Differing Strategies Despite Shared Lineages of Motor Neurons and Glia to Achieve Robust Development of an Adult Neuropil in *Drosophila*

**Highlights**
- The development of the neuropil glia and leg motor neurons is highly coordinated.
- All adult thoracic neuropil glia are born from two lineages per hemisegment.
- These lineages also give rise only to leg motor neurons.
- Unlike neurons, glia generation is plastic and exhibits interlineage compensation.

**Authors**
Jonathan Enriquez, Laura Quintana Rio, Richard Blazeski, Stephanie Bellemin, Pierre Godement, Carol Mason, Richard S. Mann

**Correspondence**
jonathan.enriquez@ens-lyon.fr (J.E.), rsm10@columbia.edu (R.S.M.)

**In Brief**
Enriquez et al. show that the glia surrounding each thoracic neuropil come from stem cells that also give rise to leg motor neurons. Unlike the hard-wired birth order and morphology of each motor neuron, the number and morphologies of the glia born from single lineages are variable, yet the structure they contribute to is highly stereotyped.
**Differing Strategies Despite Shared Lineages of Motor Neurons and Glia to Achieve Robust Development of an Adult Neuropil in *Drosophila***

Jonathan Enriquez,1,2,* Laura Quintana Rio,2 Richard Blazeski,3 Stephanie Bellemin,1 Pierre Godement,1 Pierre Mason,3 and Richard S. Mann2,4,*

1Institut de Génomique Fonctionnelle de Lyon, ENS de Lyon, CNRS, Univ Lyon 1, 46 Allée d’Italie, 69364 Lyon Cedex 07, France
2Departments of Biochemistry and Molecular Biophysics, and Neuroscience, Mortimer B. Zuckerman Mind Brain Behavior Institute, Columbia University, New York, NY 10027, USA
3Departments of Pathology and Cell Biology, Neuroscience and Ophthalmology, Mortimer B. Zuckerman Mind Brain Behavior Institute, Columbia University, New York, NY 10027, USA
4Lead Contact
*Correspondence: jonathan.enriquez@ens-lyon.fr (J.E.), rsm10@columbia.edu (R.S.M.)
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**SUMMARY**

In vertebrates and invertebrates, neurons and glia are generated in a stereotyped manner from neural stem cells, but the purpose of invariant lineages is not understood. We show that two stem cells that produce leg motor neurons in *Drosophila* also generate neuropil glia, which wrap and send processes into the neuropil where motor neuron dendrites arborize. The development of the neuropil glia and leg motor neurons is highly coordinated. However, although motor neurons have a stereotyped birth order and transcription factor code, the number and individual morphologies of the glia born from these lineages are highly plastic, yet the final structure they contribute to is highly stereotyped. We suggest that the shared lineages of these two cell types facilitate the assembly of complex neural circuits and that the two birth order strategies—hardwired for motor neurons and flexible for glia—are important for robust nervous system development, homeostasis, and evolution.

**INTRODUCTION**

Cell lineages play a critical role in generating cellular diversity during animal development yet range widely in the number and types of cells they produce. Embryonic stem cells, for example, have the potential to give rise to all cell types, whereas hematopoietic stem cells have much more limited potential, giving rise only to myeloid and lymphoid cells (Seita and Weissman, 2010; Wu et al., 2016). The wide range of developmental potentials is also reflected in the degree to which cell lineages are hardwired or plastic in the types and numbers of cells they can generate. At one extreme, each of the 959 somatic cells of the adult C. elegans hermaphrodite is derived from an invariant lineage that can be traced back to the single-celled zygote (Sulston, 1976). In contrast, cell lineages of the mammalian immune system are plastic and can be modified depending on the environmental challenges that an animal confronts (Boettcher and Manz, 2017). Similarly, intestinal stem cell lineages exhibit plasticity in response to injury and cell death (Apidianakis and Rahme, 2011; Jiang and Edgar, 2011).

Although the capacity of some cell lineages to respond to varying environmental conditions makes sense in light of the cell types these lineages generate, the underlying logic of why some lineages are invariant is usually not understood. For example, although the entire lineage is hardwired in *C. elegans*, there is almost no correlation between where a cell is born in the lineage and cell-type identity; most sub-lineages contribute broadly to endodermal, mesodermal, and nervous system tissues. Consequently, a single cell type such as a motor neuron can be born from many different sub-lineages in *C. elegans* (Hobert, 2016). In *D. melanogaster*, although there are neuroblasts (NBs), stem cells that are dedicated to the generation of neurons, during embryogenesis each NB can generate a mixture of motor neurons, interneurons, and glia that do not share an obvious function (Bossing et al., 1996; Schmidt et al., 1997). These observations raise the question of whether there is a biological purpose for the structure of invariant lineages that may be difficult to discern by only examining the final progeny. Consistent with this notion, in the adult, the neuronal progeny of individual *Drosophila* NB hemilineages share anatomical features such as stereotyped axon paths and, when activated, the progeny of individual hemilineages can evoke specific behaviors in decapitated flies (Harris et al., 2015; Truman et al., 2004). Thus, by both anatomical and behavioral criteria, there is emerging evidence that individual *Drosophila* NBs generate functionally related neurons in the adult fly. Another reason for the existence of hardwired lineages might be to facilitate the assembly of complex structures during development, which is an especially challenging problem for nervous systems. For example, the ~50 motor neurons (MNs) that innervate each leg in adult *Drosophila* are born from invariant NB lineages and have highly stereotyped birthdates and morphologies (Baek and Mann, 2009; Brierley et al., 2012).
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These stereotyped morphologies can be seen both in the muscles these motor neurons target and in their elaborate dendritic arbors that establish a very large number of synapses in dense neuropils present in each thoracic neuromere of the ventral nerve cord (VNC) (Court et al., 2017). Although each motor neuron morphology is thought to be determined by unique combinations of morphology transcription factors (mTFs; Enriquez et al., 2015), how these stereotyped morphologies form the correct synaptic connections with interneurons and sensory neurons is not understood. Interestingly, at least one of the major NB lineages that give rise to adult motor neurons in the fly also generates another important cell type in the nervous system, glia (Baek et al., 2013; Lacin and Truman, 2016), which are critical for the establishment of synapses and the maintenance of neuronal activity (Freeman, 2015; Freeman and Rowitch, 2013). The observation that glia and leg motor neurons may be derived from common progenitors (neuroglioblast [NGB]) raised the possibility that their development may be coordinated, and that being born from the same lineages might also play a role in neural circuit assembly. However, in contrast to motor neurons, very little is known about the glial types produced by these NGBs, how their morphologies are genetically specified, and how neurons and glia communicate during development to build a functional CNS.

In other contexts, Drosophila glia have been subdivided into three classes based on their functions and morphologies: surface glia, which isolate the CNS from the hemolymph, cortex glia, which send processes around neuronal cell bodies, and neuropil glia (NG), which surround neuropil and glomeruli (Omoto et al., 2016). NG are further subdivided into astrocytes and ensheathing glia (EG) (Muthukumar et al., 2014; Peco et al., 2016). EG extend flat processes that surround neuropil and glomeruli as well as a small portion of axon bundles as they enter or leave the neuropil (Omoto et al., 2015; Peco et al., 2016). More recently, EG have been shown to extend small protrusions inside the neuropil of the adult brain (Kremer et al., 2017). In the medulla (region of the brain that processes visual information), EG are associated with axons (Kremer et al., 2017) and trachea (Kremer et al., 2017; Pereau et al., 2007). In contrast, astrocytes send highly ramified processes deep into neuropil regions in close contact with synapses (Awasaki et al., 2008; Muthukumar et al., 2014; Stork et al., 2014). Although NG exhibit complex and diverse morphologies and are generally derived from stem cells that also generate neurons (Awasaki et al., 2008; Omoto et al., 2016), the developmental mechanisms leading to glial diversity remains unknown.

Here, we show that two of the ~11 lineages that generate leg motor neurons in Drosophila also generate all of the NG that populate the adult thoracic neuropils. As a consequence of being born from the same lineages, the development of the NG is physically and temporally coordinated with motor neuron development. However, unlike the hardwired birth order and unique mTF codes of the motor neurons that are born from these NGBs, individual NG do not have a stereotyped morphology, birth order, or unique transcription factor code. Moreover, using a unique mode of division, the gliogenesis phase of these lineages is plastic and highly adaptable: when gliogenesis in one lineage is compromised, other lineages compensate to maintain the correct number of NG. Thus, even though NG and motor neurons come from the same stem cells, and generate adult neuromeres with highly stereotyped structures, there are fundamental differences in how these two cell types are specified. We suggest that the combination of hardwired motor neuron generation and flexible glia production facilitates the robust construction, homeostasis, and evolution of complex neural circuits.

**Figure 1. Cellular Organization of the Adult Thoracic NG**

Panel A-C: Adult VNCs immunostained with anti-BR.P (neuronal marker, blue) (A1–C) and anti-Dll (NG marker, green) (B) with astrocytes expressing mCD8::GFP (membrane marker, green) (A1 and A2), H2B::RFP (nuclear marker, red) (B) or the multicolor system FB1.1 (single-cell marker, red, yellow, and green) (C) under the control of alrm-Gal4.

Panel B: Astrocyte (H2B::RFP*$^+*, Dll*$^+*): arrow, EG (Dll*$^-*, Dll*$^+*): arrowhead.

Panel D: Different astrocytes with processes occupying different neuropil territories: arrows. See also Figure S1 and Movie S1.

Panel F: Adult VNCs immunostained with anti-BR.P (neuronal marker, blue) (D1–F) and anti-Dll (NG marker, green) (E) with EG-expressing mCD4::GFP (membrane marker, green) (D1 and D2), H2B::RFP (nuclear marker, red) (E) or the multicolor system FB1.1 (single-cell marker, red, yellow, and green; Hadjiieconomou et al., 2011) in the control of R56F03-Gal4. (E) Astrocyte: arrow (Dll*$^-*$), EG (H2B::RFP*$^+*, Dll*$^-*$): arrowhead.

Panel G: Arrow: more than half of the samples analyzed (n = 10) a cell body of an EG (GF*P *^-*, Dll*$^-*$) was observed inside the neuropil, next to axon bundles.

Panel H: Different EG with processes occupying different neuropil territories: arrows. See also Figure S1 and Movie S1.

Panel G and H: Prothoracic neuromeres with EG-expressing mCD4::GFP and immunostained with anti-BR.P (blue) and anti-Elav (neuron marker, red) (G) or anti-N Cad (neuronal marker, red) and Nrg (axon marker, blue) (H).

Panel I and J: Prothoracic neuromeres with EG-expressing mCD4::GFP (I) and GFP::mCD8::HRP (J) immunostained with anti-BR.P (blue) (I) or labeled with DAB (brown) (J).

Panel K: Low-magnification electron microscope image of the boxed region in (J).

Panel L: Enlargement of the boxed regions in (K) (L2). Enlargement of the boxed region in (L1). Note: (L1) and (L2) show the organization of the EG processes around the neuropil while (M)–(O) show the EG processes inside the neuropil. Pink lines outline axons. P0, perineurial glia; SPG, subperineurial glia; NG, neuropil glia; C, cortex; Np, neuropil; NL, neural lamella.

Panel P: Bottom, schematic of adult CNS (blue: neuropils, gray: cortex); top, single astrocyte and EG labeled with mCD8::GFP under the control of alrm-Gal4 and R56F03-Gal4 using MARCM. Axes: green (posterior), blue (dorsal), red (medial).

Panel Q: Average number of NG in the adult VNC, in which EG or astrocytes were expressing H2B::RFP under the control of R56F03-Gal4 or alrm-Gal4, respectively, and immunostained with anti-Dll. Number of samples = 4 genotype. Error bars indicate SD.

ProN, Prothoracic neuromere; AMesoN, Accessory mesothoracic neuromere; MesoN, Mesothoracic neuromere; MetaN, Metathoracic neuromere; AN, Abdominal neuromeres; FS, frontal cross-section; TS, transverse cross-section; 3D, 3-dimensional reconstruction of confocal image stack; pMP, partial maximum projection.
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RESULTS

Cellular Organization of the Adult Thoracic Neuropil Glia

In order to characterize the cellular organization of the NG in the adult VNC, we expressed fluorescent reporters under the control of two Gal4 drivers, alrm-Gal4 (astrocyte specific; Doherty et al., 2009) and R56FO3-Gal4 (EG specific; Kremer et al., 2017; Peco et al., 2016; Pfeiffer et al., 2008), which we confirmed are expressed in all adult astrocytes and EG, respectively (Figures 1 and S1). We also used as a marker Distalless (Dll), which we found to be expressed in all NG in the adult VNC (Figure S1). As with the NG in the larval CNS (Beckervordersandforth et al., 2008; Omoto et al., 2015), the number of NG surrounding each thoracic neuropil is very consistent between animals (with a very low SD; Figures 1P and 1Q).

As described for other regions of the CNS (Awasaki et al., 2008; Muthukumar et al., 2014), adult thoracic astrocytes send processes deep into the neuropil that respect each other’s territory, thus exhibiting a tiling-like phenomenon (Figures 1A and 1C; Movie S1). EG extend processes that surround each neuropil and also respect each other’s territory with very infrequent overlaps (Figures 1D and 1F; Movie S1). In addition, the adult thoracic EG feature a more complex morphology than previously described for larval EG. First, adult EG wrap the cell bodies of neurons (Elav+) that are localized next to the neuropil (Figure 1G; Movie S2) as well as astrocyte cell bodies (Figure S1). Second, they send processes inside the neuropil where they wrap axon bundles (Neuroglian+) where no synapses are formed (BRP-negative, a marker of the active presynaptic zone) (Figures 1G and 1H; Movie S2). Interestingly, EG only wrap these axons inside the neuropil; outside of the neuropil these axons are surrounded by a distinct class of wrapping glia (Figure 1H; data not shown). Thus, EG isolate nerves inside neuropil before they make synapses with other neurons.

We next performed electron microscopy of transverse sections of prothoracic neuronomers (ProNs) (Figures 1K–1O). EG wrap the thoracic neuropils very densely with several layers (Figure 1N). These results, in combination with the tiling results described above, suggest that one EG can send processes organized in layers around the neuropil. Inside the neuropil, axon bundles and single axons can be wrapped by layers of EG processes (Figures 1M–1O). Interestingly, each nerve also contains trachea bundles that are surrounded by EG, forming a structure with all three cell types: EG, axons, and trachea (Figure 1O). Notably, axons close to the midline are not wrapped by EG (data not shown).

Developmental Origins of the Adult Thoracic NG

As described above, the adult thoracic neuropils are complex structures containing glia processes and axons/dendrites in close juxtaposition to each other, raising the question of how these structures develop. A previous study suggested that pockets of glia, marked by the general glia marker Repo, surround the immature leg neuropils in late third-instar larvae (just prior to the onset of metamorphosis) and produce adult astrocytes (Li et al., 2014), while another study suggests that these glia express Dll and may migrate to the leg to form wrapping glia, which are distinct from EG and astrocytes (Plavicki et al., 2016). In order to definitively determine what type of adult cells these Repo+ glia give rise to, we used a Dil-Gal4 enhancer trap and a Dil antibody to label these cells during development (Figure 2).

In third-instar larvae (L3), there were ~40 Repo+ Dll+ cells surrounding each immature leg neuropil (Figures 2B, 2C, and 2G), where nascent motor neuron axons enter and then exit (Figure 4A). If these cells are the progenitors of the adult NG, they should be mitotically active. To test this idea, we first confirmed that they express phosphorylated histone H3 (pH3), a marker for mitotically active cells (Figure 2D).

Although Dil protein is expressed in adult NG, Dil-Gal4 is only active in immature NG (data not shown), allowing us to mark the progeny of Dil-Gal4-expressing larval cells in lineage tracing experiments (see STAR Methods). In the first experiment, we labeled the adult progeny of Dil-Gal4-expressing L3 glia with nuclear GFP (UAS-nGFP) and co-stained with an antibody against Repo and Dil (Figure 2E). Only the adult Repo+ Dil+ NG were labeled with nGFP, demonstrating that the Dil-Gal4-expressing cells in the larval CNS only generate adult NG. In a second experiment, we used Dil-Gal4 to activate the Flybow system (UAS-FB1.1), which permanently labels cells with different fluorescent markers (Hadzieconomou et al., 2011). We co-stained the resulting adult VNCs with Prospero, which is expressed in astrocytes but not in EG (Griffiths and Hidalgo, 2004; Peco et al., 2016) (Figures 2F and S1). Based on Prospero expression and morphology, we found that both astrocytes and EG were labeled. Significantly, neither experiment labeled other classes of glia, such as surface glia or cortex glia, arguing that these glia subtypes are not derived from Dil-Gal4-expressing cells.

We confirmed this result with another driver (R31F10-Gal4) that is also expressed in immature NG (Figure S2).

Taken together, we conclude that the ~40 Repo+ Dil+ Dil-Gal4-expressing cells surrounding each immature leg neuropil in L3 are the precursors of the >280 NG that surround each adult thoracic neuropil.

Figure 2. Developmental Origin of the Adult NG

(A) 3D rendering of the thoracic segments of a L3 VNC (A1) labeled with anti-BRP (mature neuropil marker; blue) and anti-NCad (mature and immature neuropil marker; red). The glia surrounding the six immature leg neuropils express mCD8:GFP (green, left image only) under the control of Dil-Gal4. (T1, T2, and T3 indicate the three thoracic segments.) (A2) Following metamorphosis, 3D rendering of adult VNCs with astrocytes and EG-expressing GFP under the control of alrm-Gal4 (right) and R56FO3-Gal4 (left) and co-stained with anti-BRP (red).

(B–D) L3 CNSs in which glia surrounding the six immature leg neuropils express mCD8:GFP under the control of Dil-Gal4 and co-stained with anti-BRP (blue), anti-NCad (red) (B1–B3), anti-Repo (blue), anti-Dll (red) (C1–C3), or anti-Repo (blue), anti-PH3 (red) (D1–D3). (D2 and D3) Arrows point to glia expressing PH3. (E and F) Adult VNCs with expressing nuclear GFP (green) (E1–E5) or the multicolor Flybow system FB1.1 (yellow, red, and green) (see STAR Methods for details) and immunostained with anti-Repo (blue), anti-Dll (red) (E1–E5), or anti-Pros (blue) (F1–F5). Arrow points to adult NG-expressing GFP. (F1–F5) Arrow: astrocyte (mCherry*, Pros*), arrowhead: EG (mCherry*, Pros*). See also Figure S2.

(G) Left: schematic of an L3 CNS. The immature leg neuropils and proliferating NG are indicated. Right: schematic of an adult VNC with the adult leg NG. N, average number of proliferating NG in each L3 thoracic hemisegment.
Coordinated Development of the Adult NG and Neuropil

To characterize the development of the adult neuropil, we conducted several experiments to trace the origins of this complex structure using BRP to label the larval neuropils, N-cadherin to label the immature adult neuropils, and Dil-Gal4; UAS-GFP (DilG-GFP) to label the proliferating NG precursors (Figure 3; Movie S3).

In early second-instar larvae (EL2), most of the adult leg motor neurons are not yet born (Baek and Mann, 2009) and no Dll > GFP+ proliferating NG are visible. However, at this stage incoming leg sensory axons are visible and mark the location where the adult neuropil will form (Figure 3A). In early L3 (EL3; 72 hr after egg laying, AEL), 0–10 proliferating NG are observed per thoracic hemisegment (Figures 3B, 3L, and S3), and their numbers gradually expand through subsequent larval and pupal stages (Figures 3C–3E and S3). As proliferating NG are produced, they send processes that surround the growing neuropil and wrap the leg nerves (Figures 3A–3E and S3). By the mid pupal stage (48 hr APF), most of EG and astrocytes have been generated and start to differentiate by sending processes inside the neuropil (Figures 3F–3I). By the late pupal stage (96 hr APF), the adult neuropils are fully invaded by astrocyte processes and wrapped by EG (Figures 3H and 3K).

These results suggest that the adult thoracic NG are produced from early L3 to mid pupa. These glia create a structure that encompasses the immature adult neuropil (Movies S3 and S4); by L3, the motor neuron neurites are completely surrounded by the processes of the immature NG. During metamorphosis, the dendrites and axons of the neurons establish their final morphologies within the glia-defined neuropil.

The Adult Thoracic NG Are Produced by NGBs that Also Generate Leg Motor Neurons

Previous work established that the larval NG are born from a single glioblast in each hemisegment of the larval CNS (Becker-vordersandforth et al., 2008; Ito et al., 1995; Jacobs et al., 1989) and from a single glioblast or NGB in each hemisphere of the brain (Hartenstein et al., 1998; Omoto et al., 2015), while in the adult brain NG are derived from a group of uncharacterized NGBs (Omoto et al., 2015). To characterize the lineages that give rise to the adult thoracic NG, we carried out several clonal analysis experiments. Previous observations demonstrated that NB lineages such as Lin A (also called Lin 15), produce both leg motor neurons and glial cells (Baek et al., 2013; Truman et al., 2004). This observation raised the intriguing possibility that NG and leg motor neurons may share common developmental origins.

First, we used MARCM to express the membrane reporter UASmCD8::GFP with two different drivers, repo-Gal4 (expressed in all glia, Sepp and Auld, 2003) and VGlut-Gal4 (expressed in all motor neurons and some interneurons, Mahr and Aberle, 2006). We induced these clones in L1 larvae and dissected late L3 CNS and co-stained for Dil, to mark immature adult NG, and BRP to mark the mature larval neuropil. If a stem cell produces both motor neurons and NG, individual MARCM clones should contain both cell types. We identified glial cells by their position and expression of Dil, and motor neurons by their morphology, particularly motor neuron axons, which exit the CNS and target the leg imaginal discs (Baek and Mann, 2009; Truman et al., 2004). 62/62 clones that labeled NG also labeled motor neurons (Figure 4). We found 3 types of clones based on the number of motor neurons they contained. Type 1 clones (n = 36) contained ~28 leg motor neurons and ~20 immature adult NG. Based on motor neuron morphology, this lineage is Lin A (Figure 4A). Type 2 clones (n = 23) contained 2 motor neurons and ~10 immature NG (Figure 4B). In type 3 (n = 3), the clones contained 1 motor neuron and ~7 glia (Figure 4A). The total number of glia (~37) produced by these three NGBs is very close to the total number of NG progenitors that surround each neuropil at L3 (~40), suggesting that these three lineages produce most, and perhaps all, of the adult thoracic NG.

In a second approach, we generated clones using a modified QMARCM/MARC method (cis2-MARC) in which the two transcriptional repressors, GAL80 and QS, are recombined on the same chromosome arm (see STAR Methods for details). We used two drivers, VGlut-QF and repo-Gal4, allowing us to visualize VGlut+ motor neurons and Repo+ glia derived from the same stem cell with two different fluorescent markers. Using this method, and analyzing the adult legs and VNCs, we confirmed that there are three NGBs that produce leg motor neurons and adult NG. Lin A clones produce NG and ~28 motor neurons targeting the trochanter, femur, and tibia (n = 14, in ProN) (Figures 4C and 4F). Type 2 clones labeled NG and 2 motor neurons that targeted the coxa; based on the morphology of these motor neurons, we recognized this lineage as Lin D (Baek and Mann, 2009) (n = 2) (Figure 4E). Type 3 clones labeled NG and a single motor neuron that targeted a body wall muscle at the base of the coxa (n = 16) (Figure 4F). Notably, this motor neuron was previously described to be derived from Lin A (Brierley et al., 2012), a conclusion that we confirm and revise below.

The Lin A NGB Initially Produces Glioblasts and Postmitotic Motor Neurons

Because Lin A generates both glia and a stereotyped set of motor neurons, we used this lineage to investigate the birth order of these two types of progeny. In the first set of experiments we used a lineage tracing system that is restricted to Dpn+ NBs (Awasaki et al., 2014). When combined with 10C12-Gal4, which is expressed early in the Lin A lineage, this system specifically labels all Lin A progeny with GFP (Lacin and Truman, 2016). We immunostained the resulting larval CNSs with Repo, Elav, and Dpn to visualize glia, neurons, and NBs, respectively, and dissected larva at different time points during L3 (Figures 5A–5J). As a consequence of NB division patterns, progeny further from the NB tend to be older compared to progeny closer to the NB, thus potentially providing birth order information.

Using this approach, we found that Repo+ glia and Elav+ neurons are first observed at about the same time, as early as 75–78 hr AEL (Figures 5F and 5K). In some samples at this early time point, only a single Dpn+ NGB or clones with a NGB surrounded by cells not marked with Repo, Elav, or Dpn are observed (Figures 5D, 5E, and 5K). These unlabeled cells are always close to the NB and therefore may be ganglion mother cells (GMCs) or another type of intermediate cell type.
Figure 3. Coordinated Development of NG and Adult Neuropil

(A–E) Thoracic segments of larval VNCs at different stages (A, early L2; B, early L3; C, mid L3; D, late L3, E, 5 hr after pupal formation) with proliferating NG-expressing mCD8::GFP under the control of Dll-Gal4 and labeled with anti-BRP (blue), anti-NCad (red). Asterisks indicate axons of incoming leg sensory neurons. See also Figure S3 and Movies 3 and 4. (E1–E3) At this stage, the expression of BRP is almost undetectable, probably a consequence of the remodeling and/or degradation of the larval neuropils.

(F–K) ProNm of an pupal VNC with astrocytes (F–H) or EG (I–K) expressing a membrane-tagged GFP under the control of alrm-Gal4 and R56F03-Gal4, respectively, and co-stained with anti-Repo (blue) and anti-NCad (red) (F, G, I, and J), anti-BRP (blue), and anti-Dll (red) (H and K). (F and I) Processes of astrocytes (arrow) and EG (arrowhead) are first observed invading the neuropil.

(L) Average number of NG at different stages (number of samples analyzed at each stage = 4) with schematics of transverse sections of T1 or ProNm. NG (green), larval neuropils (blue), and leg neuropils (red) are indicated. Error bars indicate SD.

ATS, anterior transverse section; PTS, posterior transverse section; APF, after pupa formation.
In addition, due to the limitations of labeling recently born cells, they may be immature glia or neurons. As development proceeds, the cells close to the NGB are either unlabeled or Elav+ neurons, suggesting that starting at mid L3 (~96 hr AEL) the Lin A stem cell produces only neurons (Figures 5G–5K). Although the number of Repo+ glia increases over time, they are far from the NGB, consistent with the idea (and supported by the pH3 staining shown in Figure 2D) that early born glia continue to divide. We also note that as development proceeds, the Repo+ glia appear to migrate, such that by late L3 they surround the

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**Figure 4. NGBs Produce Leg Motor Neurons and All Thoracic NG**

(A and B) Thoracic segments of an L3 VNC labeled with anti-BRP (blue) and anti-Dll (red) containing MARCM clones expressing mCD8::GFP under the control of both VGlut-Gal4 and repo-Gal4. Lin A, Lin ?, and Lin D clones produce motor neurons (arrowhead) and glia (arrow). (A1–A5) Sample with Lin A, Lin B and Lin ? clones; note that Lin B does not produce glia. (B1–B5) Sample with a Lin D clone. Asterisks mark motor neuron axons exiting the VNC.

(C–E) Lin A (C1–C3), Lin ? (D1–D3) and Lin D (E1–E3) cis2 MARCM clones in a ProN labeled with anti-BRP (blue) (C1–C2, D1–D2, E1–E2) and in a T1 Leg (C3, D3, E3) expressing mCD8::RFP in NG (red) and mCD8::GFP (green) under the control of repo-Gal4 and VGlut-QF, respectively.

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immature adult neuropil. These results suggest that the Lin A stem cell initially produces mitotically active glioblasts and a small number of motor neurons, while at later stages it transitions to a NB that only produces neurons.

To complement these lineage tracing experiments, we used the QMARCM/MARC M twin spot system (Potter et al., 2010; see STAR Methods for details) in which both daughter lineages from a single cell division are permanently labeled with RFP and GFP, respectively. In these experiments, we induced clones during L1 to capture the first post-embryonic division and labeled motor neurons with GFP, allowing us to recognize Lin A due to the stereotyped morphology of Lin A dendrites. Sister progeny were marked with tub>RFP to label all cell types. Remarkably, in twin clones where GFP labels Lin A motor neurons, the RFP progeny are composed of Repo⁺ NG and a single motor neuron that targets a body wall muscle at the base of the coxa (Figures 5L–5M), the same motor neuron that was observed in our cis² MARCM experiments (Figure 4D). This motor neuron has also been described previously to be part of Lin A (Brierley et al., 2012). Similar results were obtained when Lin A QMARCM/MARC M clones were generated in L2 or early L3, consistent with the timing inferred from our lineage tracing experiments (data not shown).

Taken together, these results suggest that the first post-embryonic division of the Lin A NBG results in two sub-lineages (Figure 5N): one, which we refer to as Lin A.1, generates a glioblast and a single post-mitotic motor neuron, and a second (Lin A.2) generates at least one additional glioblast and the remaining 28 Lin A motor neurons. To our knowledge, this mode of division, in which a NGB generates an intermediate mother cell (IMC) that divides to produce a post-mitotic motor neuron and a glioblast, has not been observed previously (Figure 5N).

**Lin A.1 and A.2 Generate Both Astrocytes and EG**

To determine which types of NG are produced by Lin A and Lin D, we repeated the cis²-MARC M experiments with airm-Gal4 or R56FO3-Gal4 (instead of repo-Gal4), to label astrocytes and EG, respectively (Table S2). We found that Lin A.1 (n = 33) produces both types of NG, while Lin D (n = 4) only generates astrocytes (Figures 6A–6I; Movie S5; Table S2). Lin A.2 also produces both types of NG (Figures 6A–6I).

To determine whether Lin A and Lin D generate only motor neurons and NG, we repeated the MARCM experiments using a tubulin driver (tub-Gal4), to label all cell types. We stained the resulting L3 larvae with Elav, Dpn, and Repo (Figures 6J–60). The results confirmed that Lin A.2 generates motor neurons and NG. Lin A.2 clones also usually included two cells that did not stain for either Elav or Repo. Because of their proximity to the NB, they are likely to be GMCs (Figures 6J and 6M; data not shown). All non-Lin A.2 clones (n = 4) that generated Repo⁺ NG labeled only 1 (Lin A.1) or 2 (Lin D) Elav⁺ neurons (Figure 6K).

Together with our earlier experiments, these results suggest that, post-embryogenesis, Lin A and Lin D give rise to only leg motor neurons and NG. Notably, at late L3, a Dpn⁺ NB remains associated with Lin A, but not with Lin D.

We conclude that in each thoracic hemisegment, all of the adult NG are generated from only 2 NGBs, which also give rise only to leg motor neurons.

**Unlike Motor Neurons, Individual NG Born from the Same Lineage Are Not Stereotyped**

Previous studies have revealed that each of the 47 leg motor neurons have a specific birth date and morphology, characterized by their axonal targeting, dendritic arbor, and cell body position (Baek and Mann, 2009; Brierley et al., 2012). We recently characterized Lin B, a lineage that produces only 7 motor neurons, and described a post-mitotic code of mTFs that controls individual motor neuron morphologies (Enriquez et al., 2015). We identified these TFs by an expression screen using ~250 antibodies, yet, surprisingly, none of these TFs are differentially expressed in subsets of thoracic NG (data not shown). Instead, we found TFs, such as Dil, expressed in all NG. The one exception is Prospero (Pros), which is expressed in all astrocytes but not EG (Griffiths and Hidalgo, 2004; Peco et al., 2016) (Figure S1). If no single-cell TF code exists for NG, it follows that the number and morphology of the NG progeny from the same lineage may...
differ from animal to animal. Below we used the cis²-MARCM technique to test this prediction (Figures 7A–7R; Table S3). Lin A.2, D and A.1 give rise on average to 79 (n = 10), 57 (n = 4), and 33 (n = 13) astrocytes, respectively, and with a very high SD, suggesting that each lineage has the potential to generate a variable number of NG (Figure S5). We also find that the final position of NG progeny can vary from animal to animal: for each of the three lineages born in a T1 hemisegment, astrocyte processes mostly invade the ProNp (Prothoracic Neuropil) but, depending on the sample, can also send processes to the Accessory Meso-thoracic Neuropil (AMesoNp), where wing motor neuron dendrites innervate, the mesothoracic neuropil (MesoNp), and occasionally cross the mid-line to populate the contralateral neuropil (Figures 7A–7L). Similarly, although most T1-born astrocyte cell bodies remain in the ProNp (Prothoracic Neuromere), they can also end up in the Accessory Mesothoracic Neuromere (AMesoNm) and occasionally in the Mesothoracic Neuromere (MesoNm) (Figures 7A–7L, data not shown for Lin D, Table S3). Lin A astrocytes born in a T2 and T3 hemisegment have the same ability to populate neighboring neuromeres (Figure S5). Thus, unlike the highly stereotyped morphology of motor neurons born from the same lineage (Figures 7M–7R), the number, final cell body position, and neuropil regions invaded by astrocyte processes born from individual NGBs are variable from animal to animal (Figures 7S and S5; Table S3).

Although we were unable to count the number of EG produced by Lin A.1 (see STAR Methods), Lin A.2 generates ~114 EG (n = 4) with a low SD (±6), suggesting that the number of EG produced by Lin A is similar in different animals. However, as with astrocytes, the neuropil region surrounded and invaded by Lin A EG is variable from animal to animal, and these glia can populate neighboring neuromeres and cross the midline (Figures 7G and S5; Table S3). In summary, an individual NGB produces a variable number of NG with different morphologies and final locations, but the same number of motor neurons, each with a stereotyped morphology.

**Final Astrocyte Number Depends on Inter-lineage Competition**

Although the number of NG produced by a single NGB (e.g., Lin A) can be highly variable, the total number of NG produced by all lineages is very constant from animal to animal (Figures 1 and S5). In addition, the non-stereotyped morphology and final positions of NG derived from single NGBs led us to hypothesize that NG may compete during development to ensure that each neuropil is fully wrapped and innervated. We tested this hypothesis for astrocytes, by generating Dil mutant Lin A.2 MARCM clones, which results in a severely compromised number of astrocytes born from this lineage without affecting motor neuron number or morphology (Figures 7W and S6). Dil⁺ Lin A.2 clones were generated in a background where all astrocytes were labeled, thus allowing us to assess whether other lineages could compensate for the fewer number of Lin A.2-derived astrocytes. We considered three possible outcomes (Figures 7S and 7T): (1) no compensation, in which WT (non-Lin A.2) astrocytes do not respond to the reduced number of astrocytes derived from Lin A; (2) compensation where WT astrocytes respond to the lower number of Lin A.2 astrocytes by increasing the number or extent of processes invading the neuropil; and (3) compensation where WT lineages generate more astrocytes to maintain the total final number.

To distinguish between these possibilities, we generated WT or Dil⁻ mutant Lin A MARCM clones expressing mCD8::GFP under the control of repo-Gal4 and VGut-Gal4. In this genetic background, we labeled all astrocytes with a cytoplasmic QUAS-mCherry under the control of alm-QF. As a result, astrocytes produced by Lin A are labeled by mCherry and mCD8::GFP while astrocytes produced by other lineages express only mCherry. Astrocytes were counted in a ProNp containing a single Lin A MARCM clone and in the contralateral neuromere that had no clones, which served as an internal WT control. Although Dil mutant Lin A clones (n = 4) produced on average ~18 astrocytes (compared to ~80 for WT Lin A clones), the total number of astrocytes in these neuromeres was normal (~140) (Figures 7U–7X). Altogether, these results suggest that Lin D and Lin A.1 compensate for the reduced number of astrocytes produced by Lin A.2.

To determine when during development NG compensation is likely to occur, we generated Dil⁻ Lin A.2 MARCM clones (n = 4) and counted the number of Dil⁻ Repo⁺ cells in late L3 larva. We found that the number was similar to the number in WT clones (~20) (Figure S6), arguing that the absence of Dil does not affect the generation of astrocytes until the pupal stage, when NG number continue to increase (Figure 3L). It follows that compensation from WT lineages also occurs during this pupal amplification phase, long after the glioblasts are born.

These results suggest that the production of NG is extremely plastic during development, and that their progenitors continue to divide until the entire neuropil is occupied.

**DISCUSSION**

**The Logic of Lineages Producing Multiple, Functionally Related Cell Types**

Here, we show that two NGBs per thoracic larval hemisegment give rise to leg motor neurons, astrocytes, and EG. All three of
Please cite this article in press as: Enriquez et al., Differing Strategies Despite Shared Lineages of Motor Neurons and Glia to Achieve Robust Development of an Adult Neuropil in Drosophila, Neuron (2018), https://doi.org/10.1016/j.neuron.2018.01.007
these cell types are key components of each thoracic neuromere: motor neurons extend axons to innervate leg muscles and elaborate complex dendritic arbors inside the neuropil, EG wrap the developing neuropil and incoming sensory axons, and astrocytes send processes inside the neuropil where they associate with synapses between motor neuron dendrites, interneurons, and sensory neurons. Together, these cells develop in a coordinated manner to form a complex functional unit that comprises the neural circuitry used for many adult behaviors such as walking.

Glia cells are a major component of nervous systems, critical not only for the activity of neurons, but also for their development (Freeman, 2015; Freeman and Rowner, 2013). Neurons and glia are always associated with each other and exist in all bilateria, even in primitive phyla such as flatworms (Hartline, 2011). As nervous system complexity increases, there is an increase in both neuronal diversity and in the diversity of glial types and morphologies (Paredes et al., 2016). Given this close relationship, it is striking that in the thorax of the adult fly all NG are derived from lineages that also—and only—give rise to leg motor neurons. In the absence of extensive cell migration, being born from the same stem cell results in an anatomical proximity that may be important for subsequent steps in development. In particular, we propose that the shared lineages of the leg motor neurons, astrocytes, and EG facilitates the assembly of anatomically complex neuromers and neural circuits. This idea helps explain why lineage relationships appear to play less of a role in simpler nervous systems such as in C. elegans. Consistent with the idea that anatomical proximity of functionally related cell types is important for building complex nervous systems, in mammals, astrocytes and oligodendrocytes are born from the same progenitor domains as motor neurons and interneurons (Ravanelli and Appel, 2015; Rowitch and Kriegstein, 2010). Although it is currently unclear whether these cell types are derived from the same lineages in vertebrates, our results provide a striking precedent and whether these cell types are derived from the same lineages and with a specific birth order, properties that are a consequence of the unique TF code that they express. At the other extreme, based on the hundreds of TFs we have surveyed, individual astrocytes or EG appear to share the same TF code as all other astrocytes or EG, respectively, and can end up with different morphologies in multiple positions within or even in neighboring neuromers. Yet, despite this plasticity, the end result—~280 NG evenly distributed throughout each thoracic leg neuropil—is highly stereotyped. Nevertheless, these two very different modes of achieving stereotypy—hardwired (for motor neurons) and plastic (for NG)—arise from the same stem cells. We also found that, although the total number of NG in each thoracic neuropil is stereotyped (~140 astrocytes and ~140 EG), the individual contributions from the two NGB lineages can vary from animal to animal. Moreover, when one lineage is compromised the others can compensate to maintain the correct number of total NG. Although the mechanism regulating final NG number is currently unknown, we suggest that it is analogous to what has been referred to as neutral competition in mammalian and Drosophila gut homeostasis (de Navascués et al., 2012; Snippert et al., 2010). It is possible that this competition occurs via direct communication between NG. This idea is supported by the observation that individual astrocytes and EG respect each other’s territory and exhibit tiling-like behavior. Alternatively, communication between motor neuron dendrites (or other components of the thoracic neuropil) and NG may be responsible for NG stereotypy. For example, astrocytes and EG may be able to sense and adapt to the scaffold generated by motor neuron dendrites in order to fully invade and wrap each thoracic neuropil. Accordingly, NG stereotypy, both cell number and morphology, would be an indirect consequence of the mTF codes that generate the unique morphologies of motor neurons and likely other neurons.

What might be the reason for the existence of such different strategies to achieve stereotypy? One answer may be...
developmental robustness, which is especially challenging for establishing neural circuits. While motor neurons need to make precise connections (both in the CNS and in the legs), and therefore may require precise TF codes to achieve this precision, our results suggest that astrocytes and EG may have a more generic role in neuropil development. Nevertheless, NG are likely to be just as critical for neuropil development and function. However, instead of specifying each glia on a single-cell basis, the system has evolved a different strategy to achieve robustness: a generic TF code, but the ability to communicate with each other to ensure that the entire neuropil is appropriately and evenly populated by the correct number of NG. There are several potential advantages to such a strategy. One is that as the number of motor neurons or size and complexity of nervous systems vary during evolution, the number of NG can readily adapt in response. Second, NG plasticity may be crucial during normal development to adjust to natural variations in neuronal morphology. For example, in the antennal lobe of Drosophila the morphology and connectivity of local interneurons can vary between animals and may require NG to respond accordingly (Chou et al., 2010). Finally, NG may also play an important role in maintaining axons and in monitoring and pruning synapses to ensure the proper number and type of synaptic connections, as they do in invertebrates (O’Connor et al., 2017) and vertebrates (Risher et al., 2014), respectively, during normal development and after injury (Burd et al., 2016; He and Jin, 2016; Stephan et al., 2012). Glial developmental plasticity may be essential to achieve robustness and maintain homeostasis by readily adapting to these and perhaps other variations in nervous system size and morphology.

ACKNOWLEDGMENTS

We thank members of the Mann lab for comments and suggestions and Jeremy Dasen, Oliver Hobert, Laura Johnston, Emilie Peco, and Alain Vincent for comments on the manuscript. This work was supported by the Ellison Medical Foundation grant AG-SS-2945-12 and NIH grant NS070644 to R.S.M. and funding from the ALS Association (#256) and FRM (#AJE20170537445) to J.E.

AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


## Key Resources Table

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Richard Mann (rsm10@columbia.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All in vivo experiments were carried out using standard laboratory strains of D. melanogaster.

With the exception of R56F03-Gal4, UAS-CD4-tdGFP recombinant (Peco et al., 2016), all genotypes used to make recombinants and fly stocks are listed on the REAGENT or RESOURCE sheet.

Unless otherwise noted, fly stocks were obtained from the Bloomington Stock Center: alrm-Gal4-III, UAS-mCD8::GFP-III, UAS-mCD8::GFP-II, UAS-H2B::RFP-III (Mayer et al., 2005), UAS-FB1.1-II, R56F03-Gal4; UAS-GFP::CD8::HRP (Alexandre et al., 2014); R56F03-Gal4, UAS-CD4-tdGFP recombinant (Peco et al., 2016); UAS-mFlp5 (Enriquez et al., 2015); dll-Gal4, R31F10-Gal4, w hs-Flp1.22, FRT82B tub-Gal80 and FRT19A (Gary Struhl), tub-QS#0, FRT19A, Mhc-RFP, VGlut-Gal4 (also called OK371-Gal4), tub-QS, repo-Gal4, 10C12-Gal4, UAS-mCD8::RFP, VGlut-QF (this work), Dil-(Dil[SA1]) (Cohen and Jurgens, 1989), tub-Gal4, QUAS-mCD8::GFP/TM6b, QUAS-mCD8::GFP, QUAS-mcherry-III, UAS-mcherry(attP2 insertion) BDSC RRID:BDSC_52268, UAS-mcherry(attP2 insertion) BDSC RRID:BDSC_52270, alrm-QF (3rd chromosome) BDSC RRID:BDSC_66464, elav-Gal80 Rideout et al., 2010 N/A, cha-Gal80 Kitamoto, 2002 N/A, dpn > KDRT > Cre/+; act > LoxP > LexA, LexA-myrc::GFP/+; UAS-Kd/+ Awasaki et al., 2014 N/A, Recombinant DNA pCR8/GW/TOPO/VGlut vector Enriquez et al., 2015 N/A, pattb-QF-hsp70 vector Addgene cat# 24368, pattb-QF-VGlut-hsp70 vector This work N/A, Software and Algorithms Amira 3D software version 6.2 https://www.fei.com/, ImageJ version 1.48 https://imagej.nih.gov/ij/, Microsoft Excel version 2016 https://products.office.com/en-US/, Microsoft Power Point version 2016 https://products.office.com/en-US/
METHOD DETAILS

Immunostaining of L3 Larva
Inverted L3 larvae were fixed in 4% formaldehyde with PBS for 20 minutes. L3 larval CNS were dissected in PBS triton and incubated with primary antibodies for two days and secondary antibodies for one day at 4°C. Fresh PBT (PBS with 0.1% Triton X-100, 0.3%, 1% BSA) was used for the blocking, incubation, and washing steps: five times for 20 minutes at room temperature after fixation and after primary/secondary antibodies. L3 CNS mounted onto glass slides using Vectashield mounting medium (Vector Labs).

Immunostaining of adult VNC
After removing the abdominal and head segments, the thoraces of the flies were opened and fixed in 4% formaldehyde with PBS for 20 minutes at room temperature and blocked in the blocking buffer for one hour. After dissection, adult VNC were incubated with primary antibodies for two days and secondary antibodies for one day at 4°C. Fresh PBT (PBS with 0.1% Triton X-100, 1% BSA) was used for the blocking, incubation, and washing steps: five times for 20 minutes at room temperature after fixation and after primary/secondary antibodies. VNCs were dissected and mounted onto glass slides using Vectashield mounting medium (Vector Labs).

Primary and secondary antibodies
Unless otherwise noted primary antibodies were obtained from Hybridoma Bank: mouse anti-BRP, mouse anti-repo, rat anti-Elav, mouse anti-Nrg (neuronal specific form), mouse anti-Pros, rat anti-NCad, guinea-pig anti-Dll (Estella and Mann, 2008), guinea-pig anti-Dpn (gift from Jim Skeath). Secondary antibodies: Alexa 555, 647 and Alexa 405 (Invitrogen and Abcam).

Time course of NG development
We expressed a membrane reporter (UASmCD8::GFP) with Dll-Gal4 and dissected larva and pupae at different development time points (Figure 3; Movie S3). In order to specifically visualize the immature leg neuropil, larval CNS were co-stained with anti-BRP (which labels mature neuropils) and anti-N-Cadherin (NCad, which labels mature and immature neuropils at high and low levels, respectively [Kurusu et al., 2012] (Figures 3A–3E). Dll-Gal4 is also expressed in adult leg sensory neurons, which allowed us to visualize where sensory axons first enter the CNS and was used as a reference point in these developing CNS. We also stained larval CNS for Dll and BRP or Repo and Dll in order to quantify the number of glia at each development stage (Figures 3F–3J and data not shown). Finally, we used alrm-Gal4 and Rs5FO3-Gal4 to drive the expression of mCD8::GFP to determine when EG and astrocytes morphologically differentiate.

EM protocol
After removal of abdominal and head segments, the thorax of the flies was opened and fixed in 4% glutaraldehyde, washed 5 times quickly in PBS, incubated in PBS with 0.03% Triton X-100 for 30 mins and washed 5 times over 20 mins in PBS. To specifically recognize the EG in EM we expressed UAS-GFP::mCD8::HRP (HRP: horseradish peroxidase) and DAB (3,3'-Diaminobenzidine) was added to catalyze the HRP to form an electron dense product. HRP expression was revealed overnight using the DAB substrate kit (Thermofisher).

In an EM microwave (Pelco model 3451 system with coldspot), specimens were post-fixed with 1% osmium tetroxide in 0.1M phosphate buffer (PB) for 2 × 40 s (with each 40 s exposure in fresh osmium), and then washed 3 × 10 minutes in 0.1M PB. Specimens were dehydrated in ethanol grades of 50%, 70%, 95% (1 × 40 s for each grade), and 100% (2 × 40 s). Dehydrated tissue was then infiltrated with epoxy resin (Electron Microscopy Sciences Embed 812) and 100% ethanol (1:1 mixture) for 15 minutes, and then in 100% epoxy resin (2 × 15 minutes with fresh resin each time).

Specimens were mounted between 2 plastic slides with 100% epoxy resin and polymerized overnight at 60°C. The next day the polymerized resin wafers were separated from the plastic slides and placed flat on a glass slide to choose specimens for thin sectioning. An entire specimen sample was then cut out of the wafer using a razor blade and carefully placed on its side in a Dykstra flat embedding mold with the protoracic neuromere (PN) end (region of interest) closest to the beveled tip in one of the cavities of the mold. Small slits were made at the tip and floor of the silicon mold to secure the perpendicularly oriented tissue. This orientation is used so that thin sections will be cut transversely through the specimen starting at the PN. The mold was then filled with 100% epoxy resin and polymerized for 18-24 hours at 60°C.

After polymerization, the block was removed from the cavity and trimmed down at the beveled tip in the shape of a 2mm square with the specimen near the center of the blockface. With an ultramicrotome, 10 micron thick sections were cut and collected serially using a diamond Histo-knife (Diatome) and then mounted and coverslipped on a glass slide with immersion oil. The 10 micron sections were then observed with a light microscope and sections with the best areas of interest are photographed. The coverslip was carefully removed and the sections of interest are washed in 95% ethanol to remove any oil by passing them through the ethanol in 3 wells for 15 s in each well. Each 10 micron section was then carefully placed on a piece of lens tissue to dry and then remounted on a tiny drop of epoxy resin on a blank polymerized Beem capsule resin block (blockface should be flat and smooth) and polymerized.
overnight at 60°C; a spring tension apparatus was used to keep the 10 micron section flat against a plastic slide. The polymerized blockface with the 10 micron section was trimmed down to size and 60 nanometer thin sections were cut with an ultramicrotome using a Diatome diamond knife. The ultrathin sections were collected onto formvar coated slot grids and then stained with uranyl acetate and lead citrate, and examined on a JEOL 1200EX electron microscope.

Fly genetics

Crosses or stocks used to visualize NG (Figure 1, 2, 3, S1, S3):

- UAS-mCD8::GFP-II, alrm-Gal4-III
- alrm-Gal4, UAS-FB1.1-III crossed with UAS-mFlp5-II
- alrm-Gal4, UAS-H2B::RFP-III
- UAS-mCD8::GFP-II, R56F03-Gal4-III
- R56F03, UAS-FB1.1-III crossed with UAS-mFlp5-II
- R56F03, UAS-H2B::RFP-III
- R56F03-Gal4, UAS-nGFP-III crossed with alrm-Gal4
- Dil-Gal4, UAS-mCD8::GFP-II

Lineage tracing systems (Figures 2 and S2):

- Elav-Gal80; Dil-Gal4, UAS-FB1.1-III crossed with UAS-mFlp5-II
- Elav-Gal80; Dll-Gal4, UAS-Flp1/cyo; cha-Gal80/MKRS crossed with act > cd2 > Gal4, UAS-nGFP-III
- R31F10-Gal4 crossed with UAS-mFlp5; UAS-FB1.1-/; act > CD2 > Gal4
- MARCM (Figures 4, 6, 7, and S6):
- y, w, hs-Flp1,22; FRT42 tub-gal80//; repo-Gal4, UAS-mCD8::GFP/MKRS crossed with
- y, w, hs-Flp1,22; VGlut-Gal4, UAS-mCD8::GFP, Mhc-RFP FRT42D//; repo-Gal4, UAS-mCD8::GFP/Tm6b or
- y, w, hs-Flp1,22; VGlut-Gal4, UAS-mCD8::GFP, Mhc-RFP FRT42D Dll-//; repo-Gal4, UAS-mCD8::GFP/Tm6b
- y, w, hs-Flp1,22; tub-Gal4; sp/cyo; FRT82B tub-Gal80 crossed with
- y, w, hs-Flp1,22; UAS-mCD8::GFP//; FRT82B//
- y, w, hs-Flp1,22; FRT42D tub-Gal80 //; alrm-QF, QUAS-mcherry/MKRS crossed with
- y, w, hs-Flp1,22; VGlut-Gal4, UAS-mCD8::GFP, Mhc-RFP FRT42D Dll-//; repo-Gal4, UAS-mCD8::GFP/Tm6b or
- y, w, hs-Flp1,22; VGlut-Gal4, UAS-mCD8::GFP, Mhc-RFP FRT42D //; repo-Gal4, UAS-mCD8::GFP/Tm6b

cis^2 MARCM (Figures 4 and 6):

- y, w, hs-Flp1,22; FRT42 tub-gal80//; tub-QS/cyo; repo-Gal4, UAS-mCD8::GFP/TM6b or
- y, w, hs-Flp1,22; FRT42 tub-gal80, tub-QS/cyo; R56F03-Gal4, UAS-mCD8::GFP/TM6b or
- y, w, hs-Flp1,22; FRT42 tub-gal80, tub-QS/cyo; alrm-Gal4, UAS-mCherry/TM6b or
- y, w, hs-Flp1,22; FRT42 tub-gal80, tub-QS/cyo; repo-Gal4, UAS-H2B::RFP, UAS-mCD8::GFP/TM6b or
- y, w, hs-Flp1,22; FRT42 tub-gal80, tub-QS/cyo; R56F03-Gal4, UAS-H2B::RFP/TM6b or
- y, w, hs-Flp1,22; QMARC/MARM (Figure 5):
- y, w, hs-Flp1,22, tub-Gal80, FRT19A; QUAS-mCD8::GFP, UAS-mCD8::RFP/Cyo; tub-Gal4, QUAS-mCD8::GFP/TM6b crossed with
- tub-QS80, FRT19A; QUAS-mCD8::GFP, UAS-mCD8::RFP/Cyo; DVGlut-QF::hsp70term (attp86F)/MKRS

Note: MARCM/MARC clones where generated by heat shocks of first instar larva.

Lin A tracing system (Figure 5; Lacin and Truman, 2016)

10C12-Gal4 crossed with

dpn KDRT > Stop > KDRT Cre//; act LoxP > Stop > LoxP-LexA, LexA-yr::GFP//; UAS-Kd/

Cloning

pattb-VGlut-QF-hsp70. A SpeI-VGlut-Spe1 fragment containing the 5.9 kb sequence upstream of the VGlut translation start site was taken from a pCR8/GW/TOPO (Enriquez et al., 2015) vector and clone in a pattb-QF-hsp70 vector (addgene).
The resulting pattb-VGlut-QF-hsp70 construct was inserted in position 86f on chromosome III by injection into embryos carrying attP-86f landing site.

**Leg imaging**
See also Figure 4. Legs were dissected and fixed overnight at 4°C, washed five times for 20 minutes at room temperature in PBS with Triton X-100 (0.3%) and mounted onto glass slides using Vectashield mounting medium (Vector Labs). Due to their large size, final leg images may be a composite of more than one image.

**Microscopy and 2D Imaging**
Multiple 1-μm-thick sections in the z axis (dorsoventral for L3 CNS or adult VNC and mediolateral for adult legs) were imaged with a Leica TCS SP5 II or a Zeiss LSM 780 confocal microscope. Binary images for z stack images were generated using NIH ImageJ and Photoshop (Adobe Systems).

**3D Leg analysis**
See also Figure 4. Z stacks were generated using NIH ImageJ. Specific cuticle background was generated using the Argon laser 488 and by placing the confocal detector out of the range of the GFP emission wavelength. This cuticle background of the leg was used to remove the nonspecific signal from the GFP channel using ImageJ. Z stacks were used to generate 3D reconstructions of legs with Amira 3D software. The color codes used were Volrengreen to visualize mCD8::GFP, color used for cuticles was gray.

**3D images of adult VNC and larval CNS**
Ventral to dorsal Z stacks were generated using NIH ImageJ, and were used to generate 3D reconstructions using the Volren or volume rendering in Amira 3D software. The color codes used were constant color in correlation with the fluorescent proteins or the secondary Alexa used.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
All bar plots were generated with Microsoft Excel. All bar errors represent standard deviation of a minimum of 4 samples. The total number of samples used for each genotype is indicated in the text and/or the figures legends.
All Lin A cell plots were generated with Microsoft Excel. All bar errors represent standard deviation of a minimum of 7 samples. The total number of samples used for each genotype is indicated in the Figure 5 legend. The spatial coordinates were assigned to each cell using the cell counter plug-in of NIH ImageJ software. In Figure 5 (D2-J3) the coordinates of each cell were normalized with Microsoft Excel in order to have the position of Lin A NB at the origin of the plot graph.