Developmental mechanisms required for presynaptic tiling and motor circuit assembly in Drosophila

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Abstract

The mechanisms specifying neuronal diversity are well-characterized, yet it remains unclear how or if these mechanisms regulate neural circuit assembly. Here we map the developmental origin of 156 interneurons from seven bilateral neural progenitors (neuroblasts), and identify each neuron within a synapse-level TEM reconstruction of the *Drosophila* larval CNS. We find that clonally-related neurons project to unique, broad regions of the neuropil. Remarkably, each lineage produces two hemilineages ($Notch^{ON}$ or $Notch^{OFF}$) that specifically innervate sensory or motor neuropil, and this choice is regulated by Notch signaling. Thus, individual neuroblasts coordinately assemble sensory and motor neuropils, one of the fundamental organizing principles in fly and mammalian neurogenesis. Furthermore, within each hemilineage, temporal cohorts tile the neuropil, and overlapping pre- and postsynaptic domains show enriched connectivity. Thus, progenitor identity, hemilineage, and temporal identity each contribute to assembling motor circuits. We propose that mechanisms generating neural diversity are also determinants of neural connectivity. 150

Introduction

Tremendous progress has been made in understanding the molecular mechanisms generating neuronal diversity in both vertebrate and invertebrate model systems. In mammals, spatial cues generate distinct pools of progenitors which generate a diversity of neurons and glia appropriate for each spatial domain (Sagner and Briscoe, 2019). The same process occurs in invertebrates like *Drosophila*, but with a smaller number of cells, and this process is particularly well-understood. Spatial patterning genes act combinatorially to establish single, unique progenitor (neuroblast) identity; these patterning genes include the dorsoventral columnar genes *vnd*, *ind*, *msh* (Isshiki et al., 1997; McDonald et al., 1998; Weiss et al., 1998) and the orthogonally expressed *wingless*, *hedgehog*, *gooseberry*, and *engrailed* genes (Chu-LaGraff and Doe, 1993; McDonald and Doe, 1997; Sen et al., 2019; Skeath et al., 1995; Zhang et al., 1994). These factors endow each neuroblast with a unique spatial identity, the first step in generating neuronal diversity (Figure 1A, left).

The second step is temporal patterning, or the specification of neuronal identity based on birth-order, which has been observed in flies and mice (Kohwi and Doe, 2013; Rossi et al., 2017). Here we focus on embryonic neuroblasts, which generate a series of ganglion mother cells (GMCs) which acquire a unique identity based on their birth-order, due to inheritance from the neuroblast of a "temporal transcription factor"– Hunchback (Hb), Krüppel (Kr), Pdm, and Castor (Cas) – which are sequentially expressed by nearly all embryonic neuroblasts (Isshiki et al., 2001). The combination of spatial and temporal factors leads to the production of a unique GMC at each neuroblast division (Figure 1A, middle).

The third step is hemilineage specification, which was initially characterized in *Drosophila* (Truman et al., 2010), but may also be used in vertebrate neurogenesis (Peng et al., 2007). Hemilineages are formed by the asymmetric division of each GMC within a lineage into a pair of post-mitotic neurons; during this division, the Notch inhibitor Numb (Nb) is partitioned into one neuron ($Notch^{OFF}$ neuron) whereas the other sibling neuron receives active Notch signaling ($Notch^{ON}$ neuron), thereby establishing $Notch^{ON}$ and $Notch^{OFF}$ hemilineages (Figure 1A, right). In summary, three developmental mechanisms generate neuronal diversity within the embryonic central nervous system (CNS): neuroblast spatial identity, GMC temporal identity, and neuronal hemilineage identity.

A great deal of progress has also been made in understanding neural circuit formation in both vertebrates and invertebrate model systems, revealing a multi-step mechanism. Neurons initially target their axons to broad regions (e.g. thalamus/cortex), followed by targeting to a neuropil domain (glomeruli/layer), and finally forming highly specific synapses within the targeted domain (reviewed in Kolodkin and Tessier-Lavigne, 2011).

Despite the progress in understanding the generation of neuronal diversity and the mechanisms governing axon guidance and neuropil targeting, how these two developmental processes are related remains mostly unknown. While it is accepted that the identity of a neuron is linked to its connectivity, the developmental mechanisms involved remain unclear. For example, do clonally-related neurons target similar regions of the neuropil due to the expression of similar guidance cues? Do temporal cohorts born at similar times show preferential connectivity? Are neurons expressing the same transcription factor preferentially interconnected? It may be that lineage, hemilineage, and temporal factors have independent roles in circuit formation; or that some mechanisms are used at different steps in circuit assembly; or that mechanisms used to generate neural diversity could be independent of those regulating circuit formation. Here we map neuronal developmental origin, neuropil targeting, and neuronal connectivity for 78 pair of interneurons in abdominal segment 1 (A1) by identifying and reconstructing all 78 neurons within a full CNS TEM volume (Ohyama et al., 2015). This provides us the unprecedented ability to identify correlations between development and circuit formation – at the level of single neurons/single synapses – and test those

relationships to gain insight into how mechanisms known to generate diversity are coupled to mechanisms of neural circuit formation.

Results

Clonally related interneurons project to broad domains of sensory and motor neuropil

Clonally related neurons may be similar in morphology and function, or may encompass a diversity of neuronal subtypes. Here we use genetic methods to visualize clonally related neurons in the *Drosophila* newly hatched larva and identify these clones in a serial section TEM reconstruction of the entire larval CNS (Ohyama et al., 2015). Our goal was to determine whether clonally related neurons comprise one, two, or many morphological subtypes.

We took a multi-step approach to identify clonally-related neurons in the TEM reconstruction. First, we generated sparse neuroblast clones and imaged them by light microscopy. All assayed lineages had a reproducible clonal morphology including the number of fascicles entering the neuropil, cell body position, and morphology of axon/dendrite projections (Figure 1B; and data not shown). We assigned each clone to its parental neuroblast by comparing clonal morphology to that seen following single neuroblast DiI labeling (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997); the morphology of larval lineages (Birkholz et al., 2015; Lacin and Truman, 2016a); the position of the clone in the segment; and in some cases the presence of well-characterized individual neurons (e.g. the "looper" neurons in the NB2-1 clone). Note that we purposefully generated clones after production of the first-born Hb+ neurons, because the Hb+ neurons have cell bodies contacting the neuropil and do not fasciculate with later-born neurons in the clone, making it difficult to assign them to a specific neuroblast clone. In total, we mapped six neuroblast lineages, with a seventh previously mapped by our lab and others (Heckscher et al., 2015; Wreden et al., 2017), comprising 78 bilateral pair of interneurons. This is almost 1/3 of all neurons in the A1L hemisegment (78/295) (Table 1). We found that neurons in a single neuroblast clone, even without the Hb+ first-born neurons included, have broad axon and dendrite projections within the neuropil (Figure 1B).

To identify neuroblast clones in the TEM volume, we matched lineage-specific features present in both light and TEM analyses. We identified neurons that had clustered cell bodies, clone morphology matching that seen by light microscopy (Figure 1C), and one or two fascicles entering the neuropil (Figure 1C,D). The similarity in overall clone morphology between genetically marked clones and TEM reconstructed clones was striking (compare Figure 1B and 1C). We validated these assignments using two methods. First, we used neuroblast-specific Gal4 lines (Kohwi et al., 2013; Lacin and Truman, 2016b) to generate MCFO labeling of single neurons (Nern et al., 2015), and found that in each case we could match the morphology of an MCFO-labeled single neuron from a known neuroblast to an identical single neuron in the same neuroblast clone within the TEM reconstruction (data not shown). Second, we reconstructed the same seven lineages in a second hemisegment, A1R. We observed similar neuron numbers and fascicles per clone (Figure 1E) and similar clonal morphology (data not shown). Each clone projected to a unique but broad domain of the neuropil (Figure 1F). Overall, we mapped seven bilateral neuroblast clones into the TEM reconstruction (Figure 1G; Supplemental Table 1). Interestingly, most lineages projected to both dorsal and ventral neuropil (Figure 1H), which are regions of motor or sensory processing, respectively (Figure S1; Figure 1I)(Landgraf et al., 2003; Mauss et al., 2009). Taking everything together, we conclude that we have identified seven neuroblast clones and 78 interneurons in the TEM reconstruction, and that each neuroblast clone coarsely tiles the neuropil. The neuroblast-specific, highly reproducible clonal morphology suggests that neuropil

targeting of clonal projections are determined by neuroblast-specific developmental mechanisms (see Discussion)

Hemilineage identity determines neuronal axon/dendrite projections to motor or sensory neuropil

We observed that most neuroblast clones contained two pools of neurons, each projecting to either dorsal or ventral neuropil. Previous work on post-embryonic lineages showed that the Notch^{ON} hemilineage had similar morphology, whereas the Notch^{OFF} hemilineage shared a different morphology (Harris et al., 2015; Lacin and Truman, 2016b; Truman et al., 2010). We hypothesized that embryonic neuroblasts may also generate Notch^{ON} and Notch^{OFF} hemilineages with distinct projections. First, we used NBLAST (Costa et al., 2016) to compare the morphology of clonally related neurons. We found that five of the seven neuroblasts generated hemilineages with distinct projections to dorsal or ventral neuropil (Figure 2A-C; Figure S2); one made ventral neurons and glia (Figure 2D), and one was previously shown to make only a ventral hemilineage (Figure S2)(Baumgardt et al., 2014; Wreden et al., 2017). We conclude that NBLAST can identify candidate hemilineages, with one projecting to the ventral neuropil and one projecting to the dorsal neuropil.

To validate the NBLAST assigned hemilineages, we generated a Notch reporter by Crispr engineering the Notch target gene *hey*, placing a T2A:FLP exon in frame with the terminal *hey* exon, resulting in Notch^{ON} neurons expressing FLP. When we use neuroblast-specific Gal4 lines to drive expression of UAS-GFP in NB7-1 or NB5-2, we observed both sensory (ventral) and motor (dorsal) projections, as expected (Figure 2E,F), whereas a FLP-dependent reporter (*UAS-FRT-stop-FRT-RFP*) that is only expressed in Notch^{ON} neurons, showed only motor (dorsal) neuropil (Figure 2E',F'). These data support our conclusion that Notch^{ON} hemilineages project to motor neuropil, and Notch^{OFF} hemilineages project to sensory neuropil. We conclude that each neuroblast lineage concurrently generates neurons for motor processing (targeting dorsal neuropil) and for sensory processing (targeting ventral neuropil).

We next asked whether Notch activity determines hemilineage neuronal projections, or whether it is simply correlated with dorsal or ventral projections. We used neuroblast-specific Gal4 lines to drive expression of constitutively active Notch (Notch^{intra}) to transform Notch^{OFF} hemilineages into Notch^{ON} hemilineages. Misexpression of Notch in all tested individual lineages (NB1-2, NB5-2, or NB7-1) led to a loss of ventral projections and a concomitant increase in dorsal projections (Figure 2G-I). Similarly, misexpression of Notch in the NB7-4 lineage led to a loss of ventral projections and an increase in glial numbers, which derive from the Notch^{ON} hemilineage (Figure 2J). We observed that ascending and descending projection neurons were specifically generated by ventral hemilineages (Figure S3), and these projection neurons were completely lost following Notch misexpression (Figure 2G-J, insets). Thus, Notch signaling promotes neuronal projections to the dorsal neuropil, and lack of Notch signaling in sibling neurons determines neuronal projections to the ventral neuropil (Figure 2K). This is a remarkable subdivision within each lineage, as the dorsal neuropil is dedicated to motor processing and ventral neuropil is devoted to sensory processing (Figure S1)(Landgraf et al., 2003; Mauss et al., 2009); by directing sibling neurons to dorsal and ventral neuropil, both motor and sensory processing domains are coordinately assembled (see Discussion). We conclude that Notch-dependent hemilineage identity further refines lineage-specific projections to dorsal or ventral neuropil.

Hemilineage identity determines synapse localization within motor or sensory neuropil

Hemilineages synaptically tile the dorsal or ventral neuropil

Identification of 12 different hemilineages within the TEM volume allowed us to determine the location of pre- and postsynapses within each hemilineage. Synapses could be distributed evenly in overlapping domains of the neuropil, or they could be tiled with minimal overlap. We found that presynapses in each hemilineage were localized to highly restricted neuropil domains, with minimal overlap (Figure 3A,B; Figure S4). Postsynapses were also restricted to small neuropil domains, but with more overlap than presynapses (Figure 3C,D). Hierarchical cluster analysis showed that localization of presynapses in three-dimensional space was more similar for neurons within a single hemilineage than between hemilineages, again with presynapses showing tighter clustering than postsynapses (Figure 3E,F). Quantification shows a significantly greater clustering compared to random (Figure 3G). Thus, neurons in each hemilineage target pre- and postsynapses to distinct sub-domains of the neuropil (Figure 3H). We propose that hemilineage identity determines both neuronal projections and subsequent synapse localization within the neuropil (see Discussion).

Hemilineage-temporal cohorts "tile" presynapses within the neuropil

To investigate the role of temporal identity in determining neuronal projections, synapse localization or connectivity we needed to assign temporal identity to the 78 neurons within our dataset. We used two methods. First, we confirm that temporal transcription factors (Hb, Kr, Pdm, and Cas) have a radial distribution in the embryonic CNS, with early-born Hb+ neurons positioned in a deep layer adjacent to the developing neuropil, and late-born Cas+ neurons are at the most superficial position within the late embryo (Isshiki et al., 2001; Kambadur et al., 1998)(Figure 4A,B). Importantly, we show that this distribution persists for Hb and Cas reporters in the newly hatched larval CNS (Figure 4C,D). Thus, radial position can be used as a proxy for temporal identity. Second, we used MCFO to identify single Hb+ or Cas+ neurons and matched them to the morphologically identical neuron in the TEM volume (Figure 4E,F; Figure S5), and measured their "cortex neurite length" (defined as the neurite length from cell body to the edge of the neuropil). Importantly, that left/right neuronal homologs had extremely similar cortex neurite lengths (Figure 4G). We found that the experimentally validated "ground truth" Hb+ neurons were close to the neuropil, whereas "ground truth" late-born Cas+ neurons were far from the neuropil (Figure 4H). Thus, radial position can be used as a proxy for temporal identity. We next measured radial position of all 78 neurons within the seven neuroblast lineages (Figure 4I; Supplemental Table 1), and found that neurons in each temporal cohort projected widely in the neuropil, including both dorsal and ventral domains (Figure 4J), consistent with each temporal cohort containing neurons in Notch^{ON} and Notch^{OFF} hemilineages. We conclude that radial position is a proxy for temporal identity, and that each temporal cohort projects widely within the neuropil.

We have identified four temporal cohorts and 12 hemilineages, thereby generating up to 48 potential "hemilineage-temporal cohorts." This raises the question of whether hemilineage-temporal cohorts have distinct projections or synapse localization. We mapped presynapse localization for temporal cohorts in each hemilineage, and found significant positional clustering of synapses within a hemilineage-temporal cohort; this is shown for the dorsal hemilineages of NB5-2 and NB7-1 (Figure 5A,B), and other hemilineages in Figure S6A; only one of the 12 hemilineages did not show a significant correlation between birth-order and postsynaptic clustering (NB5-2 dorsal). We also observed postsynapse clustering, although with more overlap compared to presynapses (Figure S6B). Pooling data from all hemilineages revealed a positive correlation between synapse location and temporal identity (Figure 5C-E). We conclude that temporal identity maps to distinct presynaptic tiling within each hemilineage (Figure 5F). Thus, neuroblast lineage, hemilineage, and temporal identity restrict synapse localization to progressively smaller regions of the neuropil.

Hemilineage-temporal cohorts share common connectivity

We showed above that hemilineage-temporal cohorts "tile" presynapse positions within the neuropil; here we ask whether hemilineage-temporal cohorts share common connectivity. To test this idea, we analyzed the connectome of 12 hemilineages as well as the motor and sensory neurons in segment A1 left and. In total, we analyzed 160 interneurons, 56 motor neurons, and 86 sensory neurons, which corresponded to approximately 25% of all inputs and 14% of all outputs for the 12 hemilineages. We found that hemilineage connectivity is highly structured, with a higher degree of interconnectivity within dorsal and ventral hemilineages (Figure 6A), consistent with the idea that dorsal and ventral hemilineages are functionally distinct (Figure S1). Importantly, connectivity was highly similar for hemilineages on A1 left and right, validating the accuracy of the bilateral reconstructions (Figure 6B,C). Next, we generated force directed network graphs, in which neurons with greater shared connectivity are positioned closer together in network space. Examination of the network as a whole revealed an obvious division between both A1L and A1R as well as the sensory and motor portions of the network (Figure 6D). Neurons in a hemilineage showed shared connectivity (i.e. they are clustered in the network)(Figure 6E,F). Importantly, temporal cohorts within a hemilineage also showed shared connectivity, even compared to other temporal cohorts in the same hemilineage (Figure 6G,H). To quantify shared connectivity using a different method, we determined the minimum number of synapses linking neuronal pairs (a) picked at random, (b) picked from a hemilineage, or (c) picked from a hemilineage-temporal cohort (Figure 6I,J). Neuron pairs that are directly connected have a value of 1 synapse apart; neurons that share a common input or output have a value of 2 synapses apart, with a maximum of seven synapses apart. We found that neurons in a hemilineage had a much lower minimum synapse distance than random, indicating shared connectivity; similarly, neurons in a hemilineage-temporal cohort also have significantly lower minimum synapse distances, with over 60% of all neurons in the same temporal cohort being separated by two synapses or less (Figure 6I,J). We conclude that temporal cohorts share common connectivity (Figure 6K).

Discussion

Our results show that individual neuroblast lineages have unique but broad axon and dendrite projections to both motor and sensory neuropil; hemilineages further restrict projections and synapse localization to either dorsal or ventral neuropil; and temporal identity within each hemilineage correlates with more precise targeting of presynapses (and to a lesser extent postsynapses) to a small neuropil domain. Thus, all three developmental mechanisms act sequentially to progressively refine neurite projections and synapse localization. It is important to note that we chose the seven neuroblasts in this study based on successful clone generation and availability of single neuroblast Gal4 lines, and thus there should be no bias towards a particular pattern of neurite projections, synapse localization or connectivity. Our findings strongly support the hypothesis that the developmental mechanisms driving the generation of neural diversity are directly coupled to the mechanisms governing circuit organization.

Neuroblast clones have unique but broad projections. We propose that neuroblast identity (determined by the spatial position of the neuroblast) determines neuroblast-specific projection patterns. Testing this functionally would require manipulating spatial patterning cues to duplicate a neuroblast and assay both duplicate lineages for similar projections and ultimately connectivity. This has not been done for the neuroblasts characterized here, but has been done for the Otd+ brain neuroblast, where duplication of the neuroblast leads to a duplication of projection patterns and functional connectivity (Sen et al., 2014). In the future, it would be interesting to perform similar studies to determine which aspects of neuroblast clonal development are specified by neuroblast-intrinsic mechanisms. Many studies in *Drosophila* and mammals are

based on the identification and characterization of clonally-related neurons, looking for common location (Fekete et al., 1994; Mihalas and Hevner, 2018), identity (Mihalas and Hevner, 2018; Wong and Rapaport, 2009), or connectivity (Yu et al., 2009). Our results suggest that analyzing neuronal clones may be misleading due to the clone comprising two quite different hemilineages. For example, performing RNAseq on all neurons in a neuroblast lineage is unlikely to reveal key regulators of pathfinding or synaptic connectivity, due to the mixture of disparate neurons from the two hemilineages.

Drosophila third instar larval neuroblast lineages consist of two hemilineages with different projection patterns and neurotransmitter expression (Harris et al., 2015; Lacin and Truman, 2016b; Truman et al., 2010). We extend these pioneering studies to embryonic neuroblasts, and show that Notch signaling determines dorsal versus ventral projection patterns. Surprisingly, in all lineages we assayed, the Notch^{ON} hemilineage projected to the dorsal/motor neuropil, whereas the Notch^{OFF} hemilineage projected to the ventral/sensory neuropil. It is unlikely that all Notch^{ON} hemilineages target the dorsal neuropil, however, as the NB1-1 interneuron pCC is from a Notch^{ON} hemilineage (Skeath and Doe, 1998) yet projects ventrally receiving strong sensory input, whereas its sibling aCC motor neuron is from the Notch^{OFF} hemilineage (Skeath and Doe, 1998) and projects dendrites in the dorsal motor neuropil. We think it is more likely that the Notch^{ON}/Notch^{OFF} provides a switch to allow each hemilineage to respond differently to dorsoventral guidance cues. Our results reveal the striking finding that the sensory and motor processing components of the neuropil are essentially being built in parallel, with one half of every GMC division contributing to either sensory or motor networks. Hemilineages have not been well described in vertebrate neurogenesis. Notch signaling within the Vsx1+ V2 progenitor lineage generates Notch^{OFF} V2a excitatory interneurons and Notch^{ON} V2b inhibitory interneurons, which may be distinct hemilineages (Del Barrio et al., 2007; Francius et al., 2016; Peng et al., 2007; Skaggs et al., 2011). Interestingly, both V2a and V2b putative hemilineages contain molecularly distinct subclasses (Harris et al., 2019); it remains to be seen whether any of these subtypes arise from temporal patterning within the V2 lineage. In addition, Notch^{ON}/Notch^{OFF} hemilineages may exist in the pineal photoreceptor lineage, where Notch^{ON} and Notch^{OFF} populations specify cell type identity (Cau et al., 2019).

Our results suggest that all neurons in a hemilineage respond similarly to the global pathfinding cues that exist within the embryonic CNS. Elegant pioneering work identified gradients of Slit and Netrin along the mediolateral axis (Zlatic et al., 2003), gradients of Semaphorin 1/2a along the dorsoventral axis (Zlatic et al., 2009), and gradients of Wnt5 along the anteroposterior axis (Yoshikawa et al., 2003). We would predict that the palette of receptors for these patterning cues would be shared by all neurons in a hemilineage, to allow them to target a specific neuropil domain; and different in each of the many hemilineages, to allow them to target different regions of the neuropil. It may be possible to identify these receptors, as well as synaptic partner candidates, using RNAseq methods. We previously showed that expression of constitutive Notch throughout a lineage generates only Notch^{ON} cell fates, whereas expression of Numb throughout a lineage generates only Notch^{OFF} cell fates (Skeath and Doe, 1998; Spana and Doe, 1996); FACS purification and RNAseq from either genetic background should identify known guidance receptors or new candidates for hemilineage-specific axon/dendrite targeting. In addition, combining overexpression of Notch or Numb with overexpression of a temporal factor such as Hb should create single lineages with a common hemilineage/temporal identity, facilitating RNAseq experiments to identify molecules used to "tile" temporal cohorts within hemilineages.

Previous work has shown that larval and embryonic hemilineages have similar morphological features (Lacin and Truman, 2016b), suggesting the possibility that these neurons could be performing analogous functions. In support, we show that the Saaghi and Jaam neurons, previously shown to act together in a proprioceptive circuit (Heckscher et al., 2015), are derived from the dorsal and ventral hemilineages of NB5-

2, respectively. Adult activation of either of these NB5-2 hemilineages results in uncoordinated leg movement (Harris et al., 2015), consistent with a role in proprioception in both larval and adult. Similarly, larval activation of neurons in the NB3-3 lineage produced proprioceptive defects (Heckscher et al., 2015), and adult activation of the same lineage also caused proprioceptive defects (Harris et al., 2015). In the future, it will be interesting to explore further the functional and anatomical similarities of the embryonic and larval lineages.

We used the cortex neurite length of neurons as a proxy for birth-order and shared temporal identity. We feel this is a good approximation, but it clearly does not precisely identify neurons born during each of the Hb, Kr, Pdm, Cas temporal transcription factor windows. In the future, using genetic immortalization methods may allow long-term tracking of neurons that only transiently express each of these factors. Nevertheless, we had sufficient resolution to show that neurons within a temporal cohort could target their pre- or postsynapses to distinct subvolume of each hemilineage targeting domain. Temporal cohort tiling could arise stochastically due to self-avoidance mechanism (Zipursky and Grueber, 2013), by using spacing cues (Kulkarni et al., 2016; Petrovic and Hummel, 2008), or by precise responses to global patterning cues. Previous work in the mushroom body has shown how changes in temporal transcription factor expression can affect neuronal targeting, and in the optic lobe it known that altering temporal identity changes expression of axon pathfinding genes (Kulkarni et al., 2016; Zhu et al., 2006). Our data suggest a similar mechanism could be functioning in the ventral nerve cord. We find that temporal cohorts within a hemilineage share common neuropil targeting, synapse localization, and connectivity. In the future, it will be important to test whether altering neuronal temporal identity predictably alters its neuronal targeting and connectivity. We and others have recently shown that manipulation of temporal identity factors in larval motor neurons can retarget motor neuron axon and dendrite projections to match their new temporal identity rather than their actual time of birth (Meng et al., 2019; Seroka and Doe, 2019); whether they change connectivity remains to be determined.

Our results strongly suggest that hemilineage identity and temporal identity act combinatorially to allow small pools of 2-6 neurons to target pre- and postsynapses to highly precise regions of the neuropil, thereby restricting synaptic partner choice. Hemilineage information provides coarse targeting, whereas temporal identity refines targeting within the parameters allowed by hemilineage targeting. Thus, the same temporal cue (e.g. Hb) could promote targeting of one pool of neurons in one hemilineage, and another pool of neurons in an adjacent hemilineage. This limits the number of regulatory mechanisms needed to generate precise neuropil targeting for all ~600 neurons in a segment of the larval CNS.

In this study we demonstrate how developmental information can be mapped into large scale connectomic datasets. We show that lineage information, hemilineage identity, and temporal identity can all be accurately predicted using morphological features (e.g. number of fascicles entering the neuropil for neuroblast clones, and radial position for temporal cohorts). This both greatly accelerates the ability to identify neurons in a large EM volume as well as sets up a framework in which to study development using datasets typically intended for studying connectivity and function. We have used this framework to relate developmental mechanism to neuronal projections, synapse localization, and connectivity; in the future we plan on identifying the developmental origins of neurons within known larval locomotor circuits. It is likely that temporally distinct neurons will have different connectivity due to their sub-regionalization of inputs and outputs, but testing how temporal cohorts are organized into circuits remains an interesting open question.

Methods

Genetics. We used the following fly stocks: R15A05^{AD}R28H10^{DBD} (NB1-2), R70D06^{AD}R28H10^{DBD} (NB2-1), Ac^{AD} Gsb^{DBD}, 25A05^{kz} (NB7-1), *R19B03^{AD} R18F07^{DBD} (NB7-4)*, *castor-gal4* (Technau lab), *hs-Flp.G5.PEST.Opt*(FBti0161061), *13xlexAop2(FRT.stop)myr:smGfP-Flag*(FBti0169275), *13xlexAop2(FRT.stop)myr:smGfP-V5(*FBti0169272), *13xlexAop2(FRT.stop)myr:smGfP-HA*(FBti0169269), and *hb-T2A-LexA.GADfl*. Transgenic lines were made by BestGene (Chino Hills, CA) or Genetivision (Houston, TX).

Immunostaining and imaging

We used standard confocal microscopy, immunocytochemistry and MCFO methods (Clark et al., 2016; Heckscher et al., 2015; Syed et al., 2017). When adjustments to brightness and contrast were needed, they were applied to the entire image uniformly. Mosaic images to show different focal planes were assembled in Fiji or Photoshop. Primary antibodies used recognize: GFP or Venus (rabbit, 1:500, ThermoFisher, Waltham, MA; chicken 1:1000, Abcam13970, Eugene, OR), GFP or Citrine (Camelid sdAB direct labeled with AbberiorStar635P, 1:1000, NanoTab Biotech., Gottingen, Germany), GABA (rabbit, 1:1000, Sigma, St. Louis, MO), mCherry (rabbit, 1:1000, Novus, Littleton, CO), Corazonin (rabbit, 1:2000, J. Veenstra, Univ Bordeaux), FasII (mouse, 1:100, Developmental Studies Hybridoma Bank, Iowa City, IA), HA (mouse, 1:200, Cell signaling, Danvers, MA), or V5 (rabbit, 1:400, Rockland, Atlanta, GA), Flag (rabbit, 1:200, Rockland, Atlanta, GA). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA) and used according to manufacturer's instructions. Confocal image stacks were acquired on Zeiss 700, 710, or 800 microscopes. Images were processed in Fiji (https://imagej.net/Fiji), Adobe Photoshop (Adobe, San Jose, CA), and Adobe Illustrator (Adobe, San Jose, CA). When adjustments to brightness and contrast were needed, they were applied to the entire image uniformly. Mosaic images to show different focal planes were assembled in Fiji or Photoshop.

Clone generation and lineage identification

The clones were generated with the following flies: *hs-Flp.G5.PEST.Opt(FBti0161061), 13xlexAop2(FRT.stop)myr:smGfP-Flag* (FBti0169275), *13xlexAop2(FRT.stop)myr:smGfP-V5*(FBti0169272), *13xlexAop2(FRT.stop)myr:smGfP-HA*(FBti0169269), and *hb-T2A-LexA.GADfl* (see below). The embryos were collected for 7 hours in 25 ˚C, submerged in 32 ˚C water bath for 15-min heat shock, and then incubated at 25 ˚C until larvae hatched. The CNS of newly hatched larvae was dissected, stained and mounted as previously described (Clark et al., 2016; Heckscher et al., 2014; Syed et al., 2017). Antibodies used were: Dylight™405-conjugated rabbit anti-HA (Rockland), Dylight™488-conjugated rabbit anti-Flag (Rockland), and Dylight™549-conjugated rabbit anti-V5 (Rockland), and the neuropil was stained with Alexa Fluor™ 647 Phalloidin (ThermoFisher) by following manufacturer's protocol. The images were collected with Zeiss710 and processed with Imaris.

hb-T2A-LexA.GADfl was generated by in-frame fusion of T2A-LexA.GADfl to the C-terminus of the hb open reading frame with CRISPR-Cas9 gene editing. The ds-DNA donor vector for homology-directed repair was composed of left homologous arm (1098bp), T2A (Diao et al., 2016), LexA.GADfl (Pfeiffer et al., 2010), and the right homologous arm (799bp); the fragments were amplified with PCR and then assembled in pHD-DsRed (Addgene #51434) with NEBuilder (New England BioLabs). The gRNAs were generated from the vector pCFD4-U6:1_U6:3tandemgRNAs (Port et al., 2014) containing target sequence `GAAACTTAGGTCTAGAATTAG` and `GGACGCCGTCGAACTGGCAC`. The ds-DNA donor vectors and gRNA vectors were co-injected into *yw;nos-Cas9* (Kondo and Ueda, 2013) flies by BestGene. The selection marker 3xP3-DsRed was then removed in transgenic flies by hs-Cre (FBti0012692). Lineages were identified in the TEM volume by finding neurons that matched the clonal morphology, and then identifying their neuropil entry point. We then examined every neuron which

entered the neuropil in the same fascicle. In most cases, every neuron in the fascicle had a morphology that fit within the clonal morphology. In a small number of cases, the fascicles diverged slightly before the neuropil entry point. We verified the number of neurons by looking at fasciculating cell populations from at least two hemisegments (A1L and A1R). In some cases, we were able to identify a stereotyped number of cells across as many as four hemisegments, suggesting that fasciculation is stereotyped and reliable.

Morphological analysis of lineages

Morphological analysis was done using NBLAST and the NAT package (Costa et al., 2016), and analysis and figure generation was done using R. Neurons were preprocessed by pruning the most distal twigs (Strahler order 4), converting neurons to dot-props, and running an all-by-all NBLAST. For individual lineages, clusters were set using a cutoff of 3.0. In the case of NB2-1, where nearly every neuron shares a very similar morphology, we first confirmed the presence of a hemilineage using anti-Hey staining. After confirmation of a hemilineage, we next removed A02o and A02l since we could not find any clones that contained either an anterior projection (A02o) or a second contralateral projection (A02l). We reasoned that the hemilineages would represent the next largest morphological division.

Synaptic distributions and density analysis

Synapse distribution plots and density contours were generated using MATLAB. Neuron synaptic and skeleton information was imported to MATLAB using pymaid (Schlegel et al., 2016). Cross sectional synapse distribution plots were made by taking all synapse positions between the T3 and A2 segments as positional information becomes lost due to changes in brain shape beyond these bounds. Synapse distribution plots are 1D kernel density estimates. Sensory and motor density maps were made by taking the synapse positions of all sensory neurons entering the A1 nerve, and all motor neurons exiting the A1 nerve as well as all neurons with at least 3 synapses connected to one of these neurons. For sensory and motor maps as well as individual hemilineages, density plots are 2D kernel density estimates of all synapse positions across the neuropil. A cutoff of 60% was used to set the outermost contour. For lineage maps (Figure 4) , we used 80% as a cutoff. Polyadic synapses were counted as many times as they have targets. For synapse distribution plots, polyadic synapses are scaled by their number of targets.

Temporal Cohort assignment

Cortex neurite length was calculated by converting the skeletonized neuronal arbor into a directed graph away from the soma, and performing a depth-first-search of all vertices. The neuropil borders were defined by a previously created neuropil volume object (10). The neuropil entry point was defined as the first vertex within the neuropil volume object. Cortex neurite length was then the path length between the soma and the neuropil entry point. Neurons were binned into 5 groups with 6μm edges to define temporal cohorts.

Synapse similarity clustering

Synapse similarity was calculated as described previously (Schlegel et al., 2016):

$$f(is,jk) = e^{\frac{-d_{sk}^2}{2\sigma^2}} e^{\frac{|n_{is}-n_{jk}|}{n_{is}+n_{jk}}}$$

where *f(is,jk)* is the mean synapse similarity between all synapses of neuron *i* and neuron *j*. $d_{s,k}$ is the Euclidean distance between synapses *s* and *k* such that synapse *k* is the closest synapse of neuron *j* to synapse s of neuron *i*. σ is a bandwidth term that determines what is considered close. n_{is} and n_{jk} are the fraction of synapses for neuron

i and neuron *j* that are within ω of synapse *s* and synapse *k* respectively. We used parameters ω= σ = 4000nm. Clusters for dendrograms were created based on the mean distance between elements of each cluster using the average linkage clustering method. Edges with a strength of less of less than 1% of the input for a given neuron were discarded.

Electron microscopy and CATMAID

We reconstructed neurons in CATMAID as previously described (Carreira-Rosario et al., 2018; Heckscher et al., 2015; Ohyama et al., 2015). Analysis was done using MATLAB.

Figures

Figures were generated using Matlab, R, CATMAID, and FIJI, and assembled in Adobe Illustrator or Photoshop (San Jose, CA).

Statistical analysis

Statistical significance is denoted by asterisks: ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05; n.s., not significant. All statistical analysis was done in MATLAB. When comparing two groups of quantitative data, an unpaired t-test was performed if data was normally distributed (determined using a one-sample Kolmogorov-Smirnov test) and Wilcoxon rank sum test if the data was not normally distributed. Linear models were generated in MATLAB using lmfit.

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Figure 1. Individual neuroblast progeny have unique but broad neuronal projections
(A) Three mechanisms specifying neuronal diversity. Neuroblasts characterized here are shown in dark gray. Nb, Numb; N, Notch; A1L, abdominal segment A1 left side.
(B) Single neuroblast clones generated with *dpn(FRT.stop)LexA.p65* assayed in newly-hatched larvae. We recovered n>2 clones for each newly characterized lineage; NB4-1 was previously characterized (Lacin and Truman, 2016b). Posterior view, dorsal up for this and following panels; scale bar, 10 μm.
(C) The corresponding neurons traced in the TEM reconstruction. Dashed lines, neuropil border.
(D) Each clone has one or two fascicles (blue) at the site of neuropil entry (arrow).
(E) Number of neurons per clone in A1L and A1R.
(F) Pre- and postsynaptic density maps (75% threshold) for the indicated neuroblast lineages. Circles, cell bodies.
(G) Seven bilateral neuroblast lineages in segment A1 traced in the TEM reconstruction; posterior view, dorsal up. Inset: same projections, lateral view, anterior left.
(H) Presynapse density map (90% threshold) of the seven bilateral neuroblast lineages. Color code as in panel G. Cross-section view, dorsal up.
(I) Summary. Neuroblast clones have unique but broad projections. Neuroblast lineage, blue; neuropil, gray; posterior view, dorsal up.

Figure 2. Hemilineage identity determines neuronal projection to dorsal or ventral neuropil domains
(A-D) Left: NBLAST clustering reveals two morphological classes of neurons in each lineage. Cluster cutoffs were set at 3.0 for all lineages. Right: each cluster projects to dorsal (red) or ventral (blue) neuropil. Posterior view, dorsal up for this and following panels.
(E,F) Notch reporter specifically labels dorsal projections within individual neuroblast lineages. Crispr genomic engineering of the *hey* locus to express Hey:T2A:FLP, crossed to the indicated neuroblast-specific Gal4 lines driving expression of *UAS-GFP, UAS-FRT-stop-FRT-myr:RFP*. GFP shows the whole lineage, RFP shows the $Notch^{ON}$ hemilineage.
(G-J) Lineage-specific $Notch^{intra}$ mis-expression transforms ventral projections to dorsal projections. (G-J) Control neuroblast lineages project to both dorsal neuropil (red arrowhead) and ventral neuropil. NB7-4 makes glia (red arrows) and ventral projections. (G'-J') Notch mis-expression in the indicated neuroblast lineage results in a loss of ventral projections and an increase in dorsal projections or glia. Cell numbers in control and Notch mis-expression are similar (data not shown). (G"-J") Summary. n>3 for all experiments.
(K) TEM reconstruction all dorsal projecting neurons (presumptive $Notch^{ON}$; red) and all ventral projecting neurons (presumptive $Notch^{OFF}$, blue).

Figure 3. Hemilineages target pre- and postsynapses to dorsal or ventral neuropil
(A-D) Presynapse and postsynapse localization for the indicated neuroblast hemilineages (color coded); each dot represents a synapse.
(E,F) Hierarchical clustering reveals similar synapse localization for neurons within a single hemilineage. (E) Dorsal hemilineages; (F) ventral hemilineages. Presynapses (blue heat map) show more spatial clustering than postsynapses (orange heat map).
(G) Quantitation. Both presynapses (blue) and postsynapses (orange) show significantly greater spatial clustering than neurons chosen at random.
(H) Summary.

Figure 4. Mapping the temporal identity of neurons in the TEM reconstruction

(A) Schematic showing correlation between temporal identity and radial position. Posterior view, dorsal up.
(B-D) Early-born Hb+ neurons are near the neuropil and late-born Cas+ neurons are far from the neuropil in the late embryo (B) and newly hatched larvae (C,D); larval reporters are recombineered *Hb:GFP*+ and *cas-gal4 UAS-RFP*+.
(E,F) Examples of experimentally verified early-born Hb+ neurons and late-born Cas+ neurons, identified using a hb-T2A-LexA line or MCFO in a Cas-Gal4 line (see methods); additional examples in Figure S5.
(G) Cortex neurite length (from cell body to edge of neuropil) is highly reproducible for orthologous neurons (dots) on the left and right sides of segment A1.
(H) Fraction of experimentally validated Hb+ or Cas+ "ground truth" neurons at the indicated distance from the neuropil (cortex neurite length). n = 47 Cas+ neurons, and 55 Hb+ neurons from segments T3-A2.
(I) Temporal cohorts predicted based on radial position. Note that not all lineages have all temporal cohorts, mirroring experimental observation that some neuroblast lineages do not express all temporal transcription factors (Benito-Sipos et al., 2010; Cui and Doe, 1992; Isshiki et al., 2001; Tsuji et al., 2008).
(J) Reconstruction of neurons in each of the four temporal cohorts (radial bins) shown in panel I; posterior view, dorsal up.

Figure 5. Hemilineage-temporal cohorts show presynaptic tiling in motor and sensory neuropil.
(A-C) Temporal cohorts in the indicated hemilineages show presynaptic tiling in both dorsoventral and mediolateral axes; for additional hemilineages see Figure S6.
(D) Presynapses from all hemilineages show a positive relationship between neuropil position and temporal identity.
(E) Postsynapses from all hemilineages show a positive relationship between neuropil position and temporal identity.
(F) Quantification of synapse spatial clustering ("synapse similarity") in neurons chosen at random (black), a common temporal cohort (dark gray), in a hemilineage (light gray), and in a hemilineage-temporal cohort (white). The strongest predictor of synaptic clustering is hemilineage-temporal identity.
(F) Summary.

Figure 6. Hemilineage-temporal cohorts are enriched for common connectivity.
(A) Heatmap of connectivity between the indicated dorsal hemilineages (red, dorsal; blue, ventral), sensory afferents (SN), and motor neuron dendrites (MN).
(B,C) Fraction of inputs/outputs for each hemilineage. Adjacent bars of the same color represent the homologous hemilineage in the left and right hemisegments.
(D) Force directed network graph of all 156 interneurons, together with sensory afferents and motor dendrites. Neurons with similar connectivity appear closer in network space. Purple edges represent motor neuron connectivity; green edges represent sensory neuron connectivity.
(E-H) Force directed network graphs of all 156 interneurons highlighting specific lineages (E,F) or temporal cohorts (G,H). Edge colors represent outputs from given nodes.
(I) Cumulative distribution of the number of synapses between temporal cohorts of hemilineage related neurons, hemilineage related neurons, or random neurons. Neurons that belonged to a temporal cohort with only one neuron were not analyzed (16 neurons). Random neurons were selected from the same hemisegment.
(J) Quantification of the number of directly connected pairs of neurons, neurons separated by 2 synapses, and neurons separated by more than two synapses. Black circles represent pairs of neurons connected by 1 synapse (top) or two synapses (bottom).

(K) Summary.

Figure S1. The neuropil is divided into motor and sensory processing domains.
(A) Motor neuron postsynapses (purple) and sensory neuron presynapses (green) showing dorsoventral segregation. Plots are 1D kernel density estimates for dorsoventral or mediolateral axes. Purple dots represent a single postsynaptic site. Green dots represent a single presynaptic site scaled by the number of outputs from that presynaptic site.
(B) Premotor neuron postsynaptic sites (>3 synapses onto a motor neuron in segment A1), or post-sensory neuron presynaptic sites (pre >3 synapses with an A1 sensory neuron) show that motor and sensory processing neurons are also restricted to dorsal or ventral neuropil domains.
(C) 2D kernel density estimates of all pre/post synaptic sites for pre-motor and post-sensory neurons outlines the regions of sensory (green) and motor (magenta) processing in the VNC.

Figure S2. Neuroblasts make neuronal populations that target dorsal or ventral neuropil.
(A-C) NBLAST clustering for the indicated lineages reveal two morphological classes. Each cluster projects to dorsal (red) or ventral (blue) neuropil. Note that NB3-3 only generates a Notch^{OFF} hemilineage (Baumgardt et al., 2014; Tsuji et al., 2008 ; Wreden et al., 2017). Note that NB2-1 has two "outlier" neurons that fasciculate with NB2-1 progeny but have not been experimentally identified as part of the lineage. NBLAST cluster cutoffs were set at 3.0 for all lineages.
(D) NB2-1 has Notch^{ON} and Notch^{OFF} hemileages, as shown by expression of the Notch target gene Hey in a subset of the neurons in the lineage. Posterior view, dorsal up, midline at center of panel.

Figure S3. Ascending and descending projection neurons are uniquely derived from ventral Notch^{OFF} hemilineages
The indicated neuroblast lineages traced in CATMAID showing the dorsal (red) and ventral (cyan) predicted hemilineages. Note that the ventral (cyan) hemilineages contains significantly longer axons (ascending and descending projection neurons) compared to dorsal (red) hemilineage neurons consistent with what has been observed in larva (Truman et al., 2010). P = .0034, via 2-sided Wilcoxon rank sum test.

Figure S4. Individual hemilineages restrict pre- and postsynapses to dorsal or ventral neuropil.
(A) Diagram of neuropil volume analyzed for synapse spatial positions.
(B) Synapse positions are highly reproducible between left/right homologous neurons.
(C) Dorsal hemilineages have enriched connectivity to motor neurons, whereas ventral hemilineages have enriched connectivity to sensory neurons.
(D) Density plots of presynpase and postsynapse localization for dorsal (red) and ventral (blue) hemilineages. Three-dimensional synapse positions are projected onto a cross-section plane; dorsal up, midline at center, neuropil, dashed outline. Note that NB3-3 only generates a ventral hemilineage, and NB7-4 only generates a neuronal ventral hemilineage (the dorsal hemilineage produces glia).

Figure S5. Identification of "ground truth" Hb+ or Cas+ neurons in the TEM volume.
Identification of the indicated early-born Hb+ neurons and late-born Cas+ neurons in newly hatched larvae (left), with their corresponding anatomical match in the TEM volume (right). Hb+ neurons are close to the neuropil, whereas Cas+ neurons are further from the neuropil. Individual neurons were identified using a hb-T2A-LexA line or MCFO in a Cas-Gal4 line (see methods).

595 **Figure S6. Temporal cohorts within hemilineages show presynaptic tiling.**
596 Temporal cohorts in the indicated hemilineages show presynaptic tiling in both dorsoventral and mediolateral
597 axes.
598

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