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*Drosophila* voltage-gated sodium channels are only expressed in active neurons and are localized to distal axonal initial segment-like domains

Abbreviated Title: Characterizing Na\textsubscript{v} channel distribution in fly neurons

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Abstract

In multipolar vertebrate neurons, action potentials (AP) initiate close to the soma, at the axonal initial segment (AIS). Invertebrate neurons are typically unipolar with dendrites integrating directly into the axon. Where APs are initiated in the axons of invertebrate neurons is unclear. Voltage-gated sodium (NaV) channels are a functional hallmark of the AIS in vertebrates. We used an intronic MiMIC to determine the endogenous gene expression and subcellular localization of the sole NaV channel in both male and female Drosophila, para. Despite being the only NaV channel in the fly, we show that only 23 ±1% of neurons in the embryonic and larval CNS express para, while in the adult CNS para is broadly expressed. We generated a single-cell transcriptomic atlas of the whole 3rd instar larval brain to identify para expressing neurons and show that it positively correlates with markers of differentiated, actively firing neurons. Therefore only 23 ±1% of larval neurons may be capable of firing NaV-dependent APs. We then show that Para is enriched in an axonal segment, distal to the site of dendritic integration into the axon, which we named the Distal Axonal Segment (DAS). The DAS is present in multiple neuron classes in both the 3rd instar larval and adult CNS. Whole cell patch clamp electrophysiological recordings of adult CNS fly neurons are consistent with the interpretation that NaV-dependent APs originate in the DAS. Identification of the distal NaV localization in fly neurons will enable more accurate interpretation of electrophysiological recordings in invertebrates.

Significance statement

The site of AP in invertebrates is unknown. We tagged the sole NaV channel in the fly, para, and identified that Para is enriched at a distal axonal segment (DAS). The DAS is located distal to where dendrites impinge on axons and is the likely site of AP initiation. Understanding where APs are initiated improves our ability to model neuronal activity and our interpretation of electrophysiological data.
Additionally, para is only expressed in 23 ±1% of 3rd instar larval neurons but is broadly expressed in adults. Single-cell RNA sequencing of the 3rd instar larval brain shows that para expression correlates with the expression of active, differentiated neuronal markers. Therefore, only 23 ±1% of 3rd instar larval neurons may be able to actively fire NaV-dependent APs.

**Introduction**

Action potentials (AP) are generated by the sequential opening of voltage-gated sodium (NaV) and potassium channels in the axons of neurons (Sherwood, 2008). Mammalian central nervous system (CNS) neurons are typically multipolar and APs initiate at the dense concentration of NaV channels in the axonal initial segment (AIS) close to the soma, and propagate along the axon via the nodes of Ranvier (Huxley and Stämpeli, 1949; Salzer, 2003; Palmer and Stuart, 2006; Shu et al., 2006; Kole et al., 2008; Rasband and Peles, 2016). In addition to AP initiation, the AIS forms a barrier between the soma and the axon, preventing the free diffusion of organelles, proteins and lipids between the two compartments (Palay et al., 1968; Kobayashi et al., 1992; Winckler et al., 1999; Song et al., 2009). Invertebrate neurons are typically unipolar with the dendrites impinging upon the axon distal to the cell body (Rolls, 2011). Whether an AIS is present in these neurons, and where it is located along the axon is unresolved.

In order to determine the site of AP initiation, and if and where the AIS is in invertebrate neurons, we examined the location of the sole NaV channel gene in *Drosophila melanogaster*, *paralytic* (para). Unlike mammals, which have multiple NaV encoding genes (*SCN1-5A, 8-11A*) (Huang et al., 2017), the genome of *Drosophila melanogaster* encodes only 2 genes predicted to encode NaV genes, *para* and *Na channel protein 60E* (*NaCP60E*) (Suzuki et al., 1971; Okamoto et al., 1987; Tseng-Crank et al., 1991; Hong and Ganetzky, 1994). *para* is the putative NaV channel as *NaCP60E* null animals are viable with no loss of inward sodium currents detected in neurons using patch-clamp (Germeraad et al., 1992; Anholt et al.,
In contrast, *para* null animals die as 1st instar larvae with no detectable inward sodium current in neurons using patch-clamp (Loughney et al., 1989; O’Dowd et al., 1989; Hong and Ganetzky, 1994). Despite having one Na_v gene, compared to nine in mammals, it is possible that a similar degree of channel protein diversity is achieved via alternate splicing. *para* has 60 predicted isoforms, some of which have different developmental expression (Lin et al., 2009; Baines et al., 2012). Very little is known about the expression pattern or subcellular localization of Para. *In situ* hybridization studies determined that *para* is expressed in the nervous system from embryos to adults (Amichot et al., 1993; Hong and Ganetzky, 1994). Whether *para* is expressed in all or just some cells in the nervous system and where it is subcellularly localized remains to be established.

To determine the expression pattern and protein localization of Na_v channels in *Drosophila* neurons, we used previously established tools (Bateman et al., 2006; Venken et al., 2011), to develop two novel fly models. A model where the endogenous Para is tagged with GFP to determine Para subcellular localization, and another with *para* replaced with GAL4 to determine *para* gene expression.

Surprisingly, we find *para* present in a small fraction of CNS neurons in embryos and 3rd instar larvae, while it is broadly expressed in neurons in the in the adult CNS. We also generated a single cell transcriptomic of the whole 3rd instar larval brain to identify that *para* correlates with RNAs of active zone proteins and mature neuron markers, hence *para* expression is restricted to active, differentiated neurons in larvae. Neurons that co-express *para* and active zone protein RNAs are abundant in the adult CNS, but only represent 23 ±1% of neurons in 3rd instar larvae. In neurons where *para* is expressed, Para protein is enriched at an AIS-like region in axons distal to where the dendritic tree connects to the axons in a distal axonal segment (DAS). Para localized far from the soma is functionally verified electrophysiologically. In longer neurons, Para is expressed throughout the axon, likely to maintain AP propagation to the synapses.
Materials and Methods

Reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Hugo Bellen (hbellen@bcm.edu). Flies generated in this study will be deposited to the Bloomington *Drosophila* Stock Center.

Model and subject details

For experiments using gene or protein trapped *para* alleles, all stocks were kept at room temperature (22°C) and all crosses are performed at 25°C, both male and female flies were used for imaging experiments. For the single cell RNA sequencing experiments on the larval brain, flies were raised on a yeast based medium at 25°C on a 12h/12h day/night light cycle. All *Drosophila* lines used in the single-cell RNA-seq experiments are derived from the DGRP collection. One hybrid was created by crossing different DGRP lines, generating genetic diversity. Fly lines were obtained from Bloomington *Drosophila* Stock Center and the Kyoto Stock Center and are listed in Table 1.

Methods

Generating fluorescently tagged *para* flies

Tagged alleles were generated as previously described (Venken et al., 2011). In brief, two separate plasmids, pBS-KS-attB1-2-PT-SA-SD-0-EGFP-FIAsH-StrepII-TEV-3xFlag (*Drosophila* Genomic Resource Center (DGRC) #1298) and another containing PhiC31 integrase mRNA, are injected into *para*~MOR879~ embryos. These embryos are left to develop into adult flies where they are isolated after eclosion and crossed to *w*~1118~/*FM7h* flies. The loss of the yellow marker is screened for to detect successful RMCE. To check insertion orientation, DNA is isolated from yellow negative animals and using primers on either side of the original *attP* sites the orientation of the insertion is determined. Insertions
in the same orientation as para transcription were kept. This process was also used for pBS-KS-attB1-2-PT-SA-SD-0-TAGRFP-T-3XHA (DGRC#1301) and pBS-KS-attB1-2-GT-SA-GAL4-Hsp70Pa (DGRC#1412) constructs.

Confirmation of GFSTF incorporation into all para transcripts

To confirm incorporation of GFSTF into all para transcripts, we performed PCR across the GFSTF exon from para-GFSTF and para-MiMIC cDNA. cDNA was generated using SuperScript™ IV First-Strand Synthesis System (Invitrogen) from RNA isolated from 10 adult fly heads. The fly heads were ground up, using a pestle, in 30 μl Trizol. The Trizol volume was increased to 400μl and incubated at room temperature for 5 mins after brief vortexing. 80 μl of chloroform is added, and the samples are incubated at room temperature after 15 s high speed vortexing. Samples were centrifuged at 14,000 rpm for 20 min at 4°C, the top layer was added to 200 μl isopropanol and incubated at room temperature for 10 mins after mixing. The centrifugation step was repeated, and the supernatant removed. The pellet was washed with 500 μl 70% ethanol and the centrifugation step repeated once more. The pellet was then air dried and resuspended in DEPC H2O.

A forward primer in exon 1 (5’ CAGTTTGTTCCGTCCTTAC 3’) and a reverse primer in exon 4 (5’ TTCCCTGGCTACAACGTAATG 3’) were used to amplify the region. The bands were extracted using QIAquick (Invitrogen) and Sanger sequenced. To confirm the abundance of each tagged transcript a forward primer across the exon 1-2 boundary (5’ GAGAGGTCGCCGCAATATGGTC 3’) and reverse primers in the GFP sequence (5’ AACAGCTTCCGGCCCTTGC 3’) and across the exon 3-4 boundary (5’ ACACTACGAATGTCACTACATTGC 3’) were used for quantitative PCR (Q-PCR) (SYBR Green, Bio-Rad) with primers in Actin5C (F – 5’ ACACACAAATCTTACAAAATG 3’, R- 5’ CCACAATCGATGGGAAGAC 3’) used for control gene expression. Q-PCR was performed with three technical replicates for each of three biological replicates, with a negative control cDNA which was generated without adding reverse
transcriptase. Analysis was performed using Bio-Rad CFX manager 3.1 with the relative quantity ($\Delta Cq$) with reference to zero used for quantification, error bars show standard error of the mean.

Western Blot confirmation of Para-GFSTF

For Western blot analysis 20 adult fly heads of *para-MiMIC* and *para-GFSTF* animals were added to 60 $\mu$L Laemmli buffer with 5% β-mercaptoethanol and 1X GenDEPOT protease inhibitor (Fisher Scientific # 50-101-5485). Heads were ground using a pestle, then centrifuged for 10 mins at 15,000 rpm at 4°C. 10 $\mu$L of sample supernatant was then loaded into a 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad #4561096) as well as two ladders, a HiMark™ Pre-stained Protein Standard (ThermoFisher #LC5699) and a Precision Plus Protein™ Dual Color Standard (Bio-Rad # 1610374).

Samples were run at a constant 75 mV for 10 mins and then 200 mV until the 20 kDa band of the Precision Plus Protein™ Dual Color Standard reached the bottom of the gel and transferred to a nitrocellulose membrane at 400 mA for 90 mins at 4°C. The membrane was then washed in TBS-T (0.1%) and blocked for 1 hour in 5% skimmed milk. The membrane was then incubated in rabbit Anti-GFP (1:1,000) (ThermoFisher Scientific #A-11122) overnight in a TBST (0.1%) 5% skim milk solution at 4°C. The antibody was removed, and blots washed in TBST (0.1%). HRP conjugated Goat anti-Rabbit (1:10,000) (Jackson ImmunoResearch) secondary antibodies were then incubated with the blot in a TBST (0.1%), 5% skim milk solution for 2 hrs at room temperature. Blots were washed again in TBST (0.1%) and then placed in a clear plastic film and 500 $\mu$L of SuperSignal™ West Dura Extended Duration Substrate (ThermoFisher Scientific) added to the blot. The blot is then subsequently imaged using a ChemiDoc™ MP Imaging System (Bio-Rad). The blot is then stripped using a 15-minute room temperature incubation with Restore™ Western Blot Stripping Buffer (ThermoFisher Scientific) and the process is repeated from the initial wash and blocking steps using a mouse anti-Actin (1:5,000) (EMD 8
Millipore # MAB1501) primary antibody and a HRP conjugated Goat anti-Mouse (1:10,000) (Jackson ImmunoResearch) secondary antibody.

Electroretinogram (ERG) Assay

For ERG recordings para-MiMIC and para-GFSTF flies were aged to 5 days at 25°C in 12h light/12h dark cycle or in 24h darkness. ERG recordings were performed as previously described (Verstreken et al., 2003) using LabChart software (AD instruments). At least 8 flies were examined for each genotype.

Quantification was performed using Prism 8.0.

Temperature sensitive paralysis assay

para-GFSTF, para-mCherry, para-RFP-3xHA, para-MiMIC and para<sup>++</sup> males were collected at room temperature and aged for 4 days in vials of at most 10 flies. These flies were transferred to a fresh empty vial (2.5 cm diameter, 9.5 cm height) with a foam plug and given 5 mins to acclimate to the new environment. These vials were then immersed in a water bath at 40°C for 1 minute. Every 20 s the number of flies that were unable to stand was recorded. After 1 minute the flies were removed from the water bath and placed on a bench top gently where they were monitored every 20 s and the number of flies unable to stand was recorded until all flies were upright. At least 100 flies for each genotype were recorded. The results were plotted by accumulating all the data for each genotype and recording the paralysis as percentage of flies still standing. The error bars represent standard deviation.

Lethality screening of para-T2A-GAL4

para-T2A-GAL4 and two previously published para null alleles, para<sup>A</sup> and para<sup>B</sup> (Yamamoto et al., 2014) were balanced over FM7c, Kr>eGFP and placed in an embryo collection chamber on a grape juice plate with yeast to encourage egg laying. After 24 hrs a fresh plate was placed in the chamber. Flies
could lay on the plate for 24 hrs. After incubation the plates were visualized using a fluorescence microscope to look for larvae devoid of the Kr>eGFP expression pattern and staged appropriately.

**Immunofluorescent staining**

*Embryos*

Parent flies are placed in an embryo collection chamber on a grape juice plate with yeast to encourage egg laying. After 24 hrs acclimation a fresh plate is added, and flies can lay eggs on the plate for 24 hrs at room temperature to enable collection of embryos at a variety of stages. Embryos are dislodged from the plate using a paintbrush and water and transferred to a cell strainer using a Pasteur pipette. Embryos are washed with water to remove the excess yeast and grape juice and are placed in a 50% bleach 50% water solution for 3 mins to dechorinate the embryos. Embryos are then washed with water, blotted dry and then transferred via paintbrush to a glass scintillation vial containing 4ml N-heptane and 4ml modified Steffani’s fixative (4% Formaldehyde (P.A. grade, methanol free) 15% saturated aqueous solution of picric acid, 75mM disodium 1,4-Piperazinediethanesulfonic acid (PIVES) in phosphate buffered saline (PBS), pH7.4) (Stefanini et al., 1967), that has been vigorously shaken and allowed to separate into layers. The embryos float between the heptane and fixative layers. The vial is then placed on a rotating platform for 25 mins at room temperature. After 25 mins the lower fixative layer is removed and replaced with equal volume 100% methanol. The vial is shaken vigorously to remove the vitelline membrane and embryos fall to the bottom of the vial. The lower methanol layer containing the embryos is then removed and placed in a separate vial where it is washed 2 more times with 100% methanol. Embryos are transferred to an Eppendorf tube and the methanol replaced with PBS-Tween (PBST) (0.2%). Embryos are washed 3 times with PBST (0.2%) before incubating in PBST (0.2%) with 10% Normal Goat Serum (NGS) solution for 1 hour on a rotating platform at room temperature. The blocking solution is then removed and a solution containing primary antibodies, 10%
NGS and PBST (0.2%) is added and embryos are incubated overnight at 4°C on a rotating platform. Primary antibodies are then removed, and embryos are washed in PBST (0.2%) 3 times for 10 mins at room temperature before secondary antibodies are then added in a PBST (0.2%) with 10% NGS solution and incubated on a rotating platform overnight at 4°C. Secondary antibodies are then removed, and embryos are washed in PBST (0.2%) 3 times for 10 mins at room temperature. PBST (0.2%) is removed and ProLong Gold mounting medium (ThermoFisher #P36930) added to the tube. Embryos are then transferred to a glass slide and sealed with a no 1.5 coverslip and nail polish.

Primary antibodies used for imaging are mouse anti-Flag (1:200) (Sigma-Aldrich #F1804) and rat anti-Elav (1:500) (DSHB #7E8A10 (O’Neill et al., 1994)). Corresponding goat secondary antibodies were used (1:500) (Jackson ImmunoResearch) as well as Cy3 conjugated goat anti-HRP antibody (1:250) (Jackson ImmunoResearch #123-165-021).

3rd instar larvae brain dissection

Wandering 3rd instar larvae were collected from the side of vials and placed in cold PBS. The larval brains were dissected using fine forceps and the brains were placed in cold 3.7% paraformaldehyde (PFA) in 1 X PBS overnight at 4°C. The PFA solution is then aspirated off and washed twice with a 0.2% PBS-Triton-X (PBS-TX) solution and left to incubate at 4°C in 0.2% PBS-TX overnight. The 0.2% PBS-TX solution is aspirated and replaced with a primary antibody solution diluted in 0.2% PBS-TX solution with 5% NGS. Brains are left in the primary solution overnight at 4°C. The primary antibodies are then removed, and brains are washed quickly twice in 0.2% PBS-TX solution and incubated for 10 mins at room temperature in 0.2% PBS-TX solution three times. The 0.2% PBS-TX solution is then removed and replaced with secondary antibodies diluted in 0.2% PBS-TX solution and 5% NGS and incubated at 4°C overnight. The secondary antibodies are then removed, and brains are washed quickly twice in 0.2% PBS-TX solution and incubated for 10 mins at room temperature in 0.2% PBS-TX solution.
three times. The 0.2% PBS-TX solution is then removed and replaced with RapiClear 1.47 mounting medium (SUNJin Lab). The brains in the mounting medium are then placed inside a circular spacer on a glass slide. A number 1.5 coverslip is then placed on top of the solution and the coverslip is sealed with nail polish.

Primary antibodies used for imaging are Rabbit anti-GFP (FITC conjugated) (1:200) (ThermoFisher Scientific #A-21311), rat anti-Elav (1:500) (DSHB #7E8A10 (O’Neill et al., 1994)), rabbit anti-mCherry (1:200) (GeneTex #GTX59788) and mouse Anti-HA.11 (1:500) (Previously Covance catalog# MMS-101R). Corresponding goat secondary antibodies were used (1:500) (Jackson ImmunoResearch) as well as Cy3 conjugated goat anti-HRP antibody (1:250) (Jackson ImmunoResearch #123-165-021).

Adult brain dissection

For adult brain dissections animals are anaesthetized using CO₂ and using forceps transferred to a Sylgard plate where Minutien pins are inserted, firstly into the abdomen and then the thorax of the fly, positioning the fly dorsal side up with the thorax at a 45° angle to the plate. The plates are rinsed with PBS and then briefly washed with 70% ethanol to remove the waxy layer on the cuticle. The flies are then rinsed 3 times with PBS and left submerged in PBS. Adult brains are dissected by making incisions in the cuticle using forceps from the posterior of the head and slowly peeling back the cuticle, leaving the brain untouched. The trachea is then peeled of the brain using fine forceps and the brain is removed by pinching off at the posterior of the brain where it connects to the thorax. Brains are then placed in 3.7% PFA solution in PBS.

The staining protocol for adult brains is the same as the larval brain staining protocol above with the following exceptions; 1) 2% PBS-TX is used instead of 0.2%; 2) Antibodies are incubated with the brains for two days at 4°C instead of one; 3) Before addition of primary antibodies brains are placed under a vacuum 6 times for 10 mins each to remove trapped air.
Primary antibodies used for imaging are the same as for the larval brain at the same concentrations.

**Larvae Neuromuscular Junction (NMJ) dissection**

Larvae are collected the same way as for the brain dissections. On the Sylgard plate, Minutien pins are placed in the posterior and anterior most regions of the larvae dorsal side up. Fine scissors are used to insert a horizontal incision at the posterior and then used to cut from posterior to anterior of the fly between the trachea. A second horizontal incision is then made at the anterior of the larvae. The internal organs and fat are all removed leaving the brain attached. Pins are then used to secure each corner of the abdominal wall and spread the larva in a hexagonal shape. The following steps all occur with the larvae still pinned to the plate. The larvae are rinsed in PBS and fixed in 3.7% PFA for 20 mins at room temperature. The larvae are rinsed with 0.2% PBS-TX quickly twice to remove residual PFA and then 3 times for 10 mins each at room temperature. The larvae are then blocked in 5% NGS solution in 0.2% PBS-TX for 1 hour at room temperature. The larvae are incubated with a primary antibody solution diluted in 0.2% PBS-TX solution with 5% NGS. Larvae are left in the primary solution overnight at 4°C, and the plate is placed in a black box with a moist towel to prevent evaporation. The primary antibodies are then removed, and larvae are washed quickly twice in 0.2% PBS-TX solution and incubated for 10 mins at room temperature in 0.2% PBS-TX solution. The 0.2% PBS-TX solution is removed and replaced with secondary antibodies diluted in 0.2% PBS-TX solution and 5% NGS and incubated at 4°C overnight in the same box as before. The secondary antibody is then removed, and larvae are washed quickly twice in 0.2% PBS-TX solution and incubated for 10 mins at room temperature in 0.2% PBS-TX solution. The larvae are then transferred to a slide and mounted in ProLong Gold mounting medium. A number 1.5 coverslip is then placed on top of the solution and the coverslip is sealed with nail polish.
Primary antibodies used for imaging are rabbit anti-GFP (FITC conjugated) (1:200) (ThermoFisher Scientific #A-21311) and goat anti-HRP (Cy3 conjugated (1:500) (Jackson ImmunoResearch 123-165-021).

Confocal imaging

Most imaging was performed using Leica SP8 and Leica SP8X with Lightning deconvolution microscopes using a 20x or 63x oil immersion lens. NMJ imaging was performed on a Zeiss Elyra 7 with Lattice SIM using a 63x immersion lens. Image analysis processing was performed using Imaris 9.3.1 (BitPlane).

Quantification of cells expressing para

To quantify the number of cells expressing para in the 3rd instar larval and adult CNS we used the Spots feature on Imaris 9.3.1 (BitPlane). For the 3rd instar larval brain we assigned spots as having an estimated diameter of 3.8 μm with a quality score above 174. For the adult CNS a spot had an estimated diameter of 2.5 μm and a quality score above 56.1. We quantified the number of spots from 5 3rd instar larval brains and 5 1-2 day old adult animals with genotype para-T2A-GAL4; P[w[+mC]=UAS-RedStinger]4, P[w[+mC]=UAS-FLP.D]JD1, P[w[+mC]=Ubi-p63E(FRT.STOP)Stinger]9F6/CyO, and analyzed spots based on Elav and nls.RedStinger, to quantify all neurons and para expressing neurons respectively. Analysis was performed using Microsoft Excel with error bars representing standard deviation.

Quantification of Distal Axonal Segment (DAS) Length

To detect the length of axonal compartments in adult mushroom body neurons we used the measurement points feature in Imaris 9.3.1 (BitPlane) to manually trace mushroom body neurons originating from mushroom body neuroblast (MBNB) clusters a, c and d (Kunz et al., 2012) from the
soma to the anterior of the peduncle, generating intensity profiles of each fluorophore used. Tracing was performed on para-mCherry; 201Y-GAL4, UAS-mCD8::GFP (n=7) and 201Y-GAL4-UAS-mCD8::GFP/UAS-DenMark (n=4) 1-2 day old animals. Para-mCherry and DenMark fluorescence intensities were measured. The beginning of the DAS was defined by an increase to 25% of the max intensity of Para-mCherry signal and the end of the decrease in signal beneath 25%. The increase to 25% of the maximum fluorescence intensity for DenMark staining was used to determine the start of the site of dendritic innervation and the decrease below 25% was used to define the end of the site of dendritic innervation. The boundary of the somatodendritic and axonal compartments is defined as the site where DenMark signal intensity was below 5% of the maximum intensity. To get reliable measurements 3 neurons were traced from each MBNB cluster in each animal, analysis was performed in Microsoft Excel, error bars correspond to standard deviation.

**Electrophysiology**

For large ventral lateral clock neurons (lLNv) whole cell patch-clamp recording, data acquisition and analysis, methods were used as described previously in detail (Sheeba et al., 2008). Long latency NaV currents were recorded with excellent access. In order to record such small cell bodies (3-4 μm), we fashioned 10 MΩ glass pipettes using a Narishige PP-83 two-step gravity puller. Cell attached patch configuration was established by gentle negative pressure on the pipette holder. Subsequently, slightly stronger negative pressure was applied to achieve breakthrough of the membrane to the whole cell configuration with giga-ohm seals. Whole cell lLNv recordings are generally stable for 30 minutes or more. The following solution modifications were made to isolate tetrodotoxin (TTX) sensitive NaV currents. A hydroxyethyl piperazineethanesulfonic acid (HEPES)-buffered external solution was used to avoid precipitation of ion channel blockers that consisted of 110 mM NaCl, 2 mM CoCl₂, 4 mM MgCl₂, 5 mM Glucose, and 10 mM HEPES. The internal patch electrode solution consisted of 102 mM D-Gluconic acid, 102 mM CsOH, 0.085 mM CaCl₂, 1.7 mM MgCl₂, 17 mM NaCl, 0.94 mM EGTA and 8.5 mM HEPES.
Synaptic currents were blocked with 1 μM tubocurare, 10 μM picrotoxin, 5 μM CNQX and 50 μM APV. K+ currents were blocked partially with 2 mM 4-aminopyridine (4-AP) and 10 mM tetra-ethyl ammonium (TEA) (note the residual rapidly responding outward voltage evoked K+ currents from Kv channels expressed in or near neuronal cell bodies). Ca2+ currents were blocked with 2 mM CoCl2. Nav Para currents were confirmed by adding 100 nM Tetrodotoxin (TTX) to the bath solution that abolished these currents.

Single cell sequencing

Brain dissociation into single cells

Wandering 3rd instar larvae were collected, and 30 brains were dissected and transferred to a tube containing 100 μL ice cold Dulbecco’s PBS (DPBS) solution. Next, the brains were centrifuged at 800 xg for 5 min and the supernatant was replaced by 50 μL of dispase (3 mg/mL, Sigma-Aldrich_D4818-2mg) and 75 μL collagenase I (100 mg/mL, Invitrogen_17100-017). Brains were dissociated at 25°C with 500 rpm for 45-55 mins. The enzymatic reaction was reinforced by pipette mixing every 15 mins. Cells were washed with 1,000 μL ice cold DPBS solution and resuspended in 400 μL DPBS 0.04% BSA. Cell suspensions were passed through a 10 μM pluriStrainer (ImTec Diagnostics_435001050) and cell viability and concentration were assessed by the LUNA-FL Dual Fluorescence Cell Counter. All genotypes were separately dissected and dissociated.

Methanol fixation

Fixation was performed after cell dissociation. The single-cell suspension was put in a thermoshaker at 4°C with 250 rpm and ice-cold methanol was added dropwise. Next, the sample was incubated for 10 mins at 4°C, followed by freezing and storage at -20°C.
Single-cell libraries were generated using the GemCode Single-Cell Instrument and Single Cell 3’ Library & Gel Bead Kit v2 and Chip Kit (10x Genomics) according to the manufacturer’s protocol. Briefly, fly brain single cells were suspended in 0.04% Bovine Serum Albumin (BSA)–PBS. For the fresh samples, the targeted cell recovery estimate was 5,000 cells (using 8,700 as input), while for the fixed genotype mix the aim was to retrieve 9,000 cells (using 15,800 cells as input). These cells were equally taken from each genotype separately in the genotype mix. After generation of nanoliter-scale Gel bead-in-EMulsions (GEMs), GEMs were reverse transcribed in a C1000 Touch Thermal Cycler (Bio-Rad) programed at 53°C for 45 min, 85°C for 5 min, and hold at 4°C. After reverse transcription, single-cell droplets were broken, and the single-strand cDNA was isolated and cleaned with Cleanup Mix containing DynaBeads (Thermo Fisher Scientific). cDNA was then amplified with a C1000 Touch Thermal Cycler programed at 98°C for 3 min, 12 cycles of (98°C for 15 s, 67°C for 20 s, 72°C for 1 min), 72°C for 1 min, and hold at 4°C twice. Subsequently, the amplified cDNA was fragmented, end-repaired, A-tailed and index adaptor ligated, with SPRIsel ect Reagent Kit (Beckman Coulter) with cleanup in between steps. Post-ligation product was amplified with a C1000 Touch Thermal Cycler programed at 98°C for 45 s, 14 cycles of (98°C for 20 s, 54°C for 30 s, 72°C for 20 s), 72°C for 1 min, and hold at 4°C. The sequencing-ready library was cleaned up with SPRIsel ect beads. CellRanger was used to detect cells from empty droplets.

Before sequencing, the fragment size of every library was analyzed on a Bioanalyzer high sensitivity chip. The libraries were diluted to 2 nM and quantified by qPCR using primers against p5-p7 sequence. All 10x libraries were sequenced on NovaSeq6000 instruments (Illumina) with following sequencing parameters: 28 bp read 1 – 8 bp index 1 (i7) – 91 bp read 2. Sequencing information is available in extended data file 1.
Demuxlet was used to demultiplex the different genotypes used in the methanol fixed DGRP-mix sample (Kang et al., 2018). This allows us to remove doublets consisting of cells with two different genetic backgrounds. The vcf file of the DGRP project (available at http://dgrp2.gnets.ncsu.edu/) was lifted over to dm6 genome. Next, we used bulk ATAC data to update the SNPs for DGRP-639 and to add SNP profiles for the hybrid. The vcf file was then filtered to only keep SNPs unique for one of the lines used. Demuxlet was run using this vcf file and on default parameters, leading to the identification of 970 doublets and 2937 singlets. Afterwards, the datasets of the two runs were merged.

Scater was used to filter the merged dataset (McCarthy et al., 2017). First, cells were filtered using three quality characteristics: number of unique molecular identifiers (UMIs), number of genes and percentage of mitochondrial genes. Cells that were more than 3 standard deviations away from the mean for number of UMIs and number of genes and cells that were more than 4 standard deviations away from the mean for percentage of mitochondrial genes were removed. Next, a principal component analysis (PCA) was performed using quality characteristics (percentage of counts for the top 100 genes, number of genes, percentage of mitochondrial genes, number of mitochondrial genes, log10 of total counts, log10 of total mitochondrial counts), and outliers were removed. Finally, genes with an average expression below 0.01 were removed. Leading to a final dataset of 9853 genes by 5056 cells.

Seurat v3 (Stuart et al., 2019) was used to integrate the data of the two different run conditions. Datasets were separately normalized using SCTransform (Hafemeister and Satija, 2019). Next, anchors were searched and integrated using 70 dimensions. Finally, the data was scaled and principal
component analysis, t-Distributed Stochastic Neighbor Embedding (tSNE) and Louvain clustering were performed using 70 dimensions. Louvain clustering was performed using resolution 2. To distinguish astrocytes from ensheathing glia, we used resolution 2.5, in which they split in two. We tested different resolutions in the Louvain clustering algorithm ranging from 0.2 to 4. The relationship between clusters across different resolution parameters was visualized using clustree (Zappia and Oshlack, 2018). Cluster composition is available in extended data file 1.

Trajectory inference

Trajectory inference was performed using Monocle3 (Cao et al., 2019). For type 1 optic lobe neuroblasts, ordering genes for 7 principal components were used (hth, klu, ey, slp1, slp2, D and tll). For the optic lobe neuroepithelium, 50 components were used and for the whole dataset ordering, 5 principal components were used.

Loom

Data is available for visualization and downloading at http://scope.aertslab.org/#/Larval_Brain/*/welcome. Loom files of the data were generated using SCopeLoomR (https://github.com/aertslab/SCopeLoomR).

Single Cell Data analysis

To identify the cells expressing ppara we used the FeaturePlot and vlnPlot functions within the Seurat v3 package for the 3rd instar larval CNS single cell sequencing data data generated in this study and the adult single cell transcriptome atlas from Davie et al. 2018. We isolated the top 100 genes enriched in each of the 39 larval gene clusters using the FindAllMarkers function in Seurat. A Wilcoxon Rank Sum test was used to identify differentially expressed genes; the genes could not be expressed in >20% of all cells in each cluster to rule out ubiquitously expressed genes. The genes were ranked by log fold change in expression between the average expression in each cluster compared to expression in all.
other cells, and the top 100 in each dataset were selected. *para* was only present in the top 100 genes for clusters 3 and 33 (motor neurons) in the 3rd instar larval brain single cell sequencing data. The top 100 genes from both cluster 3 and 33 were submitted to [http://pantherdb.org/](http://pantherdb.org/) for gene ontology (GO) molecular function analysis (Ashburner et al., 2000; Carbon et al., 2019; Mi et al., 2019a). A PANTHER Overrepresentation test was performed using the top 100 genes in both cluster 3 and 33 separately with a reference list containing all genes in database for *Drosophila*. We annotated based on GO molecular function and identified a list of enriched terms using a Fisher’s exact test with the Bonferroni correction for multiple testing (Table 2, Table 3). A full list of genes used in each cluster and GO terms is available in extended data file 1.

To compare *para* expression with known neuronal activity genes, we collated a list of genes known to function in synaptic transmission using [http://flybase.org](http://flybase.org) (Thurmond et al 2019) and identified activity dependent genes from (Chen et al. 2018), taking the 7 genes upregulated in 2 out of 3 neuronal activity paradigms used in the study. To determine the correlation of *para* expression with the expression of active zone genes, activity regulated genes and markers of neuronal differentiation, we averaged the log(CPM+1) gene expression of all cells (including cells with zero expression) in each cluster for each gene. We then performed a Pearson’s correlation analysis to compare the average expression of each gene across clusters in both the 3rd instar larvae and adult single cell sequencing data sets. To determine the significance of each correlation we performed a two-tailed t-test with the degrees of freedom equal to the number of clusters minus 2 (100 for the adult single cell data and 39 for the larval single cell data set). We used Bonferroni correction to adjust our target p-value of 0.05 for the 210 comparisons performed giving us an adjusted p-value of 0.002381 for both data sets. A t-value of 4.0467 was used for the larval single cell larvae correlations and a t-value of 3.8124 was used for the adult correlations.
Neuron diagrams

Diagrams of neurons in Figures 9D, 10B, 12D, 13B and 14B were made using BioRender.com.

Data and code availability

Transcriptomic data is available for visualization and downloading at

http://scope.aertslab.org/#/Larval_Brain/*/welcome

and the code is released as open source code on Github. The scRNA-seq data has been deposited in GEO under accession code GEO: GSE157202. SCENIC for Drosophila is available at http://scenic.aertslab.org.

Results

Fluorescently tagged Para is a functional protein that is temperature sensitive

To generate fluorescently tagged Para alleles, we used a Minos-Mediated Integration Cassette (MiMIC) line: y1 w* Mi[y+[mDint2]=MIC]para[M108578] constructed by the Gene Disruption Project (Nagarkar-Jaiswal et al., 2015). This line has two MiMIC’s inserted in the second coding intron of the para locus (Figure 1A), which are incorporated into all 60 predicted isoforms of para. These MiMIC’s are inserted in the opposite orientation of the transcript, rendering them not mutagenic. We replaced each MiMIC through recombination mediated cassette exchange (RMCE) with a SA-EGFP-FlAsH-Strep-TEV-3xFlag-SD (Para-GFSTF) sequence to insert two artificial exons encoding several epitopes that allow visualization of the endogenous expression pattern (Figure 1B) (Venken et al., 2011). As the cassette can integrate in either orientation, we confirmed the correct orientation for both with PCR. The same process was repeated for constructs containing SA-mCherry-SD and SA-RFP-3xHA-SD sequences.

Sequencing of genomic DNA in the para-GFSTF animals revealed two GFSTF insertions 37bp apart in the second coding intron indicating all animals contain 2 GFP insertions. Using an antibody against GFP, we performed Western blots of adult fly heads and detect a major broad band of about 200-250kDa in the...
Para-GFSTF sample (Figure 1C), which is consistent with the expected molecular weight of 55 of the 60 Para isoforms. Due to the broadness of the band on the Western, we are unable to isolate bands corresponding to Para containing one or two copies of the GFSTF insert.

To validate that the GFSTF exons are spliced into all para isoforms we PCR amplified the flanking regions of the MiMIC site with primers in exons 1 and 4. Para-MiMIC cDNA had one band of ~300bp which corresponds to the expected product without any GFSTF exon (Figure 1E). The para-GFSTF sample contained 3 bands corresponding to ~300bp, ~1200bp and ~2000bp (Figure 1E). The GFSTF exon is 976bp, therefore each band corresponds to para transcripts with 0, 1 and 2 GFSTF exons incorporated respectively, likely via splicing, this was confirmed by sequencing. The presence of duplicate MiMIC insertions was also seen in para-mCherry and para-RFP-3xHA animals. Saturated PCR conditions were used to detect the GFP inserts in the RNA product (Figure 1E). To quantify the proportion of tagged versus untagged para transcripts in the para-GFSTF animals, we performed a quantitative-PCR (qPCR) using a forward primer in exons 1 and 2, and reverse primers in GFP and across exons 3 and 4 in para-GFSTF and para-MiMIC animals (Figure 1F). The qPCR showed that only 1.61% (±0.39%) of all para transcripts in para-GFSTF animals do not incorporate a GFSTF exon. Therefore, the tagged form of para generated in this study represents more than 98% all the para transcripts produced in the fly.

To generate a gene reporter allele for para, we exchanged the MiMIC cassette in para-MiMIC with a SA-T2A-GAL4-pA (T2A-GAL4) sequence using RMCE (Figure 1D). The splice acceptor is predicted to be spliced into all para transcripts and should generate a severe loss of function or null allele (Lee et al., 2018). para null alleles are lethal at the 1st instar larval stage (Loughney et al., 1989; O’Dowd et al., 1989; Hong and Ganetzky, 1994). para-T2A-GAL4 animals, in addition to two other reported para null alleles (para¹, para² (Yamamoto et al., 2014)) are homozygous lethal at the same 1st instar larval stage, and fail to complement a deficiency that uncovers the para locus (Df(1)FDD-0230908) (Figure 1G).

Finally, an 80 kB genomic rescue construct (P[acman] Dp(1;3)DC134) (Venken et al., 2010) rescues para-
 Partial loss of function \textit{para} alleles (\textit{para}^{ts1}) have been previously characterized using temperature sensitive paralysis assays (Siddiqi and Benzer, 1976; Suzuki et al., 1971). To assess if the \textit{para}\text{-GFSTF}, \textit{para}\text{-mCherry} and \textit{para}\text{-RFP\text{-3xHA}} alleles confer temperature sensitivity, we tested the effects of different temperatures on the animals. Homozygous animals that carry any of these three alleles are viable and appear healthy when raised at 25°C (Figure 1G). At 29°C, the animals exhibit temperature dependent paralysis and die after 24-36 hrs. However, they fully recover if returned to a lower temperature after 24 hrs. The temperature sensitivity of the tagged \textit{para} alleles was compared to the classical \textit{para}^{ts1} allele using an assay at 40°C for 60 s (Figure 1H). After 60 s, 80% of all \textit{para} tagged flies are paralyzed, whereas 100% of \textit{para}^{ts1} animals are paralyzed. Interestingly, the different tagged alleles vary in the rate of paralysis and recovery. This implies that the different tagged insertions have varied effects on channel protein stability and that the refolding kinetics of each tagged allele likely are variable.

To test if the \textit{para}\text{-GFSTF} allele is functional at 25°C, we measured neuronal activity by recording electroretinograms (ERG) (Figure 1I, Figure 1 –1). Loss of function alleles in \textit{para} have been shown to have a loss of off-transients (Homyk and Pye, 1989), indicating neuronal dysfunction in the visual system downstream of photoreceptors (Joesch et al., 2010). No significant differences are found between the on or off-transients in ERGs between the \textit{para}\text{-MiMIC} and the \textit{para}\text{-GFSTF} animals, suggesting that neuronal signaling is not affected by the presence of a GFSTF tag in \textit{para}. We do find a slight increase in the amplitude in \textit{para}\text{-GFSTF} animals compared to control \textit{para}\text{-MiMIC} animals (Figure 1 –1B). This data, in conjunction with mouse models of N-terminal tagged sodium channels that are physiologically comparable and in the same subcellular localization as non-tagged channels (Lee and Goldin, 2009), argues that the GFSTF tag does not significantly impair channel function when animals are raised at 23°C.
Therefore, the tagged para allele is representative of the endogenous expression and localization of untagged para.

**Para localization is sparse in the embryonic nervous system**

The subcellular localization of Para is unknown. *In situ* hybridization data have reported that para is broadly expressed in embryonic and adult neurons (Amichot et al., 1993; Hong and Ganetzky, 1994). However, the embryonic *in situ* data were presented at very low resolution (Hong and Ganetzky, 1994) and the only available Para antibody, though useful for Western blots (Xiao et al., 2017), does not work in fixed tissue in our hands. Using a brief fixation protocol, a pan-Na_v antibody raised against vertebrate Na_v channels has shown the presence of Para clusters in neuronal fascicles in the central brain of adult flies (Wang et al., 2020), however the subcellular localization of Para remains unclear. To determine Para protein localization, we performed immunofluorescent imaging of Para embryos using antibodies against the FLAG epitope. Para is not observed before stage 15. In stage 15/16 embryos Para is localized to the cell bodies of both CNS and PNS neurons, but a large portion of CNS and PNS neurons remain unmarked (Figure 2A, B). In the PNS, Para is most prominent in chordotonal neurons at the distal tip of the dendrites (Figure 2D). Throughout the remainder of embryogenesis, Para localization in the CNS remains restricted to the cell bodies (Figure 2E). In stage 17 embryos, we also observe Para protein localization in cardiomyocytes in the dorsal tube (Figure 2C). Na_v channel expression in invertebrate cardiomyocytes has not previously been reported, however mammalian cardiac muscle cells express a specific Na_v channel (Na_v 1.5) which is essential for AP propagation in the cardiac conduction system (Nuyens et al., 2001; Papadatos et al., 2002). To determine the cells that express para, we used para-T2A-GAL4 to drive UAS-mCD8::GFP. This typically reveals most or all cells as the GAL4 acts as an amplifier (Lee et al., 2018). Despite the mCD8::GFP having a membrane bound localization sequence, the marker only labels the soma. As
shown in Figure 2F, para expression in the CNS and the PNS in stage 16 embryos is restricted to a small number of neurons. In the PNS, labeling is mostly confined to the chordotonal neurons. The sparse expression of Para and non-axonal localization, together with the data that para null animals hatch as 1st instar larvae imply that para is not essential to neuronal function in the embryo.

**para expression is confined to less than a quarter of the neuronal population in the 3rd instar larval CNS but is broadly expressed in neurons in adults**

para expression is essential for larval viability (Loughney et al., 1989; O’Dowd et al., 1989; Hong and Ganetzky, 1996). To determine if expression of para is more widespread in the 3rd instar larval nervous system than in the embryonic nervous system, we used para-T2A-GAL4 to drive expression of a nuclear localized reporter UAS-RedStinger.nls (Figure 3A). Surprisingly, para expression is limited to a small population of neurons in the ventral nerve cord (VNC) (23% ±1%) when compared to the pan-neuronal marker Elav (Figure 3C). para expression is highest in the VNC while cells in the central brain exhibit lower expression. By overexposing the signal, we identify additional para positive cells in the central brain, whose expression levels are far lower than the VNC (Figure 3B).

Using GAL4 as a reporter for gene expression provides a snapshot of a gene’s current expression and does not provide information on a gene’s historical expression (Evans et al., 2009; He et al., 2019). To determine the historical expression of para in the larval brain we used UAS-G-Trace (Figure 4) (Evans et al., 2009). The G-Trace construct contains two components: 1) UAS-RedStinger to label cells currently expressing the GAL4 with a nuclear localized mCherry protein and 2) UAS-FLP, Ubi-FRT-STOP-FRT-nls.Stinger (GFP) sequence to label the nuclei of any cell that has ever expressed GAL4 with a nuclear GFP. GAL4 drives UAS-flippase that excises the STOP codon revealing cells in which GAL4 was present at any time (Figure 4A). A comparison of RedStinger.nls and G-Trace induced GFP staining shows that many central brain and VNC neurons remain unlabeled, again indicating that many neurons in the larval
CNS never express para (Figure 4B, C). Importantly, the nuclear localized RedStinger nearly fully overlaps with the G-Trace staining which indicates that once cells begin to express para, expression is maintained. To determine para expression in the adult nervous system we used para-T2A-GAL4 to drive UAS-RedStinger and the pan-neuronal nuclear marker Elav (Figure 3D, E). para expression is far broader in the adult than in the 3rd instar larvae, and almost all Elav positive neurons in the central brain and in the thoracic ganglion co-label for para expression (94% ±5%) (Figure 3C). This data indicates that very few adult neurons, but over three quarters of neurons in the 3rd instar larval nervous system, do not express Na\textsubscript{v} channels and may not be capable of generating Na\textsubscript{v} dependent APs.

**Single cell sequencing of the 3rd instar larval CNS identifies distinct neuronal, glial and neuroblast populations**

The restricted expression of para in the 3rd instar larval nervous system to only 23% ±1% of neurons, based on immunofluorescent data, indicates that the majority of neurons in the larval CNS may not fire Na\textsubscript{v}-based APs. To independently confirm this result, we isolated the CNS of whole 3rd instar larval brains, containing both the brain lobes and VNC, and performed single cell sequencing. We performed two sequencing runs (10x Chromium) for a total of ~5,000 cells from different genetic backgrounds. Seurat integrated clustering of these cells identified 39 distinct cell clusters based on gene expression (Figure 5), and these clusters show no bias for either sequencing run or fly genotypes (Figure 5 – 1A-C). Using markers for neurons (elav), glia (repo) or neuroblasts (dpn, ase), we were able to assign all cells to a major type (Figure 5). Furthermore, we annotated all glial clusters and some neuronal clusters using known markers for specific cells based on published data (Lee et al., 1999; Hassan et al., 2000; Spindler et al., 2009; Hensley et al., 2011; DeSalvo et al., 2014; Davie et al., 2018) (Figure 5).
para expression in neuronal progenitors has been implicated in cell proliferation (Piggott et al., 2019). Using *dpn* and *ase*, we can identify three major neuroblast clusters (Figure 5). Two groups are *dpn*+ *ase*+, and correspond to the type I neuroblasts, while one group is is *ase* negative but shows slight *dpn* expression (Figure 6A, B) (Bayraktar and Doe, 2013; Henson, 2017; Walsh and Doe, 2017). Furthermore, these cells express *pnt*, a marker for EGFR signaling, suggesting these are optic lobe neuroepithelium (Figure 6A, B) (Apitz and Salecker, 2014; Hakes et al., 2018). Two groups are *dpn*+ *ase*+, and correspond to the type I neuroblasts, while one group is only *dpn*+, the type II neuroblasts (Figure 6A, B) (Bayraktar and Doe, 2013; Henson, 2017; Walsh and Doe, 2017). Type I neuroblasts generate ganglion mother cells (GMCs) which only express *ase* and not *dpn*, and indeed we can identify two clusters near each of the type I neuroblasts that fulfill these requirements (Figure 6A, B). Furthermore, one of these clusters is expressing *cas*, a transcription factor that plays a role during embryonic ventral nerve cord neuroblast identity determination (Figure 6A, B) (Cui and Doe, 1992; Mellerick et al., 1992), but has also been shown to be only expressed in the central brain GMCs in the 3rd instar larval phase (Hitier et al., 2001). This suggests that these two clusters are derived from the central brain.

The other type I cluster expresses many transcription factors involved in the temporal ordering of optic lobe (OL) neuroblasts (Figure 6A, B) (Li et al., 2013; Suzuki et al., 2013). Ordering the optic lobe type I neuroblast reveals that many transcription factors are involved in the temporal ordering of optic lobe (OL) neuroblasts (Figure 7A) (Li et al., 2013; Suzuki et al., 2013). Interestingly, we also notice a branch characterized by B-H2 expression. Next, we checked the cluster of the optic lobe neuroepithelium. The optic lobe neuroepithelium generates both the lamina precursor cells (LPC) as the optic lobe type I neuroblasts previously described (Gold and Brand, 2014). The differentiation from neuroepithelial cell to neuroblast happens at the transition zone and is triggered by a proneural wave (Apitz and Salecker, 2014). Using trajectory inference, we can investigate the dynamics at this transition zone (Figure 7B). Cells are ordered from a *dpn* low state with epithelial markers *Fas2* and high Notch.
activity (Egger et al., 2010; Apitz and Salecker, 2014; Hakes et al., 2018), to a state with lethal of scute (Apitz and Salecker, 2014) and finally to neuroblast stage with earmuff expression (Hakes et al., 2018), fitting with previously described patterns. Finally, we note a cluster of Hey-positive neurons, a transcription factor found to be active in the Notch signaling pathway in newly born neurons (Figure 6A, B) (Monastirioti et al., 2010).

To determine the stage of neuronal development in which para is expressed, we performed trajectory inference on the whole dataset (Figure 7E, F). We identified a trajectory from neuroepithelium (grh+) to neuroblast (dpn+) to GMC (ase+) to developing neuron (Hey+) and then finally to mature neurons (VACHT+, VGLUT+, Gad1+) (Figure 7E, F). In addition, a separation of imp and pros positive neurons has been demonstrated at various stages of Drosophila development. (Etheredge, 2017; Davie et al., 2018; Allen et al., 2020).

Higher imp expression occurs in embryonic derived, mature neurons in the larval VNC, while higher pros expression occurs in post-embryonic, immature neurons in the larval VNC (Etheredge, 2017; Allen et al., 2020). This same separation can be observed in our trajectory (Figure 7E). We do not detect para expression in any NB or GMC cell types identified (Figure 6A, Figure 7A,C). para expression follows both the expression of Imp and markers of neuronal differentiation into cholinergic (VACHT), GABAergic (Gad1) and glutaminergic (VGLUT) neurons, therefore para is likely only expressed in mature, differentiated neurons. This contrasts with para’s previously reported role in neuronal cell proliferation (Piggott et al., 2019).

para expression is correlated with active neuronal markers in the larval and adult CNS

Using the 3rd instar larval brain transcriptome atlas, we can identify the cells expressing para and determine their identity. Plotting para expression on the larval t-SNE (Figure 8A), we identify that para is enriched in a small portion of the cells, with expression highest in neuronal clusters 3 and 33 (Figure
8C), complementing the restricted population of neurons expressing para from the imaging results (Figure 3A). To determine the identity of these clusters we selected the top 100 genes that were enriched in each cluster compared to all other clusters and performed a PANTHER Overrepresentation Test to determine enriched GO molecular functions (Ashburner et al., 2000; Carbon et al., 2019; Mi et al., 2019b). Cluster 3 is enriched in genes that have been implicated in neurotransmission, ATP synthesis and ion transport (Table 2), and cluster 33 is enriched in genes involved in ATP synthesis and ion transport (Table 3) and likely corresponds to motor neurons as these cells express target of wit (twit) and Proctolin (Proc), known markers for motor neurons (Figure 6A, 6B) (Vuilleumier et al., 2019). Both ion transport and neurotransmission are necessary for neuronal activity and ATPase activity is upregulated in active neurons (Johar et al., 2014). To determine if para expression is representative of the active neuronal population, we calculated the Pearson’s correlation between para expression and the expression of genes annotated in FlyBase as being present in the active zone (brp, cac, futsch, Nrx-1, nSyb, Rbp, RhoGAP100F, Syt1) as well as the co-expression of para and activity regulated genes (ARG) (Hr38, sr, CG14186, CG17778, CG8910, CG14024, CG13868) (Chen et al., 2016), in each cluster. We find that para expression is positively correlated with expression of active zone localized proteins and most ARG genes (Figure 8D). This data indicates that para is expressed in a restricted population of neurons that are likely to be actively firing.

Analysis of the 3rd instar larval transcriptomic atlas shows that many larval neurons express markers of immature neurons (Figure 6A,B). We analyzed the correlation of para expression with known markers for neuronal differentiation (elav), neuroblast specific markers (dpn), or ganglion mother cell markers (pros, ase) and found no or negative correlation coefficients (Figure 8D). para correlates with IGF-II mRNA-binding protein (Imp), an mRNA binding protein that is found to oppose prospero (pros) expression and is a marker of mature neurons (Davie et al., 2018). Davie et al. also found that Imp positive clusters show higher expression of genes related to oxidative phosphorylation,
hinting at an increase in neuronal activity. We did not detect \textit{para} expression in any neuroblast lineages (Figure 6A). The positive correlation of \textit{para} only with mature neuronal markers and markers of active neuron firing, suggests that a rather small fraction of neurons (23 ±1%) in the larval CNS are mature neurons that can fire Na\textsubscript{v}-based APs.

We performed a similar analysis of single cell RNA sequencing of the adult CNS (Davie et al., 2018). Seurat tSNE clustering separates the 57,000 cells into 102 unique clusters based on variable gene expression (Figure 8-1A). \textit{Para} expression is broadly distributed in the adult neurons with only a handful of clusters showing lack of \textit{para} expression, including glial cells and photoreceptors (Figure 8B, Figure 8 –1B). In addition, in the adult CNS \textit{para} has a strong positive correlation with the pan neuronal marker \textit{elav}, in agreement with the broad distribution of \textit{para} expression seen in Figure 3D. To determine if the increase in \textit{para} expression correlates with an increase in the number of active neurons, we determined the Pearson’s correlation coefficient of expression between \textit{para} and several signatures of actively firing neurons (RNA for activity regulated and active zone proteins) (Figure 8D). Similar to the larval data, \textit{para} expression is positively correlated with all active zone genes and all ARGs. Hence, active neurons as defined by expression of active zone genes and ARGs in the adult express \textit{para}. Together, these data suggest that the cells expressing \textit{para} are active, differentiated neurons, and while this describes most neurons in the adult CNS, only 23 ±1% of cells in larvae fit this criterion.

\textbf{Para is enriched in a distal axonal segment (DAS) in axons of 3\textsuperscript{rd} instar larval CNS neurons}

As the only Na\textsubscript{v} channel in flies, the subcellular localization of Para shows where AP are likely initiated and propagated. In active, differentiated 3\textsuperscript{rd} instar larval neurons Para is confined to axons; however, Para is not uniformly distributed throughout the CNS (Figure 9A, Figure 9 –1A, Movie 1). To determine where along the axon Para is enriched, we looked at the 3\textsuperscript{rd} instar larval mushroom body neurons. The anatomical structure of mushroom body neurons in the larval CNS is well defined. The
mushroom body neurons have cell bodies, located in the ventral side of the larval central brain lobes, that project a single axon, which is intersected by dendrites in the calyx, and then continues along the alpha and beta lobes where they synapse (Figure 9C,D, Figure 9-1B). We used a mushroom body specific GAL4 (201Y-GAL4) (Yao Yang et al., 1995) to drive expression of UAS-mCD8::GFP (to label the entire cell membranes) (Lee and Luo, 1999) in conjunction with Para-mCherry (Figure 9-1 B) to determine where Para is localized. Para is enriched at a segment of the axon, after the site of dendritic integration at the calyx, after which it is distributed in a decreasing concentration toward the synapses (Figure 9C, D). The same distribution of Para is also be observed in motor neuron axons exiting the VNC (Figure 9A) with Para enriched as the axons leave the VNC (after dendritic innervation) (Figure 9A, inset box i) and then decreasing towards the NMJs (Figure 9A, inset box ii). The clustering of Para at the boundary of the somatodendritic and distal axonal compartments is reminiscent of the AIS location in vertebrates (Nelson and Jenkins, 2017). However, due to the unipolar organization of fly CNS neurons, this segment is distant from the soma. Conserved components of the vertebrate AIS have been shown to accumulate in fly axons, also at the distal boundary of dendrites and the remainder of the axon, at a region called an AIS-like compartment (Trunova et al., 2011; Smith-Trunova et al., 2015; Jegla et al., 2016). However, in CNS neurons, this segment is not at the initial part of the axon, but at a distal region, thus we propose naming the segment the Distal Axonal Segment (DAS) to distinguish the invertebrate and vertebrate structures and avoid confusion.

To validate Para enrichment at the DAS across different larval CNS neuron types we used split-GAL4 drivers (Luan et al., 2006; Pfeiffer et al., 2010) to assess Para distribution in an individual pair of motor neurons. Split-GAL4s are generated by separating the activation and DNA binding domains of GAL4, then expressing each domain under a separate promoter to restrict expression. The functional GAL4 protein is only present in cells that express both promoters. Split-GAL4 line R_MB050B is restricted to the two most posterior motor neurons in the larval VNC (Figure 10A) (Aso et al., 2014). The
cell bodies of each neuron are in the posterior of the VNC, with one dorsal cell body projecting an axon that exits the VNC on the right side, and a ventral cell body that projects an axon on the left side of the VNC with the dendritic tree for each cell in the midline of the VNC. Using pan-membrane labeling (UAS-mCD8::RFP), we observe localization of Para in the axons of each cell at the DAS, distal to the dendritic tree (Figure 10B, C). We do not see the same colocalization when co-labelling with the somatodendritic marker DenMark (Figure 10D) (Nicolaï et al., 2010). Figure 11 shows cross sections through a posterior motor neuron, labelled with Split-GAL4 line R_MB050B driving UAS-mCD8::RFP, at the axon hillock (Figure 11A) (where the AIS is located in vertebrates), in the axon distal to the dendritic tree where we predict the DAS to be located (Figure 11B) and a section of the axon distal to the DAS (Figure 11C). We clearly see Para localization distal to the dendritic tree at the DAS, whereas no Para is present at the axon hillock. Para is also enriched at the DAS (Figure 11B) compared to a more distal segment of the axon (Figure 11C). These images show that 3rd instar larval neurons do contain segments of the axon where Na⁺ channels are enriched like at the AIS in vertebrates, however, the subcellular localization of the segment is not shared. In addition, in motor neurons the diffuse distribution of Para along the axon is maintained up to the first bouton of the NMJ (Figure 9B). Interestingly, small clusters of Para can be observed near each bouton along the length of the NMJ with super resolution microscopy. This data indicates that in the neurons of 3rd instar larvae, AP are likely initiated distally to the cell body, in contrast to the AIS initiation of AP in vertebrates. In addition, the low-level continual localization of Para between the DAS and synapses likely ensures AP are propagated to the synapses.

**Para is enriched in a distal axonal segment in adult CNS neurons**

*para* expression is greatly increased from the larval to the adult CNS. To determine if the localization of Para to the DAS is conserved from the larval CNS to the adult CNS we imaged the adult brain for Para-GFSTF. In the adult, CNS Para is localized to specific clusters of axons and not broadly
distributed throughout the neuropil of the adult brain (Figure 12A), and this distribution is comparable
to staining’s performed in adult fly brains with anti-Na\textsubscript{v} antibodies raised against vertebrate Na\textsubscript{v}
channels (Wang et al., 2020). In addition, diffuse Para staining can be seen in the axons of the longest
neurons of the CNS that project from the left to right brain hemisphere or vice-versa (Movie 2). The
diffuse staining in long axons is clearly observed in axons of neurons in the thoracic ganglion where we
see broad labeling of long axonal tracks (Figure 12B). The enrichment of Para in the adult CNS is
reminiscent of the DAS distribution observed in the larval CNS, not the AIS location as seen in mammals,
as the clusters are not near the neuronal cell body marker Elav (Figure 12A, B).

The DAS in larval neurons is localized after the dendrites impinge upon the axons. To determine
where along the axon the Para positive DAS clusters are localized in adult neurons, we labeled multiple
classes of neurons using split-GAL4 line \textit{R_OL0019B-GAL4} (Figure 12C-F) and \textit{201Y-GAL4} (Figure 13),
labeling the laminar columnar neurons (LC10) and mushroom body neurons respectively. In both
neuronal populations, Para is enriched in a DAS located in the axon after the last dendrite impinges
upon the axon (Figure 12E, Figure 13C). In LC10 neurons Para enrichment in the DAS is seen when
broadly labeling cells with \textit{mCD8::RFP} but not when labeling the somatodendritic region of the neurons
with DenMark (Figure 12F). In addition to the DAS, we see lower intensity Para distribution between the
DAS and the synapses (Figure 13E) for the remainder of axons. For the mushroom body neurons in the
adult, the DAS is present in the peduncle, beyond the localization of DenMark (Figure 13D), like in the
larval CNS (Figure 10B, C, Figure 13B,C). Hence, Para localization at the DAS, distal to the site where
dendrites impinge upon CNS neurons, and lower intensity Para distribution beyond the DAS, is
conserved from larvae to adults. This data shows that Para is enriched in a DAS after dendritic
innervation in multiple neuron classes throughout larval and adult development.

To determine the size of the DAS we measured signal intensity of Para-mCherry along 3 classes
of mushroom body neurons that derive from mushroom body neuroblast (MBNB) clusters (MBNBA, c
and d) (Figure 14A) (Kunz et al., 2012). In both MBNBc and MBNBd neurons the DAS starts 82.72 μm (±3.16 μm) and 72.73 μm (±5.54 μm) from the soma and is 28.69 μm (±6.12 μm) and 28.69 μm (±6.12 μm) in length respectively. The DAS in MBNBa neurons is both closer to the soma (38.43 μm (±2.94 μm)) and shorter in length (21.92 μm (±3.44 μm)) than the DAS in MBNBc and MBNBd neurons. The length of the DAS reported in this study is comparable with the length of low frequency vertebrate AISs (Adachi et al., 2015), and the size of the AIS-like region previously reported in adult mushroom body neurons (Trunova et al., 2011).

In vertebrate neurons the length of the AIS is variable depending on the size and firing properties of the neuron (Adachi et al., 2015). To determine if the size discrepancies between neuron population in the mushroom body was as a result of differences in neuron size we used UAS-DenMark (Figure 14). DenMark labels the somatodendritic region of the neuron but is enriched in the dendritic tree (Figure 13D). We can determine the length between the soma and the dendritic tree (weak DenMark signal to strong signal), the length of axon were dendrites impinge (strong DenMark signal to strong DenMark signal) and the distance from the dendritic tree to the boundary of the somatodendritic region (strong DenMark signal to no signal) using DenMark signal intensity (Figure 14). We show that in all three MBNB populations measured (a, c and d) the length of the region of the axon where dendrites impinge is similar (13.68 μm (±2.88 μm), 15.15 μm (±2.12 μm) and 13.50 μm (±1.68 μm) respectively) (Figure 14A). The distance between the soma and the boundary of the somatodendritic compartment of the cell is variable. MBNBc and MBNBd cells have a soma to somatodendritic boundary length of 74.15 μm (±8.98 μm) and 80.38 μm (±6.94 μm) respectively which is comparable to the soma to DAS distance in each neuronal population. In MBNBa cells the soma to somatodendritic boundary was shorter 55.40 μm (±6.01 μm) which corresponds to the shorter soma to DAS distance seen in MBNBa neurons. This indicates that the DAS length is shorter in neurons with a shorter soma to DAS distance,
and that the DAS does act as a boundary between the somatodendritic region of the axon and the distal axon as defined by absence of DenMark staining.

**Voltage-gated Para currents are evoked distal from the soma**

Para’s distal localization far from the soma suggest that $\text{Na}_\text{v}$-based AP in *Drosophila* neurons initiate far from the soma. $\text{Na}_\text{v}$ currents in mammalian neurons are readily evoked by voltage steps. These voltage evoked $\text{Na}_\text{v}$ currents show little or no temporal delay (1 to 2 ms) when recorded from the soma due to the close proximity of the AIS (Magistretti et al., 2006; see Figure 1). In contrast, evoked Para currents recorded at the soma should show delays following voltage steps and exhibit features of poor space clamp due to their distance from the soma. To determine this, we measured Para $\text{Na}_\text{v}$ voltage evoked currents by whole cell voltage clamp in lLNvs, pharmacologically isolated by blockers of voltage-gated calcium channels ($\text{Ca}_\text{v}$) and voltage-gated potassium channels ($\text{K}_\text{v}$) channels and synaptic currents. Depolarizing voltage steps evoke rapidly inactivating inward Para currents as shown by downward deflecting currents (Figure 15A). Para currents are abolished by the $\text{Na}_\text{v}$ specific blocker tetrodotoxin (TTX) (Figure 15A’) and show peak maximum voltage activation at -35 mV (Figure 15A’’). The short latency upward deflecting outward currents seen in Figure 15A and Figure 15A’ may be somatic or near somatic voltage-gated potassium currents that are not completely blocked by 4-aminopyridine and tetra-ethyl ammonium. Voltage evoked Para currents decrease as voltage steps approach the $\text{Na}_\text{v}$ reversal potential (Figure 15A’’). Consistent with localization originating in the DAS, TTX sensitive inward Para currents show long delays measured at the soma following voltage steps and features of poor space clamp and poor voltage control (Figure 15A, A’’). To determine Para localization in the lLNv neurons we used *Pdf-GAL4* to drive expression of $\text{mCD8::RFP}$ and DenMark, to label the neuronal membrane and dendrites respectively (Figure 15B,C), in l-LNv and s-LNv neurons. Para is enriched in the axons of l-LNv and s-LNv neurons after the integration of the dendritic tree in a DAS.
Para is not localized to the soma, proximal axon or the dendritic projections. This is consistent with the delay in evoked Para currents and indicates that the DAS is the likely site of these currents. Therefore, \( \text{Na}_V \)-evoked AP are likely generated at the DAS.

**Discussion**

The site of AP initiation in invertebrate neurons is unclear. In vertebrates, APs are generated at the AIS. The major functional hallmark of vertebrate AIS is clustering of \( \text{Na}_V \) channels. Therefore, we characterized the gene expression and subcellular localization of the sole \( \text{Na}_V \) channel in *Drosophila*, \( \text{para} \). Despite being the only \( \text{Na}_V \) channel in flies, we find that \( \text{para} \) is only expressed in 23 ±1% of neurons in the 3rd instar larval CNS. In contrast, in the adult, \( \text{para} \) expression is far broader. By generating a single-cell transcriptomic atlas of the 3rd instar larval brain we determined that \( \text{para} \) expression correlates with the expression of activity regulated genes, genes expressed in active zones and markers of mature neurons. This implies that cells expressing \( \text{para} \), while proportionally small, likely represent the active population of neurons in the 3rd instar larval CNS. This correlation occurs also in the adult CNS, indicating that the number of active neurons is likely much higher in the adult than in the 3rd instar larvae. In the neurons expressing \( \text{para} \), we find that Para distributed into axonal segments that are far removed from the soma, distal to where dendrites integrate into the axon. This structure shares some features of the mammalian AIS and whole cell patch clamp electrophysiological recordings are consistent with the interpretation that \( \text{Na}_V \)-dependent APs initiate there, however due to its distal location to the cell soma, we name this region the distal axonal segment (DAS).

The AIS identifies the origin of AP propagation (Westenbroek et al., 1989; Whitaker et al., 2000) through the high density of \( \text{Na}_V \) channels (Mainen et al., 1995). The clustering of \( \text{Na}_V \) channels at the DAS suggests this is the site of AP initiation in fly neurons. Our identification that TTX-sensitive, inward sodium currents that occur after long delays in response to somatic depolarization are also suggestive of
a distal site of AP initiation. In vertebrate neurons, TTX-sensitive inward sodium currents are measured immediately after somatic depolarization as the AIS is so close to the soma (Kuo and Bean, 1994; Magistretti et al., 2006; Lewis and Raman, 2014). Single-cell electrophysiological recordings performed on fly neurons in the CNS is a challenge due to their small size. Recordings performed in the neurons of larger invertebrates, *Aplysia*, crab and leeches also exhibit delayed activation and poor space clamp, indicating that APs are initiated at a site distal relative to the cell body (Tauc, 1962; Tauc and Hughes, 1963; Meyrand et al., 1992; Tobin et al., 2006). This suggests that AP initiation at the DAS is conserved across invertebrate species. Due to their small size, patch-clamp recordings are made on the soma (Wilson et al., 2004; Gouwens and Wilson, 2009). Therefore, the soma receives back-propagating APs from the DAS and passive fluctuations in membrane potential are readily measured in the soma as well in *Drosophila* CNS neurons (Sheeba et al., 2008). While APs appear to initiate from the DAS, the sources and ionic mechanisms of passive membrane potentials is not clear, however fluctuations in membrane potential clearly influence the pattern of AP firing in *Drosophila* neurons. For example, large fluctuations in membrane potential drive burst firing in lLNv while stable membrane potential is associated with regular tonic AP firing (Sheeba et al., 2008). Further, transition between burst and tonic firing AP firing pattern is voltage-gated calcium channel (Ca\textsubscript{v}) dependent as it is modulated by cobalt block of Ca\textsubscript{v} channels. Surprisingly, either TTX or cobalt abolishes lLNv APs altogether, suggesting complex interactions between Para and Ca\textsubscript{v} in *Drosophila* neurons (Sheeba et al., 2008). Therefore, the electrical activity recorded at the soma may not be representative of the neuron’s actual firing activity as in some cases the DAS is far away from the soma and these cell bodies may not undergo any depolarization. This emphasizes the need to establish voltage sensing reporters to simultaneously enable accurate reporting of APs and calcium events at high spatio-temporal resolution (Simpson and Looger, 2018). While genetically encoded calcium indicators are often used for a proxy for inferring APs,
intracellular calcium sources are highly diverse (CaVs, TRP channels, non-selective cation channels, multiple intracellular stores, etc.).

As an alternative to electrophysiological recordings, computational models of compartmentalized fly neurons have been used to model their electrophysiological properties. Models of different neurons in the fly CNS, predict that the site of AP propagation occurs, not proximal to the cell body like in mammals, but distally in the axon after the last dendrite innervates the axon (Gouwens and Wilson, 2009; Günay et al., 2015). The mapping of the DAS and the ability to visualize it in any fly neuron using Para-GFSTF endorses the accuracy of these models and enables the generation of models with greater precision to better predict and analyze neuron activity and dynamics.

Some studies have indicated that an AIS-like region may be present in some *Drosophila* neurons, based on GAL4 mediated overexpression of *Drosophila* homologues of vertebrate AIS proteins, such as Ank1 and Shal in mushroom body (MB) neurons (Trunova et al., 2011; Smith-Trunova et al., 2015). The overexpressed proteins were shown to cluster in axons downstream of the dendritic tree, in the MB of the 3rd instar larval and the adult CNS. Using a similar approach, it has been shown that proximal axons of ddaE neurons in the PNS also may contain AIS-like regions (Jegla et al., 2016). However, the overexpression of proteins may lead to their restriction in cellular compartments as Katsuki et al. (2009) showed that the mobility of transmembrane molecules is restricted to areas before and after dendritic branches, suggesting that there are barriers in axons that restrict protein localization (Katsuki et al., 2009). The DAS reported in this study is of comparable size and location to the AIS-like region previously reported in adult MB neurons (Trunova et al., 2011). AnkG is key for NaV channel clustering at the vertebrate AIS (Jenkins and Bennett, 2001). The similarity in the reported AIS-like localization of Ank1 localization from Trunova et al. 2011 and Na\textsubscript{v} enrichment at the DAS as defined in this study, indicates that ankyrins role in Na\textsubscript{v} clustering may be conserved in invertebrates, despite differences in ankyrin structure across species (Jegla et al., 2016). Interestingly, we found that the distance between the soma
and the length of the DAS is correlated, with a longer DAS present in neurons where the DAS is more distal. This is in contrast to what is observed in vertebrates where the more distal the AIS the shorter the length of the AIS (Adachi et al., 2015).

We observe low levels of Para distribution along long axons, suggesting that AP propagation is maintained by Para across the length of the axon. APs are all or nothing signals that rapidly propagate along axons, however, if no $\text{Na}_V$ channel is present the signal should decay rapidly. We argue that the low levels of Para distributed along the length of long axons enables the APs to reach the synapse without signal depletion. We see this clearly in neurons like motor neurons in larval and adult CNS, and neurons in the adult brain that cross from one hemisphere to the other. Note that neurons with smaller axons may also have Para continually distributed along axons at undetectable levels or may not require Para at all. However, given the expression data discussed below, the latter is unlikely.

The sparse number of CNS neurons in the 3rd instar larvae that express $\text{para}$ is an unanticipated observation. Only 23 ±1% of $\text{elav}$ positive neurons in the larval CNS express $\text{para}$, and historical gene expression tracing shows that those neurons that are $\text{para}$ negative never express $\text{para}$. These data are also in agreement with the single cell sequencing data shown in Figure 8A which document a highly restricted expression pattern for $\text{para}$ in the 3rd instar larval CNS. Neurons expressing $\text{para}$ also express genes that produce neurotransmitters as well as proteins that have been shown to be upregulated in many $\text{Drosophila}$ models of enhanced neuronal activity (Harris and Littleton, 2015; Chen et al., 2016), both in the 3rd instar larval and adult brain single cell RNA sequencing data sets. This provides evidence that this restricted cell population is likely capable of firing APs, and that $\text{para}$ expression is a potential marker for active AP firing neurons, and that only 23 ±1% of cells in the 3rd instar larvae fit this criterion. Whether the $\text{para}$ negative cells still have electrical activity in lieu of $\text{Na}_V$ channels, either through passive signaling or using $\text{Ca}_V$ is unknown. $\text{para}$ expression has been demonstrated to positively correlate with neural progenitor proliferation in 3rd instar larvae (Piggott et al., 2019). The single cell
transcriptomic atlas of the whole 3rd instar larval CNS generated in this study identifies neuronal progenitors at immature, intermediate and mature maturation steps and we failed to detect para expression in any neuroblasts lineage at any stage of maturation (Figure 6, Figure 7).

In this study we have identified the distal axonal segment, DAS, a compartment of Drosophila axons analogous to the AIS in vertebrates. We report the characterization of Na_v channels throughout development in Drosophila melanogaster, uncovering the presence of the DAS in most axons after the dendritic tree, where AP is likely initiated, and continual low-level Para distribution in long axons to maintain AP. Additionally, we performed comprehensive single cell RNA sequencing of the 3rd instar larval CNS. This single-cell atlas in conjunction with para-T2A-GAL4 revealed that only 23 ±1% of the cells in the 3rd instar larval brain express para and other genes expressed in AP firing neurons. These data are consistent with the notion that most neurons are developing in third instar larvae, especially in the brain lobes (Hartenstein et al., 2008). In contrast, para expression is broad in the adult CNS. The models generated herein should allow the mapping of DAS in any neuron and provide a GAL4 driver to target differentiated, AP firing neurons.

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**Figure legends**

**Figure 1 – Fluorescent tagged Para and para-T2A-GAL4 alleles** (A) para\(^{\text{Mg8678}}\) (para-MiMIC) contains two intronic MiMIC cassettes in the second coding intron of the para gene in the opposite orientation of transcription. (B) Using RMCE the MiMIC was replaced with two artificial exons that either encode SA-EGFP-FlAsH-Strep-TEV-3xFlag-SD (GFSTF), SA-RFP-3xHA-SD (RFP-3xHA) or SA-mCherry-SD (mCherry) to detect protein expression. These artificial exons label all 60 isoforms based on the frames of the Splice Acceptor and Splice donor sites (C) Western blot analysis of GFSTF tagged Para using an anti-GFP antibody confirms the presence of GFP tagged Para proteins at about 250 kDa which corresponds to the expected molecular weight of 55/60 transcripts. An additional band can be seen around 130kDa which corresponds to the smaller isoforms of Para in A. (D) A Splice Acceptor (SA)–T2A-GAL4-pA sequence was injected into para-MiMIC to generate a gene trap that expresses GAL4 in the same spatiotemporal pattern as para. The GAL4 produced by this artificial insertion/exon can activate the UAS-RedStinger\(\_\text{nls}\) and allows the determination of the localization of the cell body of the para expressing neurons. (E) PCR amplification of para-GFSTF and para-MiMIC cDNA, using a forward primer in exon 1 and a reverse primer flanking exons 3 and 4, reveals one predominant band of ~300bp in the MiMIC control which is the expected product sans any insertions, but three bands of ~300bp, 1600bp and 2400bp which correspond to zero, one and
two artificial exons are seen in the para-GFSTF sample. (F) Quantitative PCR of Para-GFSTF to detect the relative proportion of para transcripts incorporating or skipping the GFSTF exon shows almost exclusive expression of GFSTF containing para transcripts. Amplicons measured span from the exon 1-2 boundary to the exon 3-4 boundary, from exon 1-2 to the GFSTF cassette, and from the GFSTF cassette to exon 3-4. (Error bars = SEM) (G) para-GFSTF animals are homozygous viable at room temperature and do not display any obvious defects. However, the gene trap para-T2A-GAL4-pA is homozygous lethal and fails to complement a deficiency (Df(1)FDD-0230908) that uncovers the para locus. The first instar lethal phase of the transheterozygous animals agrees with it being a null allele. para-T2A-GAL4 lethality can be rescued with a genomic rescue construct containing the para locus (Ppacman Dp(1;3)DC134). (H) Fluorescent tagged Para animals display a temperature sensitive paralysis. Most tagged animals are paralyzed after 60 seconds at 40°C. The rate of recovery is variable amongst the constructs with para-RFP-3xHA (n=108) recovering within 60 seconds (similar rate to para-3 (n=102)) whereas para-mCherry (n=121) flies need 240 seconds to fully recover and para-GFSTF (n=140) animals need 300 seconds. Note that para-MiMIC (n=101) was not paralyzed suggesting that it does not affect the function of para, as expected (Error bars = SEM). (I) Electroretinograms show that the amplitude of off-transients of para-GFSTF and para-MiMIC are not significantly different from each other, indicating that the GFSTF tag does not impair channel function. Animals were raised in 12-hour light/dark conditions. No significant differences were seen in 24-hour dark conditions, or in the amplitude of on-transients (Figure1-1) Unpaired, two-tailed t-test (Error bars = SEM).

**Figure 1 – 1 – para-GFSTF and para-MiMIC have comparable electroretinogram recordings**

Electroretinograms (ERG) were performed on para-GFSTF and para-MiMIC flies to measure neuronal activity in the visual system. (A) Representative trace of ERGs in 24hr dark conditions, with no significant difference seen in off transients between tagged and untagged animals. (B) No significant difference was seen in the amplitude of on transients in 12-hour light/dark conditions or constant dark. The difference in phototransduction amplitude between the genotypes was not significant in 12-hour light/dark conditions; in constant dark the amplitude of para-GFSTF flies was slightly larger than para-MiMIC flies. Unpaired, two-tailed t-test (Error bars = SEM).

**Figure 2 – Para-GFSTF is present in CNS neurons, chordotonal neurons and cardiomyocytes in embryos.**
(A-B) Immunofluorescent staining of Para-GFSTF in *Drosophila* stage 15 and 16 embryos, using an antibody against the Flag epitope, shows Para localizes to the soma of some CNS and PNS neurons. (C) In stage 16 embryos Para expression is visible in the dorsal tube. (D) In the late stage PNS Para-GFSTF is localized both to the cell body and to the dendrites of chordotonal neurons. (E) In the CNS Para is restricted to the cell bodies of some CNS neurons. (F) Using membrane bound GFP (mCD8::GFP) driven by *para-T2A-GAL4* we detect *para* gene expression in a restricted portion of CNS and PNS neurons in stage 16 embryos.

Figure 3 – *para* is expressed in only 23 ±1% of neurons in the CNS of 3rd instar larvae but most neurons in the adult CNS. Immunofluorescent staining of the CNS of 3rd instar larvae and adult animals with *para-T2A-GAL4* and *UAS-RedStinger.nls* compared to the broad neuronal marker Elav. (A) *para-T2A-GAL4* driving nuclear localized *UAS-RedStinger.nls* is restricted to a limited number of CNS neurons in the larval brain with the strongest expression observed in the dorsal motor neurons in the VNC. (B) *para* is expressed in a small amount of central brain neurons that can only be seen when the signal is overexposed. (C) Quantification of neurons expressing *para* in the larvae and adult as a fraction of *elav* expressing neurons. (N = 4, error bar = SD) (D) In the adult central brain *para-T2A-GAL4* driving nuclear localized RedStinger identifies a broad range of neurons that express *para*, with a strong overlap with Elav expressing neurons. (E) The neurons in the thoracic ganglion also show almost 100% overlap with *para-T2A-GAL4* driving nuclear localized RedStinger.

Figure 4 – Many 3rd instar larval CNS neurons never express *para*. G-Trace signaling was used to determine if CNS neurons of 3rd instar larvae had historically expressed *para*. (A) The G-Trace method labels present expression of *para-T2A-GAL4* with *UAS-RedStinger.nls* and historical *para-T2A-GAL4* expression with *UAS-Flippase* and *Ubr^{PS} >FRT-STOP-FRT Stinger.nls*. (B) Comparison of Stinger and RedStinger in the VNC shows almost a complete overlap indicating that once cells activate *para* expression it remains on. (C) In the central brain most of the historical and current staining overlap; however, a small number of Stinger positive, RedStinger negative cells can be observed.

Figure 5 - Building a single-cell transcriptome atlas of the whole 3rd instar larval brain. (A) Annotated cell types on Seurat t-SNE of 5k cells from the central brain and ventral nerve chord of 3rd instar larvae. AST,
astrocyte-like glia; CTX, cortex glia; DCN, dorsal cluster neurons; ENS, ensheathing glia; SUR, surface glia; LPC, lamina precursor cells; OPC, outer proliferation center; NB, neuroblasts; GMC, ganglion mother cell; KC, Kenyon cells. The distribution of genotypes and/or fixation methods showed no bias in cluster annotation (Figure 5-1) and cluster assignment is stable (Figure 5-2). Sequencing metrics and cluster composition are listed in Extended Data file 1.

Figure 5-1 – Composition of single-cell transcriptomic clusters – Distribution of (A) genotypes and (B) sample fixation type across annotated clusters. (C) Knee-plots showing distribution of valid barcodes and unique molecular identifiers (UMI) in fresh and fixed 3rd instar larval brains.

Figure 5-2 – Clusters are stable across multiple resolutions in the Louvain algorithm. Arrows linking clusters show overlap in percentage between clusters from different resolutions. Note that cluster 11 in resolution 0.2 becomes part of cluster 3 in resolution 0.6 but only accounts for 0.05% of cluster 3, making the arrow invisible. The squared box highlights the final chosen resolution.

Figure 6 – Distribution of marker genes in the 3rd instar larval CNS single-cell transcriptomic atlas. Marker genes are used to annotate cell clusters in the 3rd instar larval brain. The selective expression of marker genes is visualized using Dot plots (A) with a blue-yellow colour scale representing min-max normalized log(counts per million (CPM)+1) gene expression and a size scale representing the number of cells in each cluster expressing the gene. Individual tSNE plots (B) for each marker gene also show enrichments in specific clusters annotated in Figure 5. NB – Neuroblast, CB – Central Brain, GMC – Ganglion Mother Cell, DCN – Dorsal Cluster Neurons, G-KC – Gamma Kenyon Cells, LPC – Lamina precursor cells, OPC – Outer proliferation center. Sequencing metrics and cluster composition are listed in Extended Data file 1.

Figure 7 - Lineage analysis of neuroblast differentiation from 3rd instar larval single cell transcriptomic data. (A) Heatmap showing expression of optic lobe (OL) temporal transcription factors in type I neuroblast (NB) populations. Min-max normalized log(CPM+1). (B) Trajectory analysis for Type I OL NB (C) Heatmap showing effect
of the neural wave in the neuroepithelium of the outer proliferation center (OPC), through phase 1 (PI) and phase 2 (PII) into NB. Min-max normalized log(CPM+1). (D) Trajectory analysis for OPC. (E) Expression profiles of marker genes for neuroepithelial (NE) cells (grh), neuroblasts (NB) (dpn), ganglion mother cells (GMC) (ase), early neuronal development (Ndev) (Hey, pros) and mature and differentiated neurons (Nmat) (Fas2, VACHT, VGluT, Gad1, Imp), plotted against pseudotime showing dynamics through neuronal maturation. para expression increases greatly once neurons have matured and differentiated. Expression shown in log(CPM+1), 10\textsuperscript{th} degree polynomial fit through 15-cell moving average. NE- Neuroepithelium, NB- Neuroblast, GMC – Ganglion Mother Cell, Ndev – Developing neuron, Nmat – Mature neuron. (F) Trajectory analysis of neuronal maturation. Sequencing metrics and cluster composition are listed in Extended Data file 1.

Figure 8 – para is only expressed in neurons in both the 3\textsuperscript{rd} instar larval and adult CNS that are likely to be actively firing. (A) Seurat t-SNE plot of 5k cells from the whole 3\textsuperscript{rd} instar larval brain shows that para is only expressed in a small number of neurons. (B) Seurat t-SNE plot from the adult brain taken from Davie et al. 2018 (Figure 8-1), shows that para is widely expressed and is present in most neuronal cells. Pseudo-color refers to para expression in Counts Per Million (CPM) in each cell. (C) Boxplots showing para expression for the different single-cell clusters from the third instar larval brain single cell transcriptomic data, para is enriched in clusters 33 and 3 (a list of other enriched genes in these clusters and gene ontology analysis is in Extended Data File 1). (D) Co-expression analysis of para with known active zone protein encoding genes, genes upregulated in response to neuronal activity in the third instar larvae (left hand side) and the adult (right hand side) CNS shows significant positive correlation (Green highlight)(Two-Tailed T-Test, $P_{adj}<0.05$). This suggests that neurons in which para is expressed are those that are actively firing. para expression positively correlates with the mature neuron marker Imp in the 3\textsuperscript{rd} instar larvae single cell sequencing data and is negatively correlated with GMC (ase), immature neurons (pros), neuroblasts (dpn) and glial cells (repo) in both data sets however the correlation doesn’t meet the threshold for significance in the larval single cell sequencing data. This trend indicates para is likely expressed only in mature neurons, which is evident by the significant positive correlation between para and elav in the adult single cell sequencing data.

Figure 8 – 1 – Distribution of para expressing cells in 3\textsuperscript{rd} instar larvae and adult transcriptomic data. (A) Annotated cell types on Seurat t-SNE plot of cells from the adult brain as previously annotated by Davie et al.
Figure 9 – Para-GFSTF is localized to Distal Axonal Segments (DAS) downstream of the site where dendrites impinge upon axons in the 3rd instar larval CNS. (A) Para-GFSTF distribution in the larval brain is most abundantly expressed in thoracic and abdominal segments, the exiting axon tracks in the VNC and the median portion of the central brain (Movie 1). Clusters of Para-GFSTF localization can be seen in axon bundles leaving the VNC (inset box i), Para is less abundant in the more distal axons (inset box ii). This pattern is also seen with other tagged Para isoforms (Figure 9-1A) (B) The distribution of Para-GFSTF extends to the neuromuscular junction where continual expression stops at the first bouton. Punctate expression of Para-GFSTF can be seen in each bouton. (C, D) 201YGAL4, driving UAS-mCD8::GFP, labels the mushroom body neurons in the 3rd instar larvae (Figure 9-1B). 201YGAL4>UAS-mCD8::GFP labelling with Para-mCherry shows that Para is enriched in the mushroom body neurons in the axon, after the site of dendritic innervation in the calyx, at a Distal Axonal Segment (DAS).

Figure 9 – 1 – Para-RFP-3xHA and Para-mCherry recapitulate the expression of Para-GFSTF in the 3rd instar larval CNS. (A) Immunofluorescent imaging of Para-RFP-3xHA and Para-mCherry subcellular localization shows the same clustered distribution as Para-GFSTF. (B) 201Y-GAL4 labels the membrane of the mushroom body neurons of 3rd instar larvae, labelling these cells with Para-mCherry identifies the cluster of Para in at the Distal Axonal Segments (DAS), yellow arrow.

Figure 10 - Para enrichment at the DAS is seen in posterior 3rd instar larval motor neurons. (A) Split-GAL4 line (R-MB050B) labels 2 motor neurons in the most posterior larval VNC. (B, C) Using mCD8::RFP we label the whole cell identifying the cell body (CB), Dendritic Tree (DT) and Axon. Colocalization with Para-GFSTF is seen with mCD8::RFP at a DAS after the dendritic tree. (D) DenMark labels only the CB and the axon. No co-localization is
seen between DenMark and Para-GFSTF showing that the DAS is not in dendrites and is distal to the site of the dendritic tree.

**Figure 11** – Para is enriched at the DAS in 3rd instar larval motor neurons. Cross sections through a posterior motor neuron labelled with Split-GAL4 line R-MB050B driving expression of UAS-mCD8::RFP. No axonal Para is detected at the axon hillock (A), where NaV channels cluster in vertebrates. (B) Para is enriched at the DAS and is less abundant at a more distal axonal compartment (C). Scale bar 5 μM.

**Figure 12** – *para* is localized to distal axonal segments in the adult CNS neurons. Immunofluorescent imaging of Para-GFSTF subcellular localization of *para* in the adult CNS. (A) In the central brain Para is enriched in axonal clusters in the neuropil (Movie 2). The Para clusters are not associated with the cell body of neurons (Elav) and are located distally to the cell body. (B) In the thoracic ganglion the Para clusters can also be observed distally to the cell body. The additional labelling of axon tracts down the midline of the thoracic ganglion highlight the lower intensity Para distribution in long axons. (C,D) Split-GAL4 line R OL0019B is specifically expressed in the LC10 neurons in the adult fly visual system. (E) Using UAS-mCD8::RFP we can identify the cell body (CB), Dendritic Tree (DT) and Axon (Ax). Para-GFSTF is localized to a Distal Axonal Segment (DAS), in the axon, downstream of the dendritic tree. (D) Using UAS-DenMark to label the soma and dendritic tree of the LC10 neurons we do not see any colocalization with Para-GFSTF indicating the DAS is localized after the somatodendritic boundary.

**Figure 13** – Para enrichment at the DAS is seen in the mushroom body neurons of the adult CNS. (A, B) 201Y-GAL4 is expressed specifically in the neurons of the mushroom body. (C) Expression of mCD8:GFP in the mushroom body clearly marks the cell body (CB), axon (Ax) and the Calyx where the neurons receive dendritic input. Para-mCherry is enriched at the DAS in the axon of the mushroom body neurons, distal to the calyx. (D) UAS-DenMark expression in the mushroom body neurons using 201Y-GAL4 colocalized with UAS-mCD8::GFP in the cell body and calyx region of the neurons but not in the peduncle beyond the DAS.

**Figure 14** – The DAS is variable in size and location and forms the boundary between the somatodendritic and the distal region of the axon. (A) Quantification of the sizes of axonal compartments (B) in neurons derived from Mushroom Body Neuroblast (MNNB) lineages a, c and d. Cell body to dendrite, dendritic
Input and dendritic input to end of DenMark staining measurements are derived from the intensity profile of UAS-
DenMark using 201Y-GAL4 with UAS-mCD8::GFP (n=4). Soma-Distal Axonal Segment (DAS) distance and DAS length
are derived from the intensity profile of Para-mCherry with 201Y-GAL4 and UAS-mCD8::GFP. The DAS length
ranges from 21.92-29.98μm, neurons with shorter soma to DAS regions have a shorter DAS. DenMark doesn’t, in
any MBNB population, extend beyond the DAS indicating that the DAS forms a boundary between the
somatodendritic proximal axon and the distal axon. Error bars = SD.

Figure 15 - Inward activating Na⁺ currents are generated distal to the soma in I-LNv neurons in the
adult CNS. (A) Depolarizing steps in whole cell patch recordings in voltage clamp mode in the presence of blockers
of synaptic currents, voltage gated potassium and calcium channels reveal the functional expression of voltage
gated Para sodium channels in I LN processes. These inward, rapidly activating currents show an average peak
value of 91 ± 3 mV, at the maximum activation voltage of –35 mV (n=5). The long delays in inward current
initiation relative to voltage steps show poor space clamp and poor voltage control, indicating that Para channels
are located distally in neuronal processes rather than in or close to the neuronal cell body. Fourth power
exponential time constant for the activation was 2.1± 0.7 ms, while the single exponential fit inactivation time
constant was 5.9 ± 2.6 (n=5). (A’) Currents were completely blocked by perfusing with 100 nM TTX. (A’’) Voltage-
current relationship for the inward sodium currents (squares) are shown, along with the effect of TTX channel
block (triangles). (B) Pdf-GAL4 is specifically expressed in the small and large lateral ventral neurons (s-LNv, l-LNv)
in the adult CNS. Using Pdf-GAL4 to drive expression of UAS-mCD8::RFP we can identify the cell body of the I LNv
neurons (CB), the dendritic tree (DT) and axons (Ax) for both s-LNv (dorsal) and I-LNv neurons. Colabelling of these
neurons with Para-GFSTF shows Para is localized only to Distal Axonal Segments (DAS) in both neuron types with
no Para seen in the soma, proximal axon or dendritic tree. (C) UAS-DenMark is localized to the soma and dendritic
projections. In the I-LNv and s-LNv neurons the dendritic projections extend across the accessory medulla.
Colabelling of Pdf-GAL4 driving UAS-DenMark and Para-GFSTF does not show any Para localization in the dendritic
tree, the soma or the proximal axon.

Extended Data file 1 – 3rd instar larvae single cell sequencing metrics, cluster composition and the gene
list and GO terms used for analysis. Related to figures 5-8.
Table 1 – Fly lines used in this study

Table 2 – para positive cluster 3 of the single cell transcriptomic atlas is enriched for genes required for neurotransmission and ion transport. PANTHER Overenrichment to determine enriched GO molecular functions in the top 100 enriched genes in cluster 3 of the single-cell 3rd instar larval brain transcriptomic atlas shows that cluster 3 is enriched for genes involved in neuronal transmission, ATP synthesis and ion transport. Process upregulated in actively firing neurons.

Table 3 – para positive cluster 33 (Motor Neurons) of the single cell transcriptomic atlas is enriched for genes required for neurotransmission and ion transport. PANTHER Overenrichment to determine enriched GO molecular functions in the top 100 enriched genes in cluster 33, which represents motorneurons, of the single-cell 3rd instar larval brain transcriptomic atlas shows that cluster 33 is enriched for genes involved in ATP synthesis and ion transport. Both of these processes are upregulated in actively firing neurons.

Movie 1 – Para-GFSTF expression in the 3rd instar larval CNS.

Movie 2 - Para-GFSTF expression in the adult CNS.

Table 1

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<td>9.97</td>
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<td>active ion transmembrane transporter activity (GO:0022853)</td>
<td>158</td>
<td>8</td>
<td>1.01</td>
<td>+</td>
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<td>7.83</td>
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<td>6.17</td>
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<td>cation transmembrane transporter activity (GO:0008324)</td>
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<td>inorganic molecular entity transmembrane transporter activity</td>
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<td>4.6</td>
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<td>Value</td>
<td>Sign</td>
<td>p-value</td>
<td>Adjusted p-value</td>
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<td>Ion transmembrane transporter activity (GO:0015075)</td>
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<td>2.32E-03</td>
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<td>Transmembrane transporter activity (GO:0022857)</td>
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<td>4.67</td>
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<td>Unclassified (UNCLASSIFIED)</td>
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<td>-</td>
<td>18.34</td>
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<td>0.00E+00</td>
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</table>
Figure 2

(A) Stage 15 Para-GFSTF

(A') Stage 15 Para-GFSTF Elav

(B) Stage 16 Para-GFSTF

(B') Stage 16 Para-GFSTF Elav

(C) Dorsal Stage 16 Para-GFSTF

(C') Dorsal Stage 16 Para-GFSTF Elav

(D) PNS Para-GFSTF

(D') PNS Para-GFSTF Elav

(E) CNS Para-GFSTF

(E') CNS Para-GFSTF Elav

(F) para-T2A-GAL4 > UAS-mCD8:GFP

(F') para-T2A-GAL4 > UAS-mCD8:GFP Elav
Figure 4

A

para | SA | T2A | GAL4

GAL4 → UAS

UAS → RedStinger.nls

Cells currently expressing para

UAS → Flippase

Ubip63 → FLP

FLP |

FRT STOP FRT

Stinger.nls

Cells that have ever expressed para

B

50µm

Stinger.nls

RedStinger.nls

50µm

Stinger.nls

RedStinger.nls

50µm

50µm

Elav

C

50µm

Stinger.nls

RedStinger.nls

50µm

50µm

Elav

RedStinger.nls Stinger.nls

Elav
Figure 6

A

CB Type I NB
CB GMC
OL Type I NB
OL GMC
OPC
LPC
Hey+ Neurons
DCN
G-KC
Motorneurons
elav
dpn
ase
pnt
cas
scro
dac
Hey
ato
aco
ey
ptw	twit
Proc
Imp
pros
para

B

log(CPM+1)

100%
75%
50%
25%
Figure 10

R_MB050B-Split-GAL4 (Posterior Motor Neurons)
Figure 11

R_MB050B_Split-GAL4
(Posterior Motor Neurons)
Figure 14

A

![Bar graph showing length in μm for different categories of MBNeurons and MBNNeurons:](image)

- Cell Body To Dendrites (n=4)
- Dendritic input into axon (n=4)
- Dendritic input to end of DenMark Staining (n=4)
- Soma-DAS Distance (n=7)
- Length of DAS (n=7)

B

![Diagram of a neuron showing Soma, Dendritic Input, DAS, and Synapses](image)
Figure 15

Pdf-GAL4 (l-LNv and s-LNv Neurons)

B C B'

D T Axon

UAS-mCD8::RFP

30μm 30μm 30μm

C'

Para-GFSTF

UAS-DenMark

30μm 30μm 30μm

C''

Isolated Colocalization

B''

DAS

Isolated Colocalization