The development and assembly of the *Drosophila* adult ventral nerve cord

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In order to generate complex motor outputs, the nervous system integrates multiple sources of sensory information that ultimately controls motor neurons to generate coordinated movements. The neural circuits that integrate higher order commands from the brain and generate motor outputs are located in the nerve cord of the central nervous system. Recently, genetic access to distinct functional subtypes that make up the *Drosophila* adult ventral nerve cord has significantly begun to advance our understanding of the structural organization and functions of the neural circuits coordinating motor outputs. Moreover, lineage-tracing and genetic intersection tools have been instrumental in deciphering the developmental mechanisms that generate and assemble the functional units of the adult nerve cord. Together, the *Drosophila* adult ventral nerve cord is emerging as a powerful system to understand the development and function of neural circuits that are responsible for coordinating complex motor outputs.

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Introduction

The central nervous systems of most bilaterian animals can be divided into two major components, an anterior brain and a more posterior nerve cord. From among its most primitive forms in annelids, to the complex spinal cord found in vertebrates, the primary function of the nerve cord is to integrate and process information from the brain to produce coordinated locomotor outputs by controlling muscle activities in the periphery. Understanding the development and assembly of circuits in the nerve cord is, therefore, crucial for understanding how animals respond to their environments by executing motor outputs.

In order to carry out these functions, the nerve cord is composed of a large number of neurons that can be classified according to their function and morphology. These include local interneurons that modulate and generate rhythmic motor patterns, ascending and descending neurons that relay information to and from the brain, and motor neurons that synapse onto muscles and are directly responsible for causing muscle contractions and movements. In addition, the nerve cord receives numerous inputs from peripheral sensory neurons. As will be highlighted below, these populations can be further subdivided based on their specific functions and anatomy.

To assemble complex neural circuits, biological systems employ a range of developmental mechanisms that regulate and specify the number, birth order and unique identities of the component neurons. To link the developmental origins of neural circuits with their functional outputs, it is advantageous to use a model where sophisticated tools are available for both types of analyses. The *Drosophila* model system is well renowned for the range of genetic tools available to dissect the development and functions of individual and small groups of neurons [1–5]. Moreover, adult *Drosophila* execute a large range of complex motor outputs using multi-jointed legs for walking and grooming, and wings and halteres for flying. Additional behaviors that use these appendages are courtship by males and aggression between individuals. Notably, rudimentary forms of some motor programs, including walking, can be executed in the absence of a brain, indicating the autonomy of the circuits in the nerve cord for executing rhythmic motor outputs [6••,7]. However, intact adult *Drosophila* simultaneously integrate visual, chemosensory, and proprioceptive inputs while executing these motor behaviors [8]. The combination of behavioral complexity, plasticity, and abundance of genetic tools sets this system apart as an exceptionally powerful model to understand the development and function of neural circuits.

In *Drosophila*, most of the neurons that make up the nerve cord, known as the ventral nerve cord (VNC) due to its ventral position, are born post-embryonically in the larval stages in an immature form [9]. Because the larval and adult stages execute significantly different behaviors, the

Figure 1
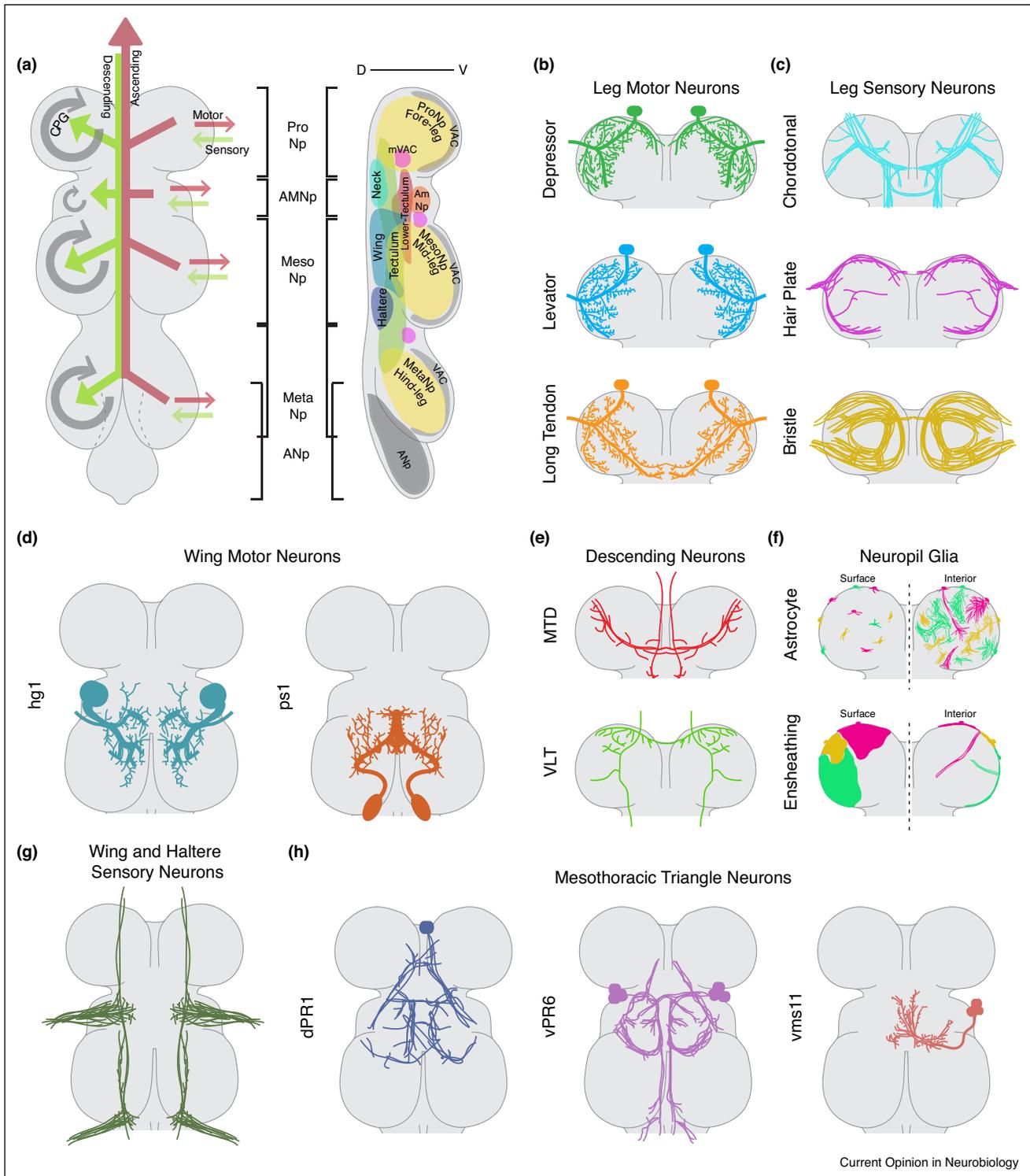
Structural and functional organization of the *Drosophila* adult VNC.
(a) Frontal (left) and lateral (right) views of the *Drosophila* adult VNC depicting five major neuropils — prothoracic, mesothoracic and metathoracic neuropils corresponding to the three thoracic segments and the accessory mesothoracic neuropil (AMNp), and abdominal neuropil (ANp). For nomenclature refer to Ref. [19].
Left — Neuronal subtypes projecting in and out of the *Drosophila* adult VNC with information flow depicted in the form of arrows. Motor and ascending neurons project axons out of the VNC (red arrows); sensory and descending neurons project into the VNC (green arrows); and Central Pattern Generator (CPG) neurons are local interneurons contained within the VNC (grey arrows).

nervous system undergoes a striking transformation during metamorphosis during which adult-specific neural circuits develop into their mature forms. In comparison to the adult brain of *Drosophila* [10–14] much less is known about the functional organization and development of distinct adult VNC circuits. In this review we highlight recent studies that begin to describe the diverse population of cell types and neuropils that make up the *Drosophila* adult VNC together with the specific tools and techniques that enable a more thorough understanding of their developmental origins and functional contributions to various motor outputs.

Structural and functional organization of the *Drosophila* adult VNC

In order to understand how distinct neurons find their place in a functional circuit, it is crucial to characterize the anatomy and organization of the nervous tissue. This involves identifying specific landmarks, such as groups of neuronal cell bodies, neuronal tracts and commissures, anatomically distinct neuropils, and the organization of non-neuronal glial cells, which contribute to the assembly, growth, and homeostasis of nervous tissue [15,16].

In the insect nerve cord, neuronal cell bodies are located in an outer cortex, while their projections converge into densely packed neuropils that can be recognized by staining for Bruchpilot and N-cadherin, two well-established markers of mature synapses [17,18]. Further, tightly fasciculated nerve bundles enter and exit specific neuropils through distinct tracts and commissures, which are clearly identified by anti-Tubulin and anti-Futsch staining. Notably, the organization of the *Drosophila* adult VNC differs dramatically from its larval counterpart. While the larval VNC is organized as repeating neuropils in each of the thoracic and abdominal segments, the adult VNC is dominated by three major thoracic neuropils, corresponding to the three thoracic segments (prothoracic, mesothoracic and metathoracic), and a fused posterior abdominal ganglia [18,19] (Figure 1a). Shepherd *et al.* used a developmentally-based 'hemilineage' approach to define the anatomical framework of the entire adult VNC

during the course of metamorphosis [20]. Specifically, most adult VNC neurons arise during post-embryonic neurogenesis from 25 distinct neuroblast (NB) progenitors in each thoracic neuromere, and each lineage gives rise to both Notch-ON and Notch-OFF hemilineages, some of which undergo programed cell death [9,21]. Of the 33 hemilineages that survive apoptosis, each extends primary neurites into specific tracts with stereotyped points of entry into an immature neuropil. These tracts, readily discerned by Neuroglian staining, remain tightly fasciculated and largely intact throughout metamorphosis, thereby providing a consistent reference for neuropil organization. For example, certain lineage tracts define the boundaries of two smaller neuropils — the accessory mesothoracic neuropil (AmNp), which receives wing sensory afferents, and a dorsal compartment called the tectulum, which consists of intersegmental projecting hemilineages (Figure 1a). Interestingly, this hemilineage-based organization of VNC anatomy is likely to be functionally relevant as distinct hemilineages represent functional 'modules' that contribute to specific motor outputs [6••] (Table 2).

According to the detailed description of the anatomy of the adult VNC and associated nerves, the *Drosophila* adult VNC is currently viewed as containing sixteen distinct neuropils [22••] (Figure 1a, Table 1). The definition of these neuropils and their anatomical boundaries has depended on multiple complementary efforts to characterize the function and development of the diverse populations of cell-types that project into the VNC (Figure 1b–h). These include, but are not restricted to, the motor neurons (MNs) controlling movements of the legs, wings, and neck [23••,24,25] (Figure 1b,d); sensory neurons (SNs) such as the proprioceptive chordotonal organ, mechanosensory and chemosensory neurons from the legs, wings and halteres [26,27••,28,29] (Figure 1c,g); descending neurons (DNs) that bring command-like information from the brain [30••,22••,31] (Figure 1e); ascending neurons that relay somatosensory information back to the brain [32]; the mesothoracic triangle neurons that are CPG-like neurons responsible for generating

Right — Spatial boundaries of the sixteen distinct neuropils that make up the *Drosophila* adult VNC (adapted from Namiki *et al.* [22••]). For the complete list of neuropils and associated neuronal hemilineages refer to Table 1.

(b) Dendritic projections of a subset of prothoracic leg motor neurons subtypes in the prothoracic VNC segment, including depressor, levator and long tendon muscle targeting motor neurons [57,24,25].

(c) Axonal projections of a subset of sensory neuron subtypes projecting into the prothoracic VNC segment from the corresponding legs, including those from the chordotonal organs, hair plates and bristles [27••].

(d) Dendritic projections of selected wing motor neuron subtypes, hg1 and ps1 in the wing neuropil compartment of the VNC. Corresponding axon targets are in the fourth axiallary and pleurosternal muscles respectively [23••].

(e) Axon projections of a subset of leg neuropil targeting descending neurons in the prothoracic VNC segment, belonging to two distinct cervical connective tracts — the median tract of dorsal cervical fasciculus (MTD; also known as the dorsal medial tract (DMT)) and the ventral lateral tract (VLT) [22••].

(f) Structural organisation of astrocyte and ensheathing neuropil glia, both on the surface (left of midline) and interior (right of midline), of the prothoracic VNC segment [35••].

(g) Axon projections of both wing and haltere sensory neurons in the haltere and accessory mesothoracic neuropil regions [26].

(h) Projections of local intersegmental CPG-like mesothoracic triangle neurons dPR1, vPR6 and vms11 in the VNC that control distinct features of wing extension and pulse song [33,34].

Table 1

Neuropils of the *Drosophila* adult VNC

Neuropil	Defining projections	Contributing hemilineages
Leg (Pro, Meso, Meta)	Leg MNs	15B, 24B, 1B, 3A, 9A,12B, 13A, 19A, 20A, 21A, 22A, 23B
Neck[a]	Neck MNs	2A
Wing[a]	Wing MNs	3B, 12A, 9B
Haltere[a]	Haltere SNs	8B
Tectulum	Commissural INs	2A, 6A, 6B, 7B, 8A, 3B, 11A/B, 12A, 18B, 19B
Lower tectulum[a]	Peripheral sensing INs	10B, 11A/B, 18B
Accessory mesothoracic	Wing SNs	12A ♂, 23B
Ventral Association Centers[a]	SNs	13B, 14A
Medial Ventral Association Centers[a]	SNs	N/A
Abdominal	N/A	N/A

Neuropils of the *Drosophila* adult VNC as denoted in Namiki *et al.* [22••], along with defining projections and contributing hemilineages as denoted in Shepherd *et al.* [20] and Harris *et al.* [6••].
Refer to Figure 1a for spatial boundaries of each neuropil.
[a] Hemilineages contributing to these neuropils were separately inferred from Harris *et al.* [6••].

male-specific courtship songs [33,34] (Figure 1h); and glia that wrap and ensheath cell bodies and projections in the VNC [35••,36] (Figure 1f).

Characterizing the morphologies of functionally distinct neurons, especially individual cells within each subtype, has been greatly facilitated by the generation of cell-specific markers that generally consist of transcriptional regulatory elements that drive expression of a downstream reporter in a spatially restricted manner [37–40,5]. Combinations of regulatory elements can be genetically intersected to generate more limited expression patterns in distinct cell types. For example, we now have genetic access to ~50% of ~100 distinct descending neuron subtypes, 100 distinct subtypes of sex-specific neurons, as well as individual pairs of wing MNs that innervate the flight muscles [22••,23••,34]. Another recent study described different subtypes of proprioceptive neurons that respond to distinct types of mechanical stimulation, such as vibration and joint angles [41••]. Together, these observations underscore the idea that the stereotyped anatomy of VNC neuropils, a consequence of VNC development, underlies its ability to produce distinct motor behaviors.

The ability to label and genetically manipulate small groups of neurons in the adult VNC with high resolution is also essential to map or 'register' neurons onto a standardized VNC [42••,43,17] and can eventually be used alongside a detailed connectome of the VNC derived from electron microscopy (EM) to assemble a more complete picture of the circuits making up the adult VNC. Such an EM connectome is well on its way for the fly brain [10,44] and one for the VNC will hopefully not be far behind.

Developmental logic of *Drosophila* adult VNC neurons and glia

During *Drosophila* embryogenesis the neuroepithelium gives rise to neural stem cells, neuroblasts (NBs), which first give rise to neurons that are involved in larval function and behavior (embryonic lineages). Most NBs then enter a quiescent phase, and reinitiate divisions during the early larval stages to give rise to adult-specific neurons (post-embryonic lineages) [45••,46,47]. These adult neurons project their neurites into the immature adult neuropils in the larval VNC (Figure 2a). Interestingly, the majority of post-embryonic progeny exhibit a coordinated switch from Chinmo to Broad-Complex (BrC) transcription factor (TF) expression at ~60 hours after larval hatching such that ~30% of the entire lineage consists of Chinmo expressing cells while the remaining ~70% express BrC genes [48] (Figure 2b). While Chinmo and BrC are known to regulate neuronal cell-fates in the mushroom body of the brain [49], the purpose of this coordinated switch has not been described in the adult VNC. Interestingly, the Chinmo to BrC switch correlates with a twofold decrease in cell-size across all thoracic post-embryonic lineages [48], which might be necessary to accommodate the large number of BrC expressing progeny. As mentioned above, in many lineages one of the two hemilineages undergoes Notch-dependent programmed cell death [9,21], suggesting that Notch helps regulate final cell number. The execution of such coordinated events must involve many molecular changes at the progenitor level, and indeed, the Chinmo to BrC transition is controlled by temporal TFs in the early VNC NBs [48] and has been linked to opposing gradients of RNA binding proteins in brain NBs [50••]. Similarly, Notch expression in progeny neurons is also determined by temporal patterning in the NBs [51]. Importantly, extrinsic cues such as ecdysone signaling also play an important role in coordinating temporal transitions in these NB progenitors [52,53,54••].

Apart from synchronizing the generation and assembly of neurons arising from multiple progenitors, developmental mechanisms must also contribute to the diversity of neuronal cell identities. In the adult fly VNC, additional

Figure 2
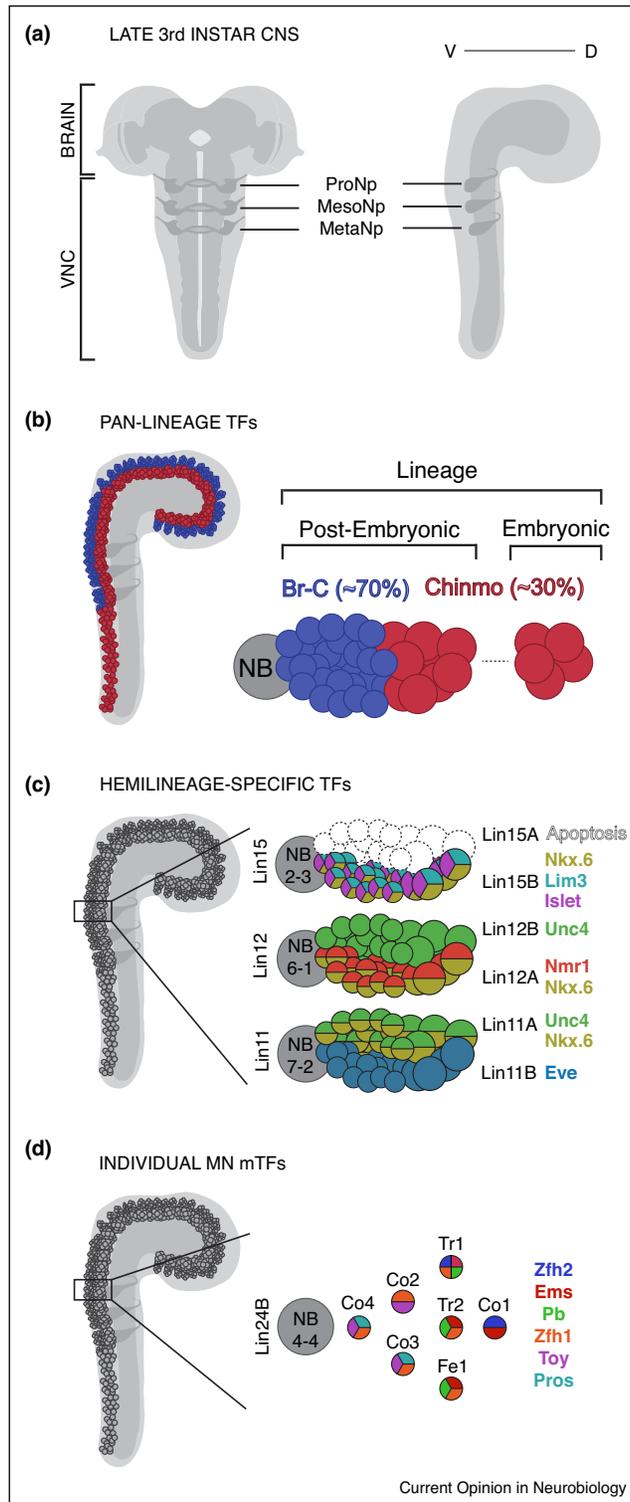
Transcription factor expression in post-embryonic VNC lineages.

(a) Immature adult thoracic VNC neuropils depicted in the late third instar larval CNS. Frontal (left) and lateral (right) views.

(b) Lateral view (left) of the third instar larval CNS depicting cell bodies in the cortex expressing Chinmo (red) and Broad-Complex (Br-C) (blue) TFs.

NB lineage (right) comprising embryonic and post-embryonic progeny; depicting proportions of Chinmo and Broad-Complex (Br-C) TF expression and corresponding to approximately a twofold decrease in cell size [48].

(c) Unique combinations of TF expression identify post-embryonic hemilineages of NB2-3 (Lin15A-B), NB6-1(Lin12A–B) and NB7-2(Lin11A–B).

Table 2

Hemilineage-specific TFs and corresponding motor outputs

Hemilineage	TF expression		Motor output
0A	En		N/A
0B	N/A		N/A
1A	Msh		Walking
1B	Nmr1		N/A
2A	Toy		Wing buzz, takeoff
3A	Nkx6, Nmr1		N/A
3B	Dbx		Change in posture
4A	N/A		N/A
4B	Nkx6, Lim3, Hb9		N/A
5B	Vg, Cut, Toy		Change in posture
6A			Uncoordinated leg movement
6B	Vg, Cut, En	Toy[a]	Uncoordinated leg movement
7B	Unc4		Wing buzz, takeoff
8A	Ey[b], Ems		Change in posture
8B	Lim3[b], Acj6		N/A
9A	Msh		Change in posture
9B	Lim3, Isl	Ems[a]	N/A
10B	Nkx6, Lim3, Hb9		Walking
11A	Nkx6, Unc4		Wing buzz, takeoff
11B	Eve[b]		Wing buzz, takeoff
12A	Unc4		Wing buzz, wing wave, walking
12B	Nkx6, Nmr1	Dbx[a]	Change in posture
13A	Dbx		N/A
13B	D, Vg		Change in posture
14A	Msh		N/A
14B	Lim3, Isl		N/A
15B	Nkx6, Isl, Lim3		N/A
16B	Lim3, Hb9		N/A
17A	Unc4, Isl		N/A
18B	Unc4		Wing buzz, takeoff, walking
19A	Dbx		Uncoordinated leg movement
19B	Unc4		N/A
20/22A			
20/22B	BarH		Change in posture
21A	Msh		Uncoordinated leg movement
21B		Ey[a]	N/A
23B	Unc4, Acj6		Change in posture, uncoordinated leg movement
24B	Ems, Toy, Nkx6		Repetitive leg movements

Post-embryonic hemilineages of the *Drosophila* adult VNC, along with corresponding TF expression patterns described in Lacin and Truman [45••] and Lacin *et al.* [55] and corresponding motor outputs upon hemilineage-specific activation described in Harris *et al.* [6••]. Hemilineages 2B, 5A, 7A, 10A, 15A, 16A, 17A, 18A, 23A, and 24A are not shown because they undergo apoptosis.

[a] Hemilineage-specific expression is unknown for these TFs.

[b] Personal communication H.Lacin, Truman Lab.

mechanisms likely define distinct sub-populations of post-mitotic neurons. For instance, although hemilineages are defined by Notch expression across all post-embryonic lineages, each hemilineage can also be uniquely identified by a combination of TFs [45,55] (Figure 2c, Table 2). More recently, multiple lineage-tracing approaches were used to identify the embryonic origins of each post-embryonic lineage [45••,1,46,3]. The tools generated to uniquely label each adult VNC NB lineage [45••], along with powerful clonal analyses like

Mosaic Analysis with a Repressible Marker (MARCM) [56], prove to be essential in understanding the developmental logic of distinct neuronal subtypes.

Using MARCM-based approaches, the major lineages that give rise to motor neurons (MNs) that innervate adult leg muscles have been characterized in detail, and reveal a stereotyped topographic organization of leg MN dendrites that correlate with their axon projections to distinct muscles in the adult legs [24,25]. Further,

(Figure 2 Legend Continued) Refer to Table 2. for the currently known list of hemilineage-specific TFs [45••,55].
(d) Individual leg MNs belonging to hemilineage 24B from NB4-4, express unique combinations of morphological TFs (mTFs) that control their dendritic projections and axon targeting morphologies [57].

the dendritic projections and axon targeting morphologies of individual leg MNs were shown to be controlled by unique combinations of TFs termed morphological TFs (mTFs) [57] (Figure 2d). Interestingly, while individual leg MNs can be identified by their unique morphologies, their dendritic innervation patterns tightly cluster based on axon targeting to one of four segments (coxa, trochanter, femur and tibia). Leg MNs also display stereotyped dendritic projection patterns based on the type of muscle being innervated irrespective of leg segment. For instance, prothoracic leg MNs innervating the long tendon muscles (present in both the tibia and femur) project some dendrites across the midline in the corresponding thoracic neuropil, several depressor targeting leg MNs extend projections into the antero-medial region of the neuropil, and levator and reductor targeting leg MNs tend to project their dendrites into the lateral compartments of the thoracic neuropils (Figure 1b). This topological organization suggests that dendritic projections within each leg neuropil are functionally compartmentalized to receive the correct inputs. Consistently, descending neurons that project into the leg neuropils also have highly stereotyped projections and use one of two tracts to project into more lateral or medial compartments [22**] (Figure 1e). Interestingly, one such descending neuron specifically triggers backward walking of the adult fly [58,59**], while other sets of descending neurons produce distinct behavioral responses when activated [30**,60,61**]. The ability to merge functional studies with neuronal activity measurements, which have recently become possible in the *Drosophila* VNC, will be essential to further test these hypotheses [62].

Apart from molecular mechanisms that define cell-diversity in post-mitotic progeny, other mechanisms controlling birth-order and modes of divisions also help diversify post-embryonic lineages. For instance one of the major leg MN lineages, Lin15B (also called LinA), exhibits a strong correlation between birth-order and proximal-distal axon targeting in the femur and tibia leg segments [24,25]. Notably, the Hox TF Antennapedia, which is strongly expressed in the Lin15 distal-targeting MNs is sufficient to increase axon targeting in the distal femur when ectopically expressed in the entire lineage [63] and Olig, another TF expressed in a subset of Lin15 MNs is important for proper axons targeting in the femur and tibia [64]. This suggests that the birth order of Lin15 MNs might be important in establishing the post-mitotic mTF code and is likely regulated by the mechanisms described at the progenitor level.

Interestingly, the Lin15 NB also gives rise to leg neuropil glia (Figure 1f) that remain closely associated with the Lin15 leg MNs, suggesting that the shared lineage between MNs and glia may help assemble complex neuropils containing multiple cell types [35**]. These glia also appear to play a direct role in guiding the growth of MN dendrites through Plexin/Semaphorin signaling [65]. Also noteworthy is that even though they come from the same NB lineage, the mode of division of the glial progenitors differs dramatically from those of the leg MNs, as the glia greatly increase in number during metamorphosis while the MNs do not. Moreover, unlike the stereotyped morphologies of the leg MNs, the astrocyte-like neuropil glia adopt varied morphologies but strictly 'tile' with one another in a non-overlapping fashion. In accordance to these differing developmental strategies, distinct mTF codes have not been identified in the neuropil glia, suggesting that such TF codes may only be required when unique and stereotyped individual cell-type morphologies are being generated.

Future directions
While the above studies have established a solid anatomical framework and some of the developmental mechanisms that operate in the VNC, many questions remain. For example, multiple distinct NB temporal windows, such as those observed in embryonic VNC NBs and the post-embryonic brain [66–68], have yet to be identified in the post-embryonic VNC and likely regulate birth order, number and diversity of NB progeny. Further, since many of the above studies have been conducted in lineages that generate leg MNs and neuropil glia, it is important to map each neuronal subtype to its specific hemilineage. Third, in order to identify and understand the mechanisms controlling neuronal cell diversity in post-embryonic lineages, it is essential to compare the entire transcriptome across hemilineages as well as between individual cells belonging to a single lineage, as has been demonstrated in the *Drosophila* brain and larval VNC [54**,69,70,71].

Conflict of interest statement
Authors declare no competing interests.

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