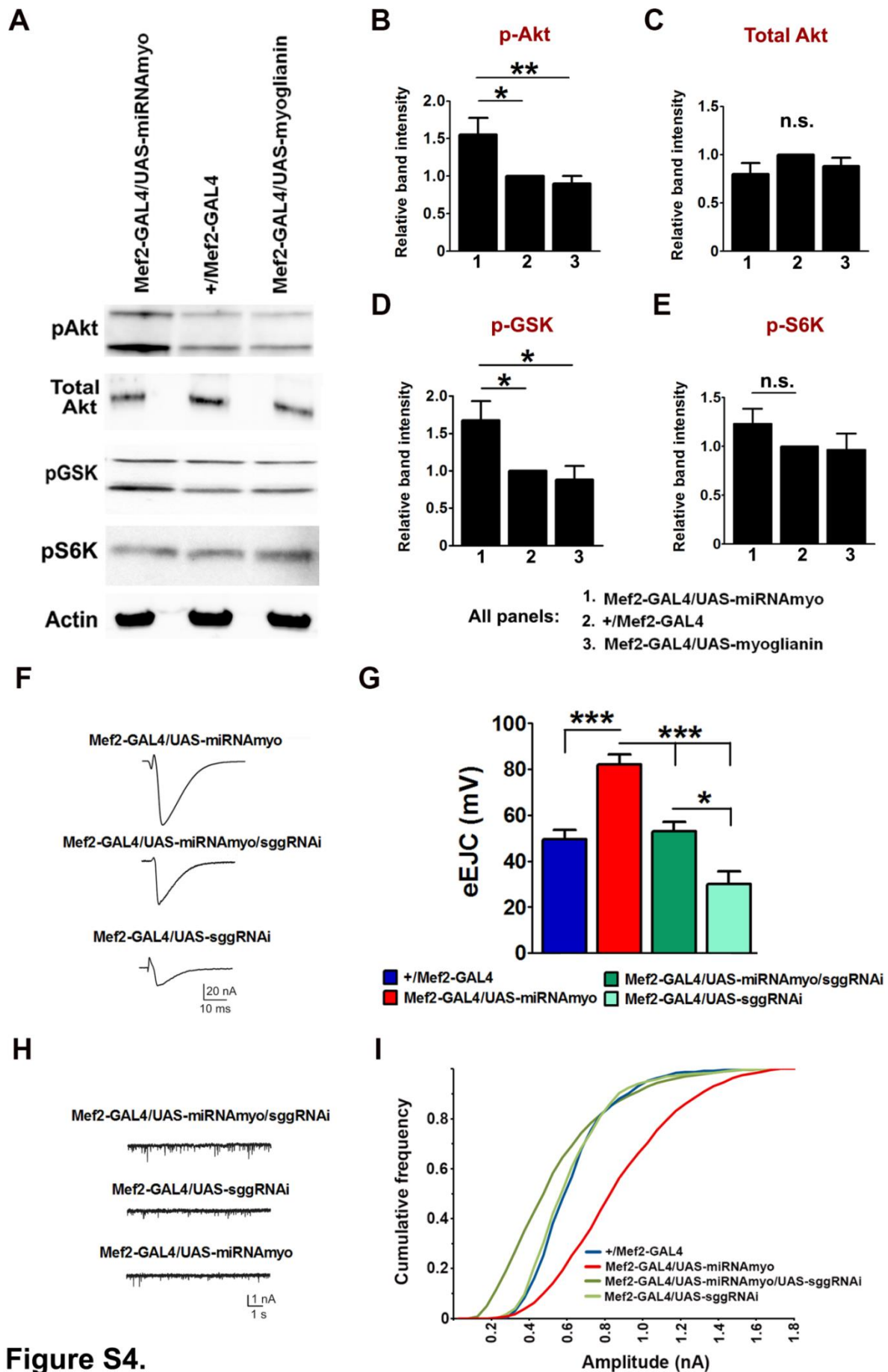


Figure S4.

Figure S4. (A) Representative Western blots from *Mef2-GAL4/UAS-miRNA_{myo}*, *Mef2-GAL4/UAS-miRNA_{myo}* and *+Mef2-GAL4* (control) larval muscles using different antibodies (see Supplemental Experimental Procedures). (B-E) Quantification of Western blots performed on larval muscle preparations ($n = 5-12$). (F) Representative eEJC traces for (G). (G) Quantification of eEJC amplitudes in the *myo/Shaggy* epistasis experiment ($n = 6-12$). (H) Representative 'miniature' EJC traces for (I). (I) Cumulative frequency diagram of mEJC amplitudes ($n = 6-16$). All panels: error bars indicate SEM (ANOVA + Tukey's post-test: * $p < 0.05$, ** $p < 0.01$, *** $P < 0.001$, n.s = not significant).

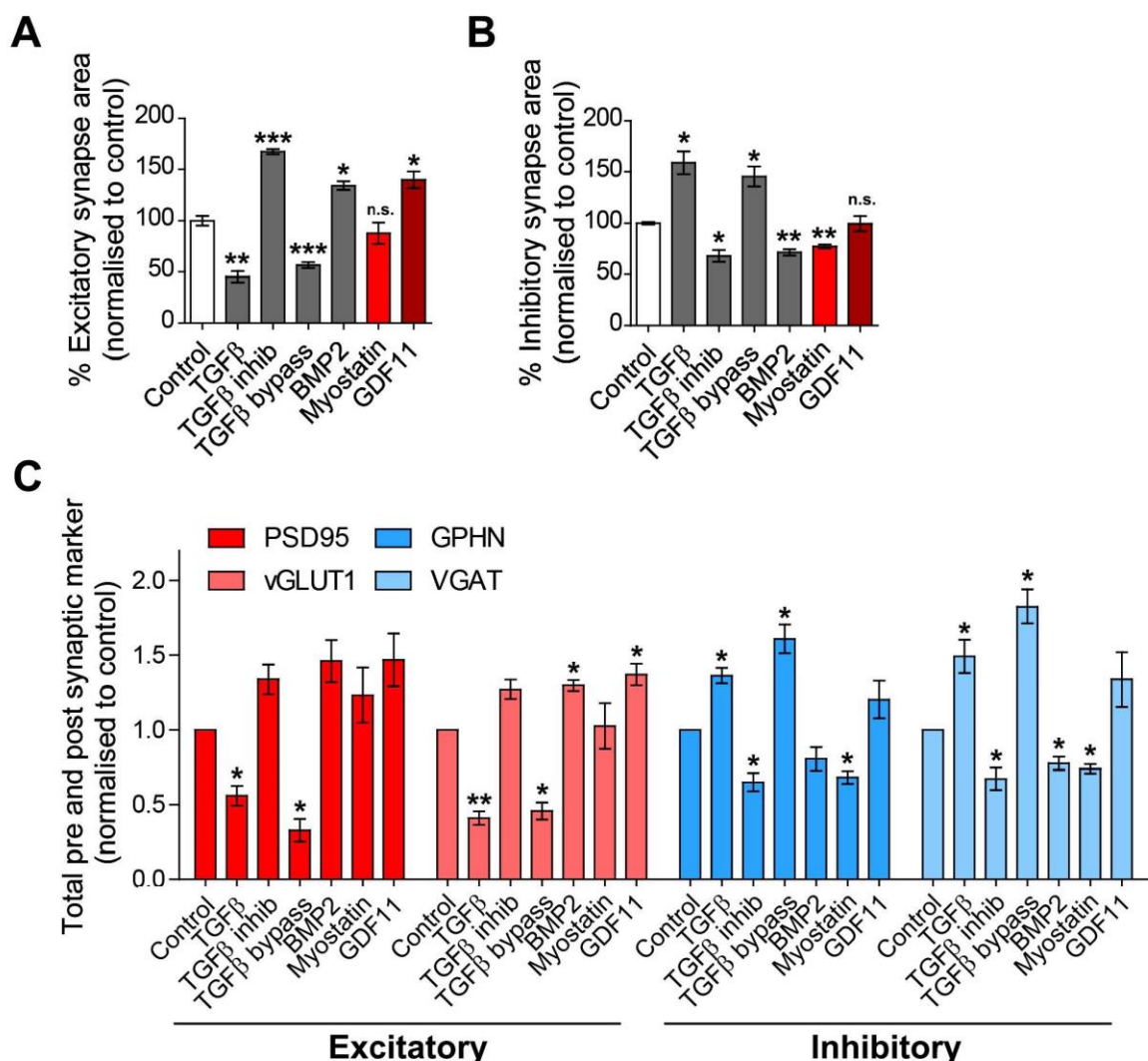


Figure S5.

Figure S5. (A) Microscopy image quantification of median excitatory synapse area occupied per image, normalised to control after indicated treatments as in Fig. 6C. Synapses are marked by co-labelling with vGLUT1 and PSD95 localized to neurites (MAP2) ($n = 3$ independent experiments). (B) Microscopy image quantification of median inhibitory synapse area occupied per image normalised to control after indicated treatments as in Fig. 6C. Synapses are marked by co-labelling with VGAT and GPHN localized to neurites (MAP2), ($n = 3$ independent experiments). (C) Microscopy image quantification of rat brain isolated cortical neuron culture treated as indicated with either DMSO (Control), 5 ng/ml TGF- β 1 (TGF- β), 400 nM TGF- β 1 signalling antagonist (A83), 400 nM TGF- β 1 signalling agonist (Alento), 10 ng/ml BMP2 (BMP2), 10 ng/ml Myostatin or 10 ng/ml GDF11 (GDF11) for 5 days commencing from 6 DIV. Cultures were immunostained for excitatory pre (vGLUT1) and post (PSD95) and inhibitory pre (VGAT) and post (GPHN) synaptic density markers in addition to a neuronal marker (MAP2, blue). Quantification represents the median punctae frequency per neurite area per image of the indicated marker normalised to control ($n = 3$ independent experiments). Error bars represent SEM. (ANOVA + Dunnett + test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, n.s. = not significant).

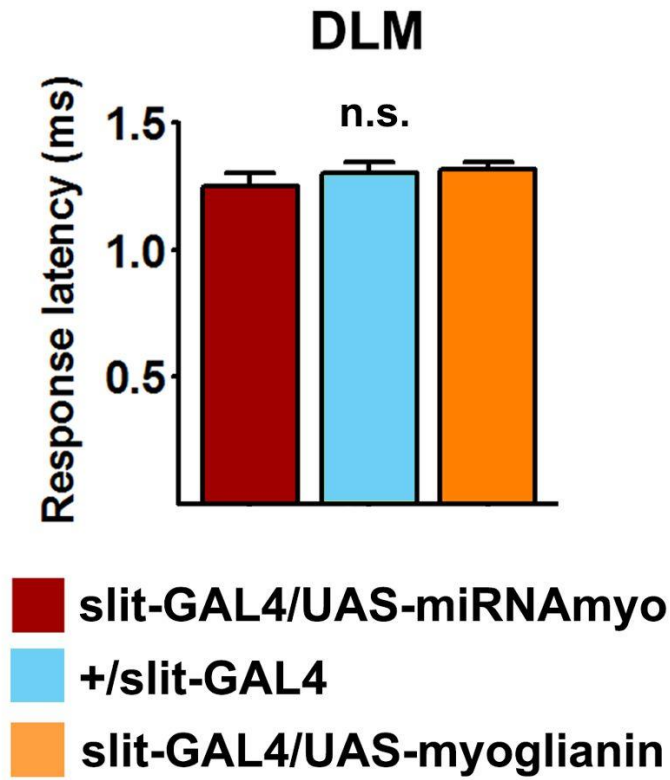
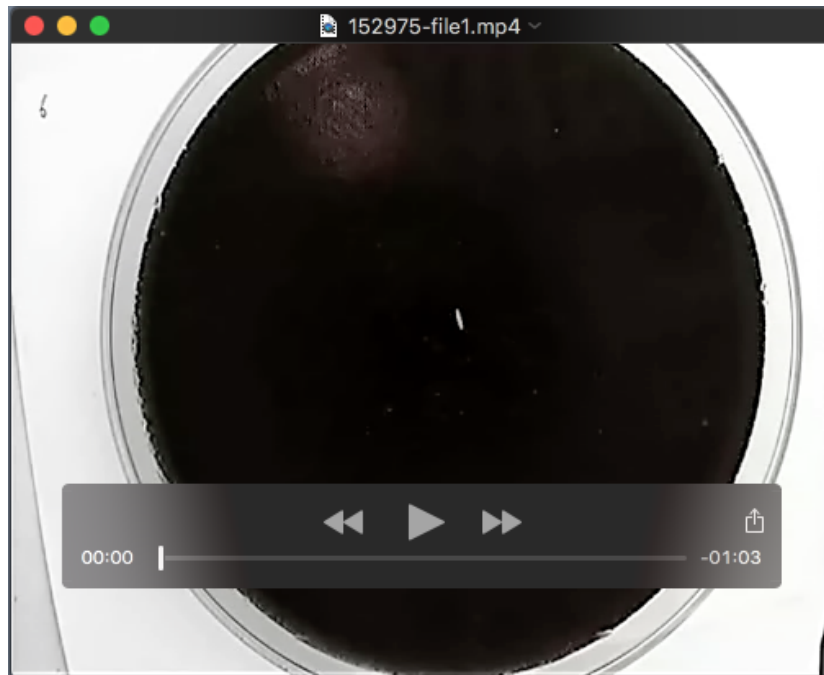


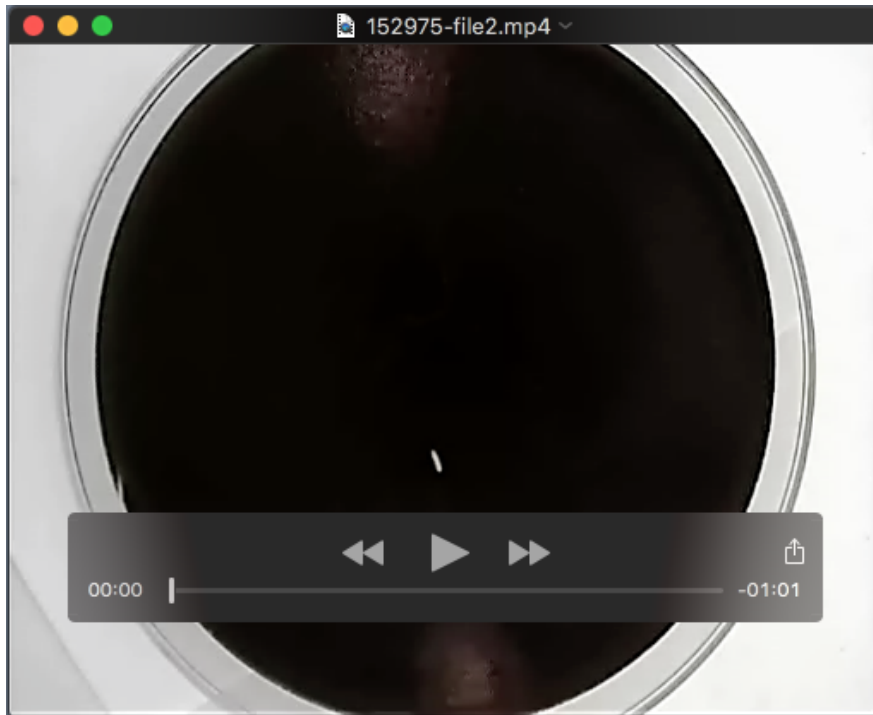
Figure S6.

Figure S6. Quantification of response latencies in the DLM branch of the GF circuit ($n = 5-7$). (ANOVA + Tukey's post-test: n.s. = not significant).

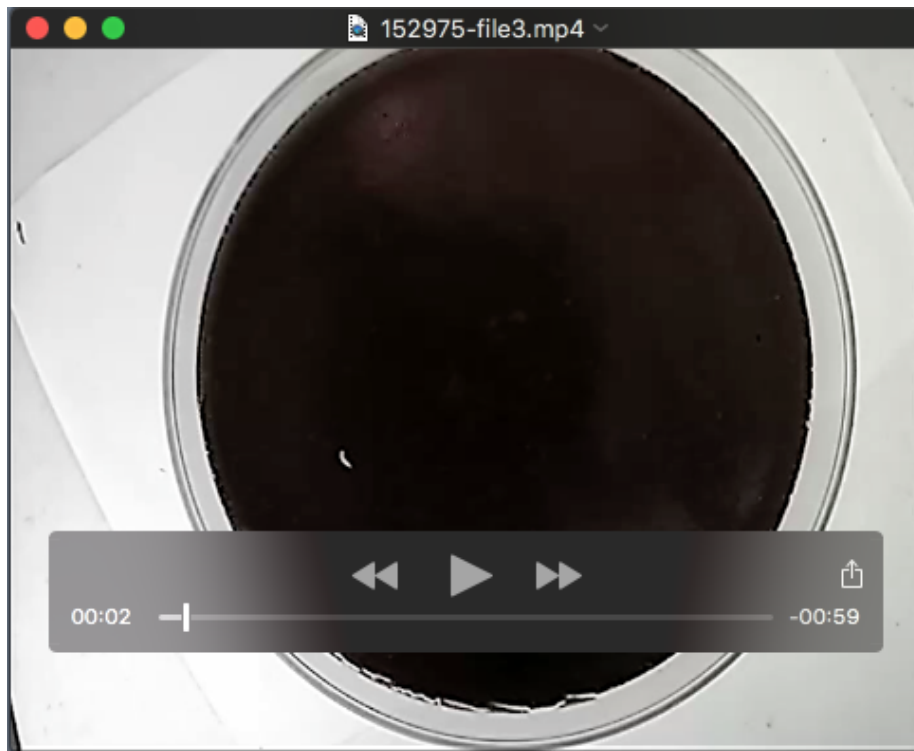
Movies



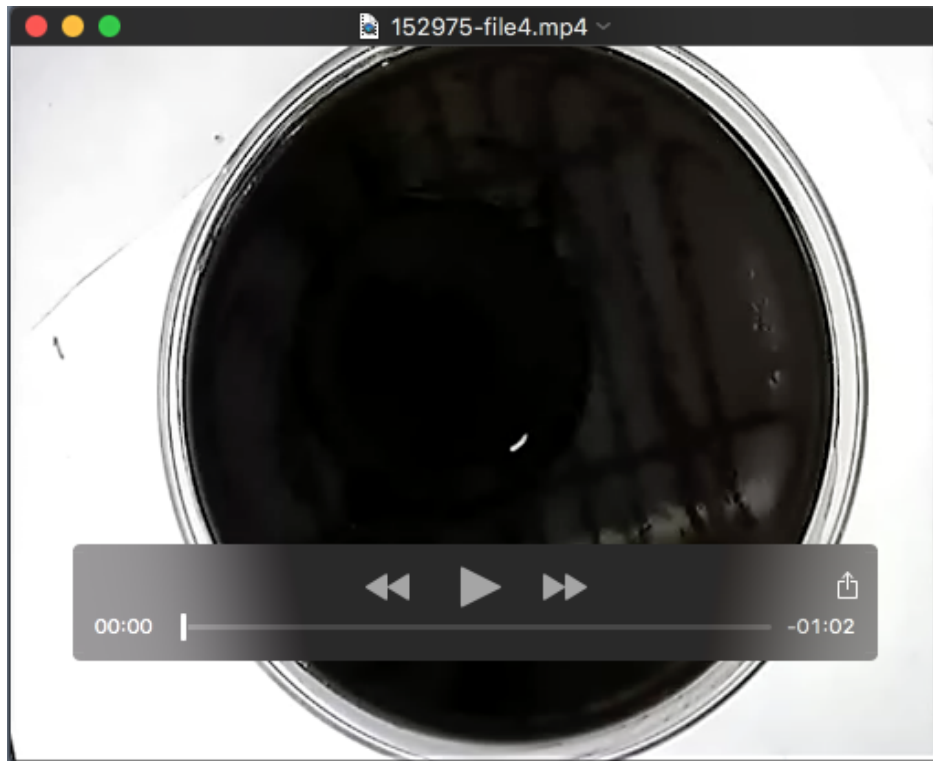
Movie 1. Larval crawling in +/-Mef2-GAL4



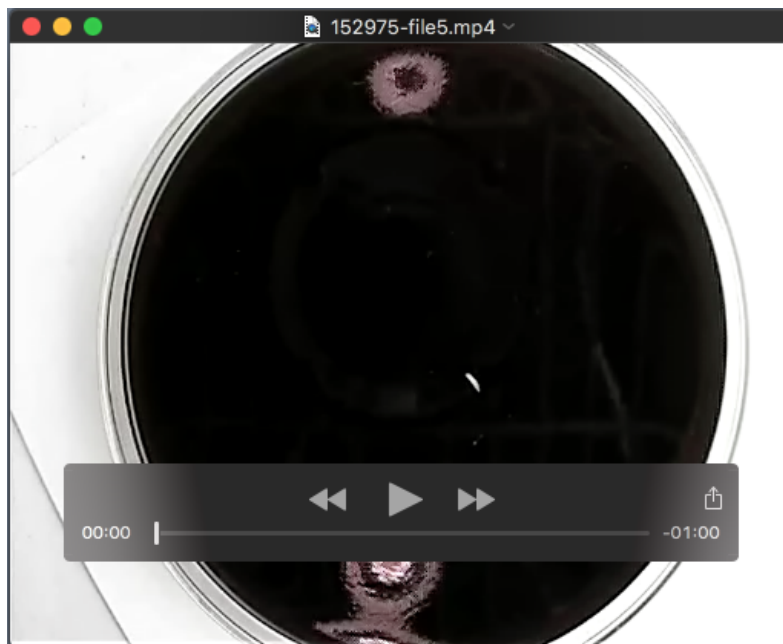
Movie 2. Larval crawling in Mef2-GAL4/UAS-miRNAmyo



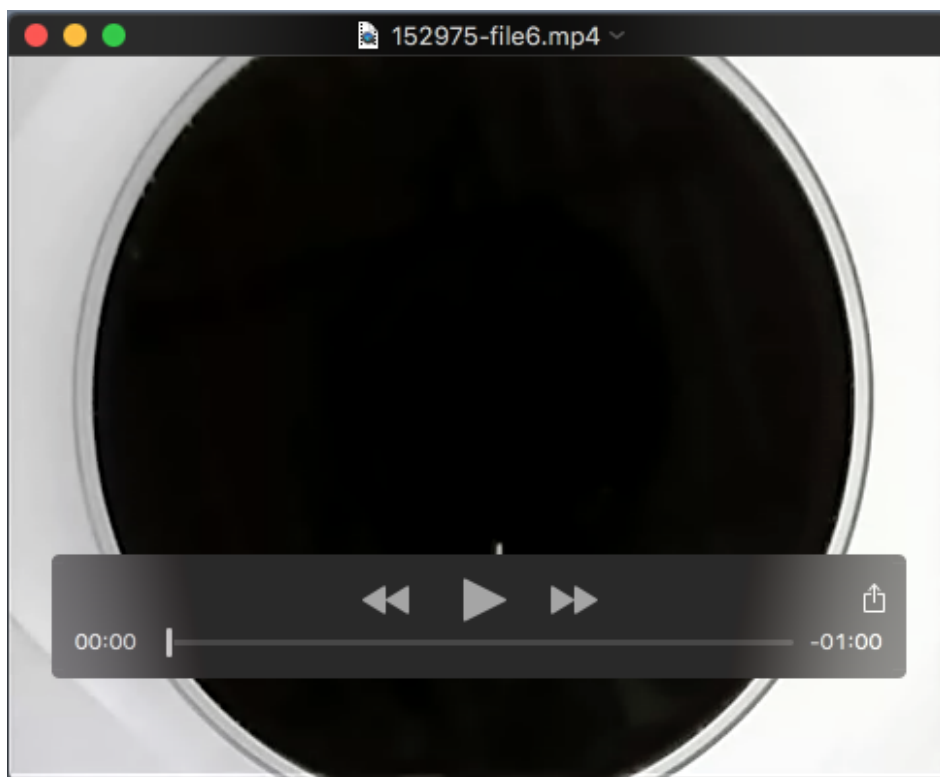
Movie 3. Larval crawling in Mef2-GAL4/UAS-myoglianin



Movie 4. Larval crawling in +/-repo-GAL4



Movie 5. Larval crawling in repo-GAL4/UAS-miRNAmyo



Movie 6. Larval crawling in repo-GAL4/UAS-myoglianin