

1 **The role of lineage, hemilineage and temporal identity in establishing neuronal connectivity in the**  
2 ***Drosophila* larval CNS**

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20 **Abstract**

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22 The mechanisms specifying neuronal diversity are well-characterized, yet it remains unclear how or if these  
23 mechanisms regulate neuronal morphology and connectivity. Here we map the developmental origin of 78  
24 bilateral pairs of interneurons from seven identified neural progenitors (neuroblasts) within a complete TEM  
25 reconstruction of the *Drosophila* newly-hatched larval CNS. This allows us to correlate developmental  
26 mechanism with neuronal projections, synapse targeting, and connectivity. We find that clonally-related  
27 neurons from project widely in the neuropil, without preferential circuit formation. In contrast, the two  
28 Notch<sup>ON</sup>/Notch<sup>OFF</sup> hemilineages from each neuroblast project to either dorsal motor neuropil (Notch<sup>ON</sup>) or  
29 ventral sensory neuropil (Notch<sup>OFF</sup>). Thus, each neuroblast contributes both motor and sensory processing  
30 neurons. Lineage-specific constitutive Notch transforms sensory to motor hemilineages, showing hemilineage  
31 identity determines neuronal targeting. Within a hemilineage, temporal cohorts target processes and synapses  
32 to different sub-domains of the neuropil, effectively “tiling” the hemilineage neuropil, and  
33 hemilineage/temporal cohorts are enriched for shared connectivity. Thus, neuroblast lineage, hemilineage,  
34 and temporal identity progressively restrict neuropil targeting, synapse localization, and connectivity. We  
35 propose that mechanisms generating neural diversity are also determinants of neural circuit formation.

36

## 37 Introduction

38  
39 Tremendous progress has been made in understanding the molecular mechanisms generating neuronal  
40 diversity in both vertebrate and invertebrate model systems. In mammals, spatial cues generate distinct pools  
41 of progenitors which generate a diversity of neurons and glia appropriate for each spatial domain (1). The  
42 same process occurs in invertebrates like *Drosophila*, but with a smaller number of cells, and this process is  
43 particularly well-understood. Spatial patterning genes act combinatorially to establish single, unique  
44 progenitor (neuroblast) identity; these patterning genes include the dorsoventral columnar genes *vnd*, *ind*, *msb*  
45 (2-4) and the orthogonally expressed *wingless*, *hedgehog*, *gooseberry*, and *engrailed* genes (5-8). These factors endow  
46 each neuroblast with a unique spatial identity, the first step in generating neuronal diversity (Figure 1A, left).  
47 Here we focus on the left and right sides of abdominal segment 1 (A1L, A1R) and so segment-specific  
48 patterning due to Hox gene expression is not relevant. The second step occurs as each neuroblast “buds off”  
49 a series of ganglion mother cells (GMCs) which acquire a unique identity based on their birth-order, due to  
50 inheritance from the neuroblast of a “temporal transcription factor” – Hunchback (Hb), Krüppel (Kr), Pdm,  
51 and Castor (Cas) – which are sequentially expressed by nearly all embryonic neuroblasts (9). The combination  
52 of spatial and temporal factors leads to the production of a unique GMC with each neuroblast division  
53 (Figure 1A, middle). The third and final step in generating neuronal diversity is the asymmetric division of  
54 each GMC into a pair of post-mitotic neurons; during this division, the Notch inhibitor Numb (Nb) is  
55 partitioned into one neuron (Notch<sup>OFF</sup> neuron) whereas the other sibling neuron receives active Notch  
56 signaling (Notch<sup>ON</sup> neuron), thereby establishing two distinct hemilineages (10-13)(Figure 1A, right). In  
57 summary, three developmental mechanisms generate neuronal diversity within the embryonic CNS:  
58 neuroblast spatial identity, GMC temporal identity, and neuronal hemilineage identity.

59 A great deal of progress has also been made in understanding neural circuit formation in both vertebrates  
60 and invertebrate model systems, revealing a multi-step mechanism. Mammalian neurons initially target their  
61 axons to broad regions (e.g. thalamus/cortex), followed by targeting to a neuropil domain (glomeruli/layer),  
62 and finally forming highly specific synapses within the targeted domain (reviewed in 14).

63 Despite the progress in understanding the generation of neuronal diversity and the mechanisms  
64 governing axon guidance and neuropil targeting, how these two developmental processes are related remains  
65 unknown. While it is accepted that the identity of a neuron is tightly linked to its connectivity, the  
66 developmental mechanisms involved remain unclear. For example, do clonally-related neurons target similar  
67 regions of the neuropil due to the expression of similar guidance cues? Do temporal cohorts born at similar  
68 times show preferential connectivity? Are neurons expressing the same transcription factor preferentially  
69 interconnected? It may be that lineage, hemilineage, and temporal factors have independent roles in circuit  
70 formation; or that some mechanisms are used at different steps in circuit assembly; or that mechanisms used  
71 to generate neural diversity could be independent of those regulating circuit formation. Here we map  
72 neuronal developmental origin, neuropil targeting, and neuronal connectivity within a whole CNS TEM  
73 reconstruction (15). This provides us the unprecedented ability to identify correlations between development  
74 and circuit formation – at the level of single neurons/single synapses – and test those relationships to gain  
75 insight into how mechanisms known to generate diversity might be coupled to mechanisms of neural circuit  
76 formation. We find that lineage, hemilineage, and temporal identity are all strongly correlated with features of  
77 neuronal targeting that directly relate to establishing neural circuits.

## 78 Results

### 79 Clonally related neurons project widely within the neuropil

82

83 It is not possible to determine the clonal relationship of neurons in the TEM volume based on anatomical  
84 features alone; for example, clonally-related neurons are not ensheathed by glia as they are in grasshopper  
85 embryos or the *Drosophila* larval brain (16, 17). We took a multi-step approach to identify clonally-related  
86 neurons in the TEM reconstruction. First, we generated sparse neuroblast clones and imaged them by light  
87 microscopy. All neuroblasts assayed had a distinctive clonal morphology including the number of fascicles  
88 entering the neuropil, cell body position, and morphology of axon/dendrite projections (Figure 1B-G; and  
89 data not shown). The tendency for neuroblast clones to project one or two fascicles into the neuropil has also  
90 been noted for larval neuroblast clones (11-13). We assigned each clone to its parental neuroblast by  
91 comparing our clonal morphology to that seen following single neuroblast DiI labeling (18-20), and what has  
92 been reported previously for larval lineages (21, 22), as well as the position of the clone in the segment, and in  
93 some cases the presence of well-characterized individual neurons (e.g. the “looper” neurons in the NB2-1  
94 clone). Note that we purposefully generated clones after the first-born Hb<sup>+</sup> neurons, because the Hb<sup>+</sup>  
95 neurons have cell bodies contacting the neuropil and do not fasciculate with later-born neurons in the clone,  
96 making it difficult to assign them to a specific neuroblast clone. We found that neurons in a single neuroblast  
97 clone, even without the Hb<sup>+</sup> first-born neurons included, project widely throughout the neuropil, often  
98 targeting both dorsal motor neuropil and ventral sensory neuropil, as well as widely along the mediolateral  
99 axis of the neuropil (Figure 1B).

100 Next, we used these neuroblast lineage-specific features to identify the same clonally-related neurons in  
101 the TEM reconstruction in A1L. We identified neurons that had clustered cell bodies, clone morphology  
102 matching that seen by light microscopy (Figure 1C), and one or two fascicles entering the neuropil (Figure  
103 1D,E). The similarity in overall clone morphology between genetically marked clones and TEM reconstructed  
104 clones was striking (compare Figure 1B and 1C). We used two methods to validate the clonal relationship  
105 observed in the TEM reconstruction. We used neuroblast-specific Gal4 lines (13, 23) to generate MCFO  
106 labeling of single neurons, and found that in each case we could match the morphology of an MCFO-labeled  
107 single neuron from a known neuroblast to an identical single neuron in the same neuroblast clone within the  
108 TEM reconstruction (data not shown). We also validated the reliability of clone morphology and neuron  
109 numbers by tracing the same seven lineages in A1R, where we observed similar neuron numbers and fascicles  
110 per clone (Figure 1D, E), and similar clonal morphology (data not shown). Overall, we mapped seven  
111 bilateral neuroblast clones into the TEM reconstruction (Figure 1F,G; Supp. Table 1). Note that we chose  
112 these seven neuroblasts based on successful clone generation and availability of single neuroblast Gal4 lines,  
113 and thus there should be no bias towards a particular connectivity or circuit. We conclude that each  
114 neuroblast clone has stereotyped cell body positions, 1-2 fascicles entering the neuropil, and widely projecting  
115 axons and dendrites.

116

### 117 **Lineages generate two morphologically distinct classes of neurons, which project to motor or** 118 **sensory neuropil domains.**

119

120 After mapping seven lineages into the EM volume, we observed that most lineages seemed to contain two  
121 broad classes of neurons with very different projection patterns. Recent work has shown that within a larval  
122 neuroblast lineage all Notch<sup>ON</sup> neurons have a similar clonal morphology (called the Notch<sup>ON</sup> hemilineage),  
123 whereas the Notch<sup>OFF</sup> hemilineage shares a different morphology (11-13). We hypothesized that the observed  
124 morphological differences may be due to hemilineage identity (Figure 2). First, we used NBLAST (24) to  
125 compare the morphology of clonally related neurons. We observed that five of the seven neuroblast lineages  
126 generated two highly distinct candidate hemilineages that each projected to a focused domain in the dorsal or

127 ventral neuropil (Figure 2A-D). A sixth neuroblast lineage, NB7-4, generated neurons projecting to the  
128 ventral neuropil, and a pool of glia (Figure 2E). The seventh neuroblast lineage, NB3-3 (Figure 2F), has  
129 previously been shown to directly generate a single Notch<sup>OFF</sup> hemilineage due to direct differentiation of the  
130 neuroblast progeny as neurons, bypassing the terminal asymmetric cell division (25, 26). We conclude that  
131 NBLAST can identify candidate hemilineages, with one projecting to the ventral neuropil, and one projecting  
132 to the dorsal neuropil (Figure 2G). This is a remarkable subdivision within each lineage, because the dorsal  
133 neuropil is the site of motor neuron dendrites and premotor neurons while the ventral neuropil is the site of  
134 sensory neuron presynapses and post-sensory neurons (27, 28) (Fig. S1). Additionally, neurons from the same  
135 candidate hemilineage are morphologically related, but different from the neurons in the other candidate  
136 hemilineage from the same parental neuroblast (Figure 2H). Thus, each neuroblast lineage generates two  
137 totally different classes of neurons, doubling the neuronal diversity generated in a single lineage. We conclude  
138 that neuroblasts produce two types of neuronal progeny: one targeting motor neuropil and one targeting  
139 ventral neuropil.

140

### 141 Hemilineage identity determines axon projection targeting

142

143 We next wanted to (a) validate the NBLAST hemilineage assignments, (b) determine whether Notch<sup>ON</sup>  
144 hemilineages always project to dorsal/motor neuropil domains (ventral/sensory neuropil for Notch<sup>OFF</sup>  
145 hemilineages), and (c) to experimentally test whether hemilineage identity determines neuropil targeting. We  
146 can achieve all three goals by using neuroblast-specific Gal4 lines to drive expression of constitutively active  
147 Notch (Notch<sup>intra</sup>) to transform Notch<sup>OFF</sup> hemilineages into Notch<sup>ON</sup> hemilineages.

148 There are Gal4 lines specifically expressed in NB1-2, NB7-1, and MB7-4 (13, 29) which we used to drive  
149 Notch<sup>intra</sup> expression. Notch<sup>intra</sup> expression in NB1-2 or NB7-1 led to a loss of ventral projections and a  
150 concomitant increase in dorsal neuropil projections (compare Figure 3A,B to Figure 3D,E). Similarly,  
151 Notch<sup>intra</sup> expression in the NB7-4 lineage led to a loss of ventral projections and an increase in the number  
152 of glia (Figure 3C). For all lineages, the loss of ventral neurons is also visible in dorsal views (Figure 3A-F  
153 insets). In addition, we generated a Notch reporter by Crispr engineering the Notch target gene *hey*, placing a  
154 T2A:FLP exon in frame with the *hey* exon, resulting in Notch<sup>ON</sup> neurons expressing FLP. When we use NB7-  
155 1-Gal4 to drive expression of UAS-GFP we see the full NB7-1 clone (Figure 3G), whereas a FLP-dependent  
156 reporter (*UAS-FRT-stop-FRT-RFP*) will only be expressed in Notch<sup>ON</sup> neurons innervating the dorsal  
157 neuropil (Figure 3G'). Taken together, our Notch experiments strongly support the NBLAST assignments of  
158 neurons into two distinct hemilineages, and show that all tested neuroblast lineages make a Notch<sup>ON</sup>  
159 hemilineage that projects to dorsal/motor neuropil (or makes glia), and a Notch<sup>OFF</sup> hemilineage that projects  
160 to ventral/sensory neuropil. In conclusion, we show that NBLAST can be used to accurately identify  
161 neuroblast hemilineages; that Notch<sup>ON</sup>/Notch<sup>OFF</sup> hemilineages project to motor/sensory neuropil domains,  
162 respectively; and most importantly, that hemilineage identity determines neuronal targeting to the motor or  
163 sensory neuropil.

164

### 165 Hemilineage identity determines synapse targeting

166

167 Here we use motor and sensory domains (Fig. S1) as landmarks to map synapse localization for different  
168 hemilineages. We observed that dorsal hemilineages localize both pre- and post-synaptic sites to the motor  
169 neuropil, whereas ventral hemilineages localize both pre- and post-synaptic sites to the sensory neuropil  
170 (Figure 4A-D; Fig. S3), but see Discussion for caveats. Consistent with these observations, the vast majority  
171 of sensory output is onto ventral hemilineages, and the vast majority of motor neuron input is from dorsal

172 hemilineages (Figure 4E). We conclude that within the seven assayed neuroblast lineages, Notch<sup>ON</sup>  
173 hemilineages target synapses to the motor neuropil, whereas Notch<sup>OFF</sup> hemilineages target synapses to the  
174 sensory neuropil (Figure 4F).

175 After showing that hemilineages target synapses to restricted domains of dorsal or ventral neuropil, we  
176 asked if individual hemilineages tile the neuropil or have overlapping domains. We mapped the pre- and post-  
177 synaptic position for six ventral hemilineages and four dorsal hemilineages. Each of the dorsal hemilineages  
178 targeted pre-synapses and post-synapses to distinct but overlapping regions of the neuropil (Figure 5A,C).  
179 Similarly, each of the ventral hemilineages targeted pre-synapses and post-synapses to distinct but overlapping  
180 regions of the neuropil (Figure 5B,D). Clustering neurons by synapse similarity (a measure of similar position  
181 in the neuropil volume) confirms that most neurons in a hemilineage cluster their pre- and post-synapses  
182 (Figure 5E). We conclude that neuroblast hemilineages contain neurons that project to distinct but  
183 overlapping neuropil regions, strongly suggesting that the developmental information needed for neuropil  
184 targeting is shared by neurons in a hemilineage (see Discussion).

### 185 186 **Mapping temporal identity in the TEM reconstruction: radial position is a proxy for neuronal birth-order**

187  
188 Most embryonic neuroblasts sequentially express the temporal transcription factors Hb, Kr, Pdm, and Cas  
189 with each factor inherited by the GMCs and young neurons born during each window of expression  
190 (reviewed in 30). Previous work has shown that early-born Hb+ neurons are positioned in a deep layer of the  
191 cellular cortex adjacent to the developing neuropil, whereas late-born Cas+ neurons are at the most  
192 superficial position, with Kr+ and Pdm+ neurons positioned in between (Figure 6A)(9, 31). Thus, in the late  
193 embryo, radial position can be used as a proxy for temporal identity (Figure 6B). To determine if this  
194 relationship is maintained in newly hatched larvae, we could not simply stain for temporal transcription  
195 factors, as their expression is not reliably maintained in newly hatched larvae. Instead, we used more stable  
196 reporters for Hb (a recombinered Hb:GFP transgene) and Cas (*cas-gal4* line driving *UAS-histone:RFP*). We  
197 confirm the radial position of Hb:GFP and Cas>RFP in the late embryonic CNS, and importantly, show that  
198 the same deep/superficial layering is maintained in newly hatched larvae (Figure 6C,D). Note that although  
199 we are not attempting to map Hb+ neurons to specific lineages (see above), here we use Hb+ neurons in a  
200 lineage-independent way to help validate the use of radial position as a proxy for temporal identity.

201 Additionally, we generated a new Hb-LexA construct in order to identify additional Hb+ neurons, which  
202 we then traced in the EM volume (Figure 6E,F, cyan neurons). We also used *cas-gal4* to drive MCFO in order  
203 to identify new late-born neurons (Figure 6E,F magenta neurons). In total, we identified 18 neurons in the  
204 EM volume with known birthdates (Figure 6E,F; Fig.S4). In order to quantify distance from the neuropil, we  
205 measured the neurite length between the cell body and the neuropil entry point. We found that all confirmed  
206 Hb+ neurons were located close to the neuropil, whereas late-born neurons were located more distantly  
207 (Figure 6G,H). We also confirmed that left/right neuronal homologs had extremely similar cortex neurite  
208 lengths (Figure 6I). Thus, we confirm that neuronal cortex neurite length is consistent across two  
209 hemisegments, and can be used to approximate the temporal identity of any neuron in the TEM  
210 reconstruction.

### 211 212 **Temporal cohorts “tile” hemilineage neuropil domains**

213  
214 In order to determine the role of temporal identity in neuronal targeting and connectivity we first used cortex  
215 neurite length to map the birthdates of all neurons in 10 hemilineages (Fig. S5). Unlike the striking dorsal-  
216 ventral division observed from mapping hemilineages, the synaptic distributions of individual temporal

217 cohorts appeared far more overlapping (Fig. S5). To quantify this, we compared the synapse similarity of  
218 hemilineage-related neurons and temporal-related neurons and found that neurons related by hemilineage  
219 were more similar than those related by birthdate (Fig. S6). We conclude that hemilineages, not temporal  
220 cohorts, are more important determinants of neuropil targeting.

221 We next asked whether temporal identity is linked to more precise sub-regional targeting or “tiling” of  
222 neuronal projections and synapses within a hemilineage. Here we focus on NB3-3. Previous work has shown  
223 that temporal identity in NB3-3 plays a role in segregating neurons into distinct circuits: early-born neurons  
224 (A08x/m) are involved in escape behaviors while late-born neurons (A08e1/2/3) are involved in  
225 proprioception (25). We confirmed the identity of early- and late-born neurons in this lineage using radial  
226 position (Figure 7A), and found that these five previously characterized neurons projected to different  
227 regions of the neuropil, and different regions of the central brain (Figure 7B,C). We grouped the remaining  
228 neurons in this lineage into temporal cohorts based on their radial position, and found a striking correlation  
229 between birth-order and synapse similarity (Figure 7E,F). We conclude that neurons in the proprioceptive or  
230 nociceptive circuits target their synapses to different regions of the neuropil.

231 We next tested whether other lineages contained hemilineage/temporal cohorts that “tile” neuronal  
232 projections and synapse localization. Indeed, examination of the NB5-2 ventral hemilineage showed that  
233 early- and late-born neurons targeted their projections to “sub-regional” domains of the full hemilineage  
234 (Figure 8A,B). Additionally, both pre- and post-synaptic distributions were strongly correlated with birth-  
235 order (Figure 8C-H). Similar results were observed for pre-synaptic targeting (but not post-synaptic targeting)  
236 in the NB5-2 dorsal hemilineage (Figure 8I-P). Examination of the remaining hemilineages found that only  
237 one did not have a significant correlation between birth-order and presynaptic targeting (NB1-2 dorsal) and  
238 only one hemilineage did not show a significant relationship between birth-order and post-synaptic targeting  
239 (NB5-2 dorsal). Pooling data from all hemilineages reveals a positive correlation between synapse location  
240 and temporal identity (Figure 8Q). We conclude that temporal identity subdivides hemilineages into smaller  
241 populations of neurons that target both projections and synapses to different sub-domains within the larger  
242 hemilineage targeting domain (Figure 8R). Thus, hemilineage identity provides coarse targeting within  
243 neuropil, and temporal identity refines targeting to several smaller sub-domains.

244

## 245 **Temporal cohorts share common connectivity**

246

247 Temporal cohorts share restricted neuronal projections and synapse targeting within each hemilineage,  
248 raising the possibility that temporal cohorts may also share connectivity. To test this idea, we analyzed the  
249 connectome of 12 hemilineages as well as the motor and sensory neurons in segment A1 left and right (Figure  
250 9A-C). In total, we analyzed 160 interneurons, 56 motor neurons, and 86 sensory neurons, which  
251 corresponded to approximately 25% of all inputs and 14% of all outputs for the 12 hemilineages. We found  
252 that hemilineage connectivity is highly structured, with a higher degree of interconnectivity within dorsal and  
253 ventral hemilineages (Figure 9A), consistent with the idea that dorsal and ventral hemilineages are functionally  
254 distinct (SFig. 1). Next, we generated force directed network graphs, in which neurons with greater shared  
255 connectivity are positioned closer together in network space (Figure 9D-H). Examination of the network as a  
256 whole revealed an obvious division between both A1L and A1R as well as the sensory and motor portions of  
257 the network (Figure 9D). Neurons in a hemilineage showed increased shared connectivity (i.e. they are  
258 clustered in the network). Importantly, temporal cohorts within a hemilineage also showed increased shared  
259 connectivity, even compared to other temporal cohorts in the same hemilineage (Figure 9E-J). To quantify  
260 shared connectivity using a different method, we determined the minimum number of synapses linking  
261 neuronal pairs (a) picked at random, (b) picked from a hemilineage, or (c) picked from a temporal cohort

262 within a hemilineage (Figure 9I,J). Neuron pairs that are directly connected have a value of 1 synapse apart;  
263 neurons that share a common input or output have a value of 2 synapses apart, with a maximum of seven  
264 synapses apart. We found that neurons in a hemilineage had a much lower minimum synapse distance than  
265 random, indicating shared connectivity; similarly, neurons in a temporal cohort within a hemilineage also have  
266 significantly lower minimum synapse distances, with over 60% of all neurons in the same temporal cohort  
267 being separated by two synapses or less (Figure 9I,J). We conclude that temporal cohorts share common  
268 connectivity.

## 269 Discussion

270  
271  
272 Our results show that individual neuroblast lineages have unique but broad axon and dendrite projections to  
273 both motor and sensory neuropil; thus, each neuroblast contributes neurons to both sensory and motor  
274 processing circuits. In contrast, the two hemilineages within a neuroblast clone have highly focused  
275 projections into either the sensory or motor neuropil, with all Notch<sup>ON</sup> hemilineages assayed projecting to the  
276 motor neuropil and all Notch<sup>OFF</sup> hemilineages assayed projecting to sensory neuropil. Conversion of  
277 Notch<sup>OFF</sup> to Notch<sup>ON</sup> identity by lineage-specific misexpression of constitutively active Notch redirects  
278 sensory hemilineages into the motor neuropil, showing that Notch signaling regulates dorsal/ventral choice in  
279 axon projections; it is unknown whether connectivity is also changed from sensory to motor circuits. Most  
280 importantly, we show that temporal cohorts within each hemilineage “tile” their projections and synapses to  
281 neuropil subdomains, and each temporal cohort has shared connectivity. Our results strongly support the  
282 hypothesis that the developmental mechanisms driving the generation of neural diversity are directly coupled  
283 to the mechanisms governing circuit organization

284 Previous work on *Drosophila* larval neuroblasts show that the pair of hemilineages have different  
285 projection patterns and neurotransmitter expression (11-13). We extend these pioneering studies to  
286 embryonic neuroblasts, and show that pairs of hemilineages not only have different projection patterns, but  
287 also target pre- and post-synapses to distinct regions. Surprisingly, in all lineages where we performed Notch  
288 mis-expression experiments, neurons in the Notch<sup>ON</sup> hemilineage projected to the dorsal neuropil, whereas  
289 Notch<sup>OFF</sup> neurons projected to the ventral neuropil. It is unlikely that all Notch<sup>ON</sup> hemilineages target the  
290 dorsal neuropil, however, as the NB1-1 interneuron pCC is from a Notch<sup>ON</sup> hemilineage (10) yet projects  
291 ventrally and receives strong sensory input, and its sibling aCC motor neuron is from the Notch<sup>OFF</sup>  
292 hemilineage (10) and projects dendrites in the dorsal motor neuropil. We think it is more likely that the  
293 Notch<sup>ON</sup>/Notch<sup>OFF</sup> provides a switch to allow each hemilineage to respond differently to dorsoventral  
294 guidance cues: in some cases the Notch<sup>ON</sup> hemilineage projects dorsally, and in some cases it projects  
295 ventrally. Nevertheless, our finding that neuroblasts invariably produce both sensory and motor hemilineages  
296 reveals the striking finding that the sensory and motor processing components of the neuropil are essentially  
297 being built in parallel, with one half of every GMC division contributing to either sensory or motor networks.  
298 This has not been observed in larval hemilineages, and may be the result of an evolutionary strategy to  
299 efficiently build the larval brain as fast as possible.

300 While we do observe some differences between embryonic and larval hemilineages, the similarities are far  
301 more striking. Previous work has shown that larval and embryonic hemilineages have similar morphological  
302 features (13), suggesting the possibility that these neurons could be performing analogous functions. Here we  
303 show that two components of a proprioceptor circuit, the Jaam and Saaghi neurons (32), are derived from  
304 two hemilineages of NB5-2 (also called lineage 6 (21)). Activation of either of these hemilineages in adults  
305 results in uncoordinated leg movement, consistent with the idea that these hemilineages could be involved in  
306 movement control. Similarly, adult activation of the NB3-3 lineage (also called lineage 8 (21)) caused postural

307 effects, again consistent our previous findings that activation of this lineage in larvae cause postural defects  
308 (32). In the future, it will be interesting to further explore the functional and organizational similarities of the  
309 embryonic and larval nervous systems.

310 Our results suggest that all neurons in a hemilineage respond similarly to the global pathfinding cues that  
311 exist within the embryonic CNS. Elegant previous work showed that there are gradients of Slit and Netrin  
312 along the mediolateral axis (33), gradients of Semaphorin 1/2a along the dorsoventral axis (34), and gradients  
313 of Wnt5 along the anteroposterior axis (35). We would predict that the palette of receptors for these  
314 patterning cues would be shared by all neurons in a hemilineage, to allow them to target a specific neuropil  
315 domain; and different in each of the many hemilineages, to allow them to target different regions of the  
316 neuropil. Expression of constitutively-active Notch in single neuroblast lineages will make two Notch<sup>ON</sup>  
317 hemilineages (see Figure 3), or expression of Numb will make two Notch<sup>OFF</sup> hemilineages. In this way it will  
318 be possible to obtain RNAseq data on neurons with a common neuropil targeting program.

319 Many studies in *Drosophila* and mammals are based on the identification and characterization of clonally-  
320 related neurons, looking for common location (36, 37), identity (37, 38), or connectivity (39). Our results  
321 suggest that analyzing neuronal clones may be misleading due to the clone comprising two quite different  
322 hemilineages. For example, performing RNAseq on individual neuroblast lineages is unlikely to reveal key  
323 regulators of pathfinding or synaptic connectivity, due to the mixture of disparate neurons from two  
324 hemilineages.

325 We used the cortex neurite length of neurons as a proxy for birth-order and shared temporal identity. We  
326 feel this is a good approximation (see Figure 5 for validation), but it clearly does not precisely identify  
327 neurons born during each of the Hb, Kr, Pdm, Cas temporal transcription factor windows. In the future,  
328 using genetic immortalization methods may allow long-term tracking of neurons that only transiently express  
329 each of these factors. Nevertheless, we had sufficient resolution to show that neurons within a temporal  
330 cohort could target their pre- or post-synapses to distinct sub-domains of each hemilineage targeting domain.  
331 Temporal cohort tiling could arise stochastically due to self-avoidance mechanism (40), by using spacing cues  
332 (41, 42), or by precise responses to global patterning cues. Previous work in the mushroom body has shown  
333 how changes in temporal transcription factor expression can affect neuronal targeting, and in the optic lobe it  
334 known that altering temporal identity changes expression of axon pathfinding genes (42, 43). Our data  
335 suggest a similar mechanism could be functioning in the ventral nerve cord. We find that temporal cohorts  
336 within a hemilineage share common neuropil targeting, synapse localization, and connectivity. It will be  
337 important to test whether altering neuronal temporal identity predictably alters its neuronal targeting and  
338 connectivity. We have recently shown that manipulation of temporal identity factors in larval motor neurons  
339 can retarget motor neuron axon and dendrite projections to match their new temporal identity rather than  
340 their actual time of birth (29). For example, mis-expression the early temporal factor Hb can collapse all five  
341 sequentially-born U motor neuron axons to the U1 early temporal identity, with axon and dendrite  
342 projections matching the endogenous U1 motor neuron (29); whether they change connectivity remains to be  
343 determined.

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



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

361

### 362 **Methods summary**

363

364 For detailed methods see Supplemental File 1. Fly stocks are mentioned in the text and described in more  
365 detail in the Supplemental Methods. We used standard confocal microscopy, immunocytochemistry and  
366 MCFO methods (32, 44, 45). When adjustments to brightness and contrast were needed, they were applied to  
367 the entire image uniformly. Mosaic images to show different focal planes were assembled in Fiji or  
368 Photoshop. Neurons were reconstructed in CATMAID as previously described (15, 32, 46). Analysis was  
369 done using MATLAB. Statistical significance is denoted by asterisks: \*\*\*\* $p < 0.0001$ ; \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ;  
370 \* $p < 0.05$ ; n.s., not significant.

371

### 372 **Acknowledgements**

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377 Bloomington *Drosophila* Stock Center (NIH P40OD018537) were used in this study. Funding was provided  
378 by HHMI (CQD, BM, LM, AAZ), NIH HD27056 (CQD), and NIH T32HD007348-24 (BM).

379

380 **Figure 1. Individual neuroblast progeny project widely within the neuropil**

381 (A) Three mechanisms specifying neuronal diversity.  
382 (B) Single neuroblast clones generated with *dpn(FRT.stop)LexA.p65* in newly-hatched larvae. We recovered  $n > 2$   
383 clones for each lineage except NB4-1 whose lineage morphology is well characterized in (13); posterior view; scale  
384 bar, 20  $\mu\text{m}$ .  
385 (C) The corresponding neurons traced in the TEM reconstruction. Dashed lines, neuropil border.  
386 (D) Each clone has one or two fascicles at the site of neuropil entry (blue). Number of neurons per clone  
387 show below for A1L and A1R.  
388 (E) Quantification of fascicle number at neuropil entry by light and EM microscopy.  
389 (F,G) Seven neuroblast lineages traced in the TEM reconstruction; posterior view (F), lateral view (G).

391 **Figure 2. Lineages generate two morphological distinct classes of neurons which project to dorsal  
392 and ventral regions of the neuropil.**

393 (A-F) NBLAST clustering for the indicated neuroblast progeny typically reveals two morphological groups  
394 (red/cyan) that project to dorsal or ventral neuropil; these are candidate hemilineages. Cluster cutoffs were set  
395 at 3.0 for all lineages.  
396 (G) Superimposition of all dorsal candidate hemilineages (red) and all ventral candidate hemilineages (cyan).  
397 (H) Dendrogram showing NBLAST results clustering neurons based on similar morphology. Clustered  
398 neurons were all from hemisegment A1L. Colored bars denote lineage identity.

400 **Figure 3. Hemilineage identity determines axon projection targeting to dorsal or ventral neuropil**

401 (A-C) Wild type. Posterior view of three neuroblast lineages expressing GFP using single NB-Gal4 drivers (see  
402 methods for genetics). Note the projections to dorsal neuropil (red arrowhead) and ventral neuropil (cyan  
403 arrowhead). Insets, anterior view of A1-A8 segments. Note: NB7-4 makes neurons (cyan arrowhead) and glia  
404 (red arrowhead). Below: summaries. Blue channel is either FasII or phalloidin.  
405 (D-F) Notch<sup>intra</sup> mis-expression. Posterior view of three neuroblast lineages expressing GFP and  
406 constitutively active Notch<sup>intra</sup>. Note loss of the ventral projections and expansion of dorsal projections (red  
407 arrowhead). Insets, anterior view of A1-A8 segments.  $n > 3$  for all experiments. Below: summaries.  
408 (G,G') Crispr genomic engineering of the *hey* locus to create a Notch reporter. The *hey* locus was engineered  
409 to express Hey:T2A:FLP, crossed to *NB7-1-Gal4 UAS-GFP UAS-FRT-stop-FRT-myr:RFP*, and stained for  
410 GFP (G, whole lineage) and RFP (G', Notch<sup>ON</sup> hemilineage) in a newly hatched larva. Dorsal up, midline,  
411 dashed; arrows indicate neuronal processes in the dorsal or ventral neuropil.

413 **Figure 4. Hemilineage identity determines synapse targeting to motor or sensory neuropil domains**

414 (A,B) Each lineage generates a sensory targeting hemilineage and a motor targeting hemilineage, represented  
415 here by NB1-2 and NB5-2 (other neuroblasts shown in SFig. 3). Pre- and post-synaptic sites displayed as 2D  
416 kernel density. Note the restricted domains, and how both pre- and post-synaptic sites remain in the same  
417 functional neuropil domain. Purple and green regions are the contour line denoting the greatest 40% of all  
418 pre-motor (purple) or post-sensory (green) synaptic densities.  
419 (C) Pre-synaptic density maps for all hemilineages tile the neuropil.  
420 (D) Post-synaptic maps for all hemilineages tile the neuropil.  
421 (E) Connectivity diagram showing sensory neurons preferentially connect to neurons in ventral hemilineages,  
422 while motor neurons preferentially connect to neurons in dorsal hemilineages. Edges represent fractions of  
423 outputs for sensory neurons, and fraction of inputs for motor neurons.  
424 (F) Summary showing that lineages generate a sensory and a motor processing hemilineage in a Notch-  
425 dependent manner.

426

427 **Figure 5. Hemilineages target synapses to distinct but overlapping motor or sensory neuropil**  
428 **domains**

429 (A,B) Presynaptic distributions of four hemilineages (A) or five ventral hemilineages (B) shown in posterior  
430 view. Dots represent single pre-synaptic sites with their size scaled by the number of outputs from a given  
431 pre-synaptic site.

432 (C,D) Postsynaptic distributions of four dorsal hemilineages (C) or five ventral hemilineages (D) shown in  
433 posterior view. Dots represent single postsynaptic sites.

434 (E) Neurons with similar synapse positions tend to be in the same hemilineage. Dendrogram clustering  
435 neurons based on combined synapse similarity. Combined synapse similarity was determined by calculating a  
436 similarity matrix for pre-synapses and post-synapses separately and then averaging similarity matrices.

437

438 **Figure 6. Mapping temporal identity in the TEM reconstruction: radial position is a proxy for**  
439 **neuronal birth-order**

440 (A) Schematic showing correlation between temporal identity and radial position. Posterior view.

441 (B-D) Immunostaining to show the radial position of Hb+ and Cas+ neurons at embryonic stage 16 (B),  
442 recombinered *Hb:GFP* (C), or *cas-gal4 UAS-RFP* (D) newly-hatched larvae (L0).

443 (E) Single cell clones of either Hb or late-born neurons. Hb neurons were labeled using *hb-T2A-LexA* (see  
444 methods). Late-born neurons were labeled using *cas-Gal4; MCFO*. We use the term late-born as we can not  
445 rule Gal4 perdurance into neuroblast progeny born after Cas expression ends.

446 (F) Neurons identified in the TEM reconstruction that match those shown in E.

447 (G) All Hb+ and late-born neurons identified in the TEM reconstruction.

448 (H) Distribution of cortex neurite lengths for known Hb+ and late-born neurons shows that late-born  
449 neurons are further from the neuropil than Hb+ neurons.

450 (I) Left/right homologous pairs of neurons with verified birthdates show highly stereotyped cortex neurite  
451 lengths across two hemisegments. Solid red line represents a linear fit, with dotted red lines representing 95%  
452 CIs.  $R^2 = .87$ ,  $p = 1.4e-8$ .

453

454 **Figure 7. Temporal cohorts in the NB3-3 lineage have distinct synapse targeting domains.**

455 (A) Plot of mean cortex neurite lengths across bilateral pairs of NB3-3 neurons. Colors are assigned by  
456 dividing the lineage into two temporal cohorts. Mean cortex neurite length for the lineage was 18 $\mu$ m, with  
457 four neurons having less than the mean (cyan cells). A08m has a mean length greater than 18 $\mu$ m, but has been  
458 shown previously to be early-born. Asterisks denote neurons with confirmed birthdates matching their color  
459 assignment. 6/7 previously birthdated neurons had cortex neurite lengths consistent with their birthdate.

460 (B-D) Full 11 cell clone of NB3-3 in hemisegments A1L and A1R. Colors were assigned by dividing the  
461 lineage into two temporal cohorts on the basis of cortex neurite length with the exception of A08m, which  
462 has been shown previously to be born early.

463 (E) Presynaptic similarity clustering of NB3-3 neurons again shows a clustering of early and late-born neurons  
464 with the exception of A08m. Presynaptic distributions of these two populations of cells show both a  
465 dorsoventral split in the VNC as well as differential target regions for the projection neurons in the brain.

466 (F) Postsynaptic similarity clustering of NB3-3 neurons shows two groups divided by temporal cohort.  
467 Postsynaptic distributions of these two populations of cells show a dorsoventral division consistent with their  
468 differential input from chordotonal neurons (early-born NB3-3 neurons) or proprioceptive sensory inputs  
469 (late-born NB3-3 neurons).

470

471 **Figure 8. Temporal cohorts in multiple neuroblast lineages have distinct synapse targeting domains**  
472 (A-H) NB5-2 ventral hemilineage. (A) NB5-2 ventral hemilineage (cyan, early-born; magenta, late-born).  
473 (B) Cortex neurite lengths of neurons in the hemilineage. (C-D) Presynaptic distributions of neurons in NB5-  
474 2V colored by birth-order. Little separation in the dorsoventral or mediolateral axes in the VNC was  
475 observed, but early-born neurons project axons to the brain while late-born neurons do not. (E-F)  
476 Presynaptic (E) and postsynaptic (F) similarity clustering of NB5-2V neurons shows neurons of a similar  
477 birth-order have similar synaptic positions. (G-H) Presynaptic (G) and postsynaptic (H) similarity plotted  
478 against birth order similarity. Birth-order similarity was defined as the pairwise Euclidean distance between  
479 cell bodies divided by the greatest pairwise distance between two cell bodies in the same hemilineage. Solid  
480 lines represent linear fits while dotted lines represent 95% CIs.  
481 (I-L) NB5-2 dorsal hemilineage. (I) NB5-2 dorsal hemilineage (cyan, early-born; magenta, late-born). (J)  
482 Cortex neurite lengths of neurons in NB5-2D. (K-L) Presynaptic distributions of neurons in NB5-2D colored  
483 by birth-order. Little separation in A/P axis in the VNC was observed, early-born and late-born neurons  
484 segregate in the D/V and M/L axes. (M-N) Presynaptic (M) and postsynaptic (N) similarity clustering of  
485 NB5-2D neurons shows neurons of a similar birth-order have similar synaptic positions. (O-P) Presynaptic  
486 (O) and postsynaptic (P) similarity plotted against birth order similarity. Birth-order similarity was defined as  
487 the pairwise Euclidean distance between cell bodies divided by the greatest pairwise distance between two cell  
488 bodies in the same hemilineage. Solid lines represent linear fits while dotted lines represent 95% confidence  
489 interval. For NB5-2D, a significant relationship between postsynaptic targeting and birth-order was not  
490 observed.  
491 (Q) Presynaptic (blue) and postsynaptic (red) similarity plotted against birth order similarity across nine  
492 hemilineages. NB1-2V was excluded as it only contained two neurons. When examined separately, only one  
493 hemilineage (NB1-2D) did not show a significant relationship between presynaptic similarity and birth-order  
494 similarity, and only one hemilineage (NB5-2D) did not show a significant relationship between postsynaptic  
495 similarity and birth-order similarity. Solid lines represent linear fits, and dashed lines represent 95%  
496 confidence interval.  
497 (R) Summary showing hemilineage targeting setting up broad neuropil targeting and temporal information  
498 sub-regionalizing hemilineage targeting.  
499

500 **Figure 9. Temporal cohorts within hemilineages have shared connectivity**

501  
502 (A) Heatmap of connectivity between hemilineages and A1 sensory and motor neurons shows structure in  
503 hemilineage interconnectivity. Entries indicate the degree of connectivity (not the number of synapses)  
504 between each hemilineage. Edges with a strength of less than 1% of the input for a given neuron were  
505 discarded.  
506 (B,C) Fraction of inputs/outputs for each hemilineage. Adjacent bars of the same color represent the  
507 homologous hemilineage in the left and right hemisegments.  
508 (D) Force directed network graph of all neurons in the dataset highlighting the sensory and motor  
509 subdivision. Neurons with similar connectivity appear closer in network space. Purple edges represent all  
510 incoming connections to motor neurons, while green edges represent all outgoing connections from sensory  
511 neurons.  
512 (E-H) Force directed network graphs of all neurons highlighting specific lineages (E,F) or temporal cohorts  
513 (G,H). Edge colors represent outputs from given nodes.  
514 (I) Cumulative distribution of the number of synapses between temporal cohorts of hemilineage related  
515 neurons, hemilineage related neurons, or random neurons. Neurons that belonged to a temporal cohort with

516 only one neuron were not analyzed (16 neurons). Random neurons were selected from the same  
517 hemisegment.

518 (J) Quantification of the number of directly connected pairs of neurons, neurons separated by 2 synapses, and  
519 neurons separated by more than two synapses. Black circles represent pairs of neurons connected by 1  
520 synapse (top) or two synapses (bottom).

521 (K) Summary.

522  
523 **Fig. S1. The dorsal neuropil contains motor neuron post-synapses and premotor neurons pre- and post-**  
524 **synapses, whereas the ventral neuropil contains sensory neuron pre-synapses and post-sensory neuron**  
525 **pre- and post-synapses**

526 (A) Motor neuron post-synapses (purple) and sensory neuron pre-synapses (green) showing dorsoventral  
527 segregation. Plots are 1D kernel density estimates for dorsoventral or mediolateral axes. Purple dots represent  
528 a single post-synaptic site. Green dots represent a single pre-synaptic site scaled by the number of outputs  
529 from that presynaptic site.

530 (B) Premotor neuron post-synaptic sites (>3 synapses onto a motor neuron in segment A1), or post-sensory  
531 neuron pre-synaptic sites (pre >3 synapses with an A1 sensory neuron) show that connecting neurons are still  
532 restricted to dorsal or ventral neuropil domains.

533 (C) 2D kernel density estimates of all pre/post synaptic sites for pre-motor and post-sensory neurons outlines  
534 the regions of sensory (green) and motor (magenta) processing in the VNC.

535

536 **Fig. S2. Ventral hemilineages have projection neurons**

537 The indicated neuroblast lineages traced in catmaid showing the dorsal (red) and ventral (cyan) predicted  
538 hemilineages. Note that the ventral (cyan) hemilineages contains significantly longer axons (ascending and  
539 descending projection neurons) compared to dorsal (red) hemilineage neurons consistent with what has been  
540 observed in larva (Truman, 2010).  $P = .0034$ , via 2-sided Wilcoxon rank sum test.

541

542 **Fig. S3. Hemilineage identity determines synapse targeting to motor or sensory neuropil domains**

543 2D kernel density estimates for all hemilineages not shown in Figure 4. Density maps are of post-synaptic and  
544 pre-synaptic densities for four neuroblast lineages. Note the restricted domains, and how both pre- and post-  
545 synaptic sites remain in the same functional neuropil domain. Green and magenta regions represent density  
546 estimates for the pre-motor and post-sensory neurons for segment A1. Posterior view, dorsal up, midline  
547 dashed line.

548

549 **Fig. S4. Known Hb+ or Cas+ neurons identified in the TEM reconstruction**

550 Cyan: neurons known to be Hb+. Magenta, neurons known to be Cas+. Posterior view, midline, dashed line;  
551 inset, dorsal view, anterior up.

552

553 **Fig. S5. Neurons with a common temporal identity project widely within the neuropil**

554 (A-F) Skeletons of 6 lineages colored by inferred birth order (cyan, early-born) to (magenta, late-born).  
555 Posterior view, dorsal up.

556 (G) Quantification of cortex neurite length in each neuroblast lineage.

557 (H) Overlay of all six lineages; note the intermingling of early- and late-born neuronal projections.

558 (I,J) Pre- or post-synapse distributions of neurons position labeled by neuronal temporal identity; note the  
559 intermingling of synapses from early- and late-born neurons.

560

561 **Fig. S6. Neurons in a hemilineage have more similar synaptic targeting than neurons in a temporal**  
562 **cohort**

563 (A) Combined synapse similarity clustering similar to Figure 5E. Neuron names are colored either by  
564 hemilineage or by temporal cohort. Note the lack of coherent clusters of temporally-related neurons from  
565 different hemilineages.

566 (B) Mean combined synapse similarity of neurons from hemilineages or temporal cohorts. Mean similarity  
567 was calculated by randomly selecting pairs of neurons in the same hemilineage or the same temporal cohort  
568 100 times.  $p < .0001$  via 2-sided Wilcoxon rank sum test.

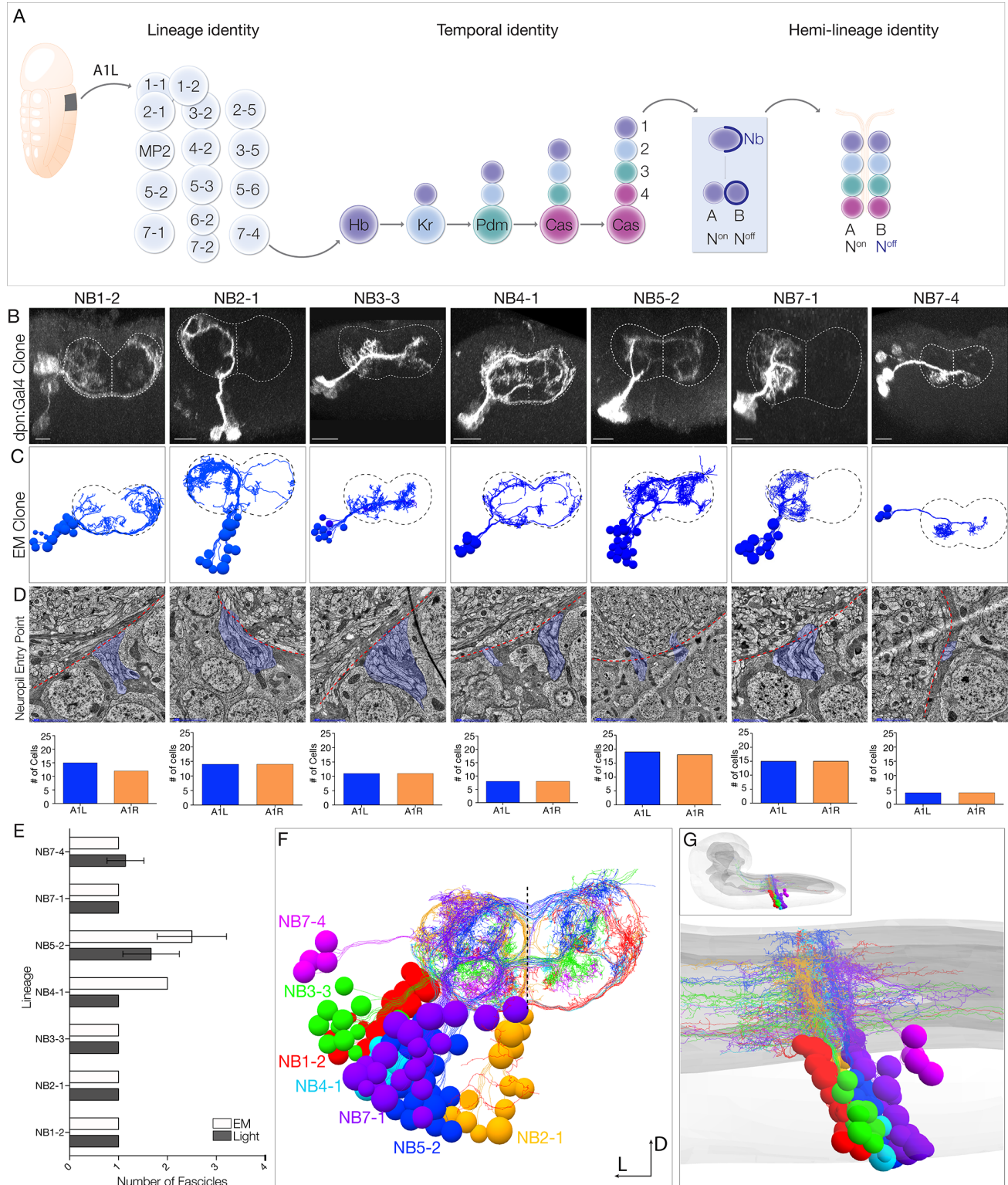
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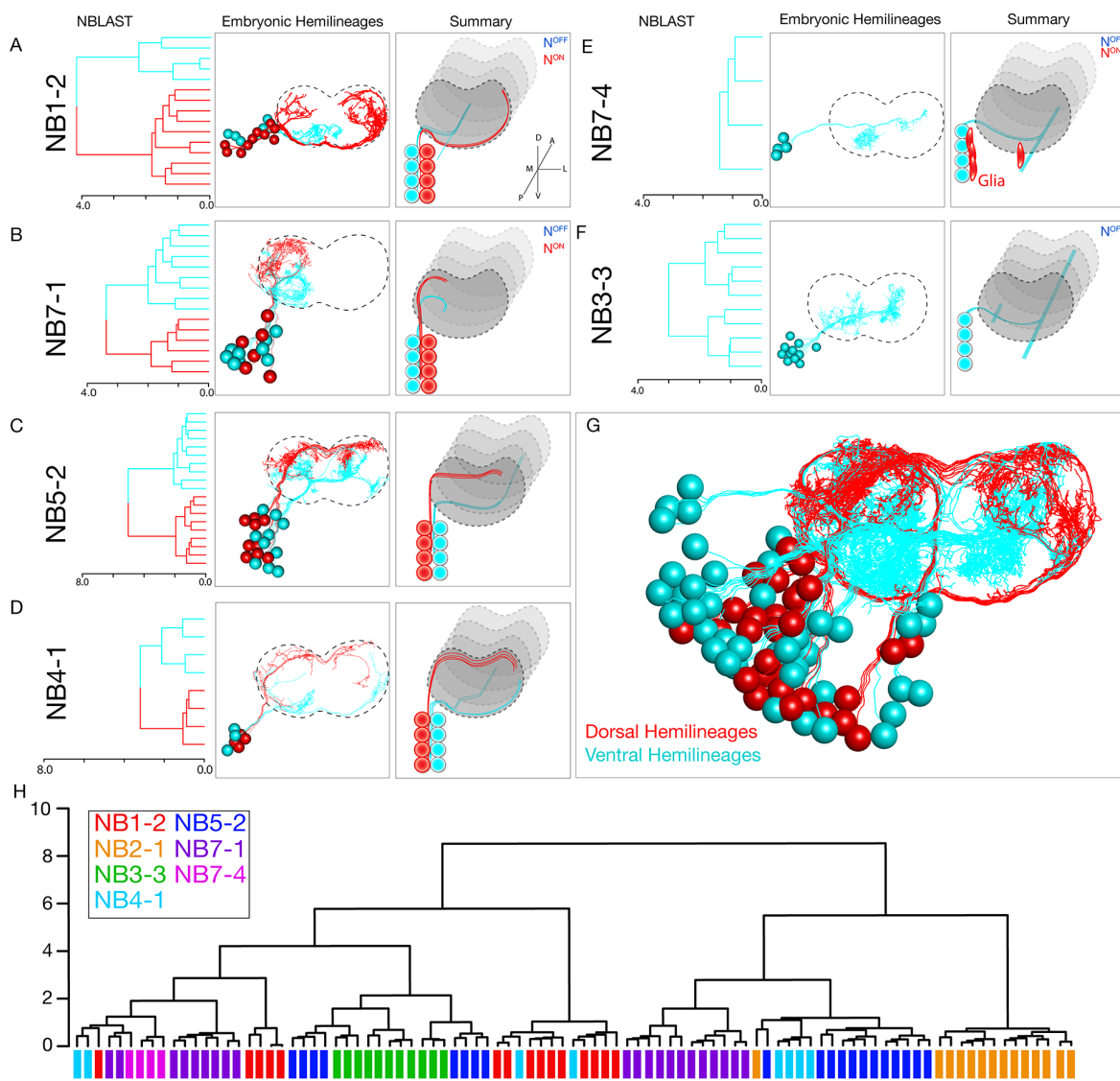
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Figure 1



671 Figure 2

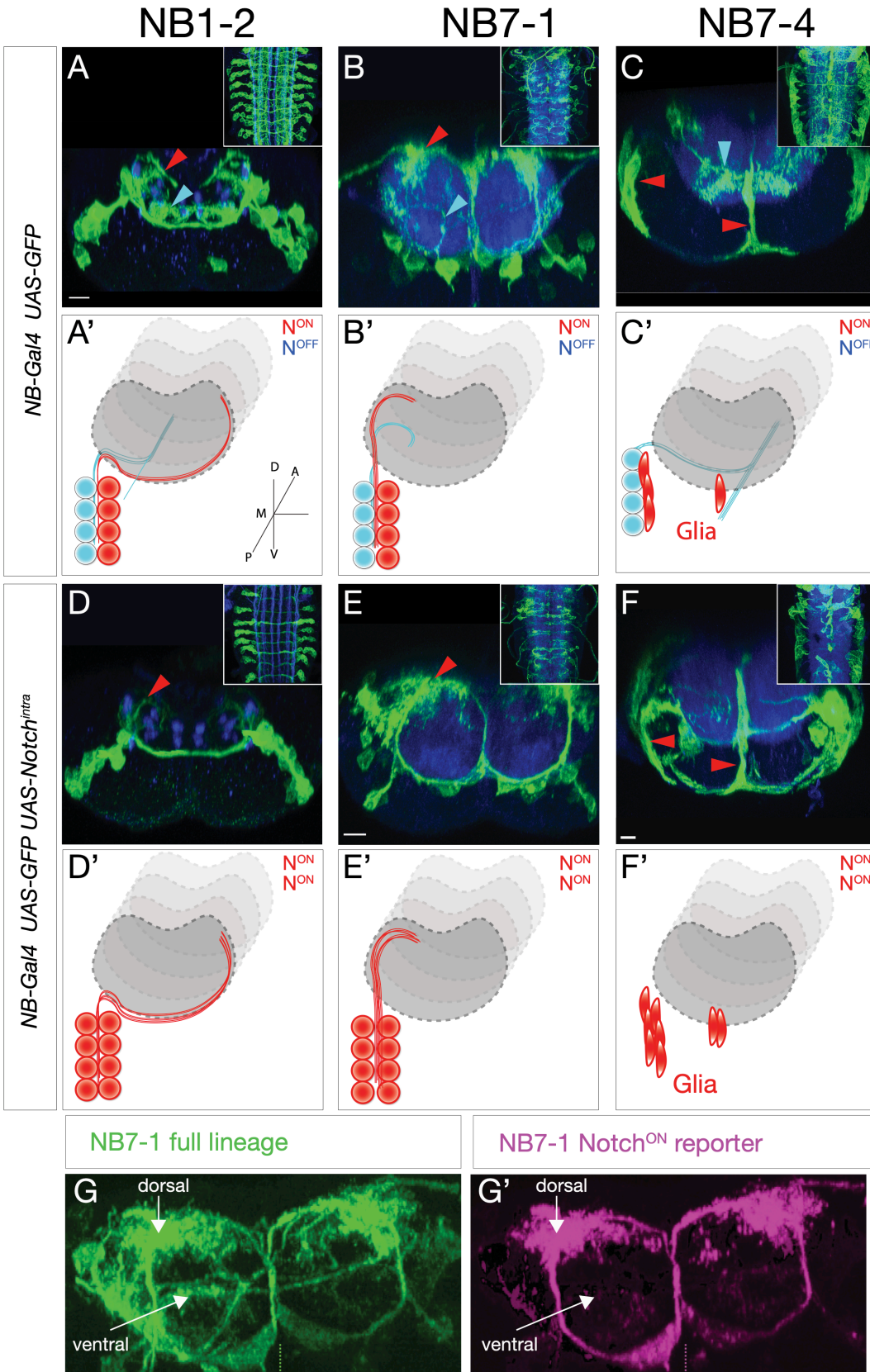
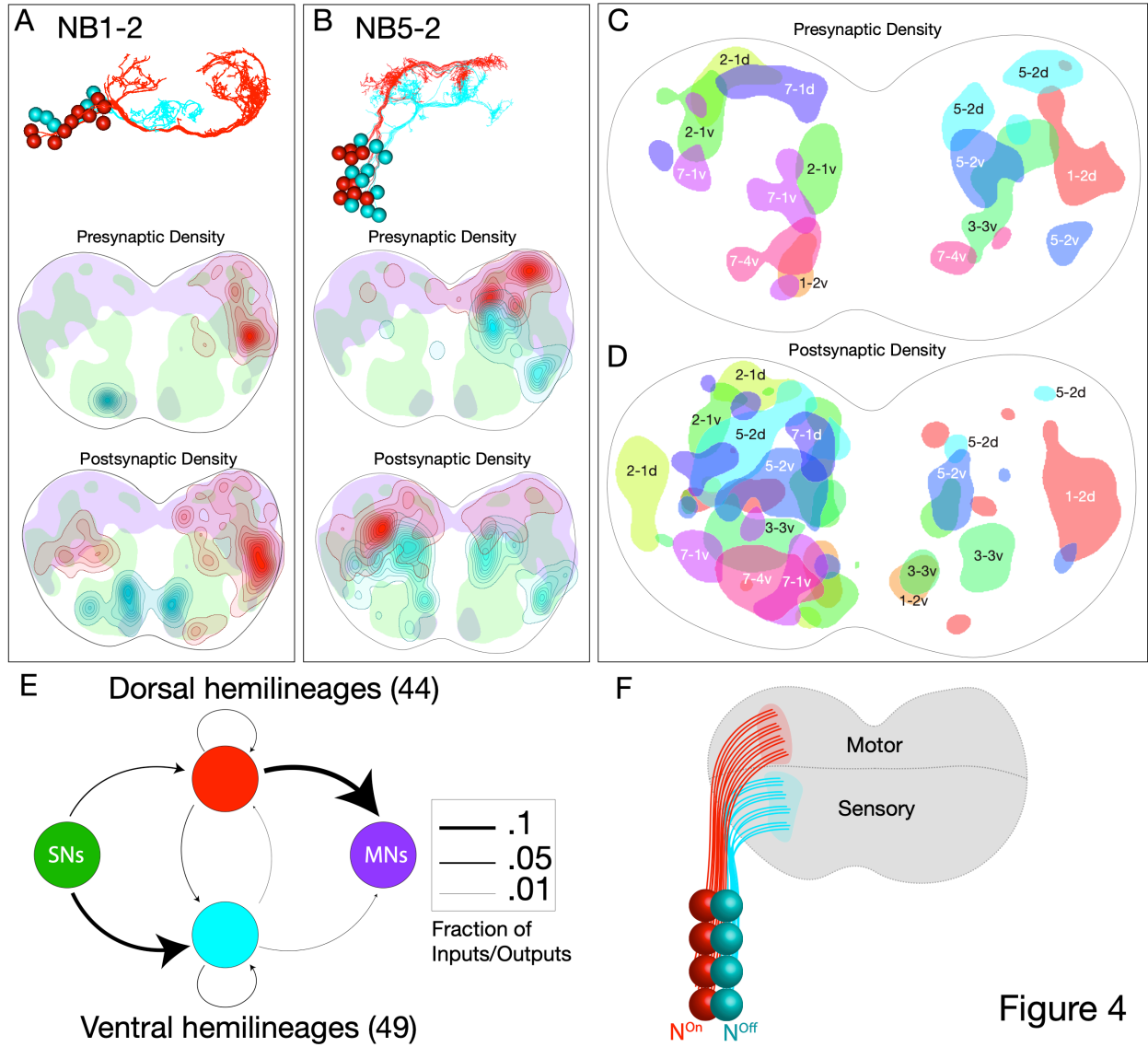
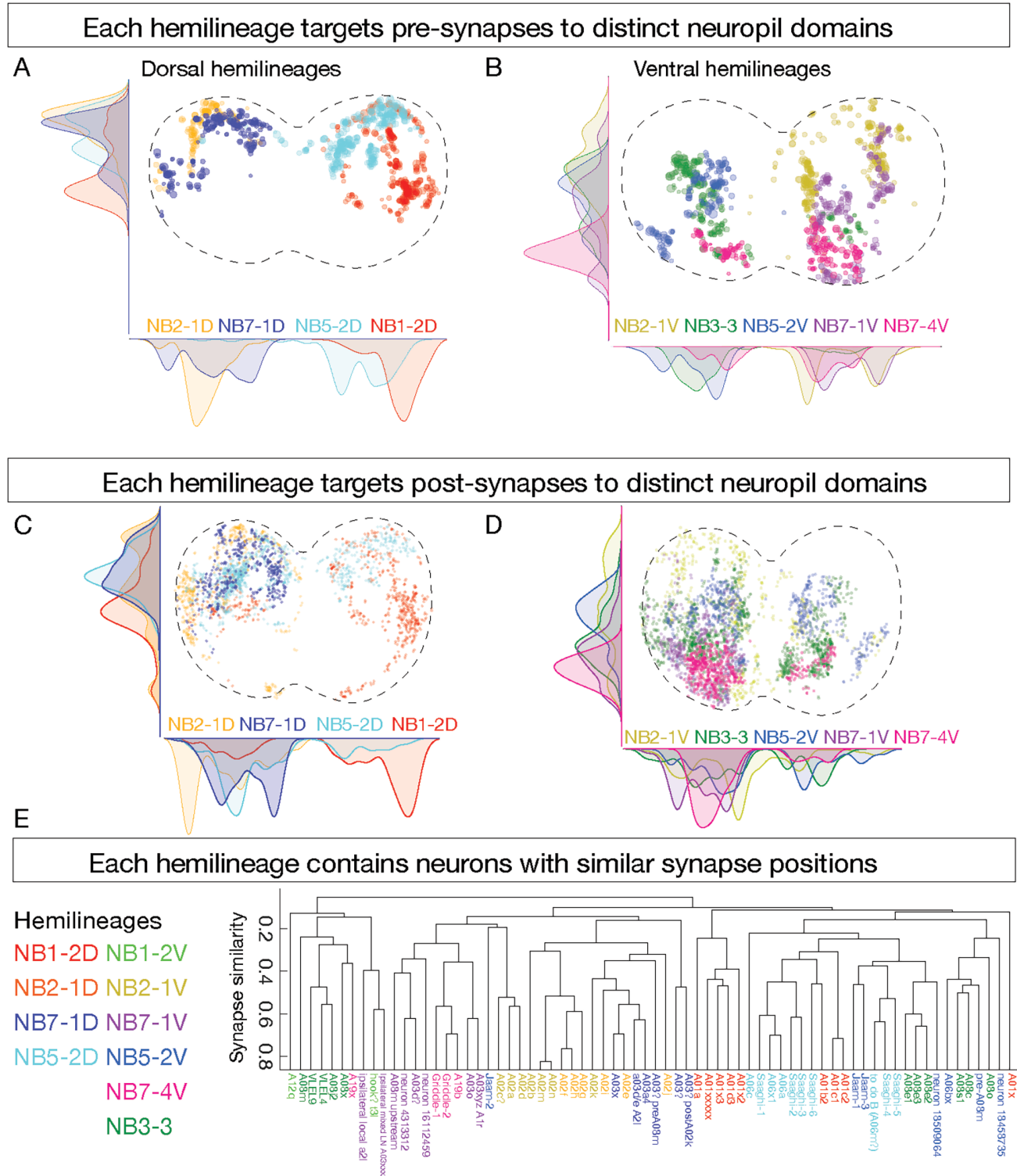


Figure 3

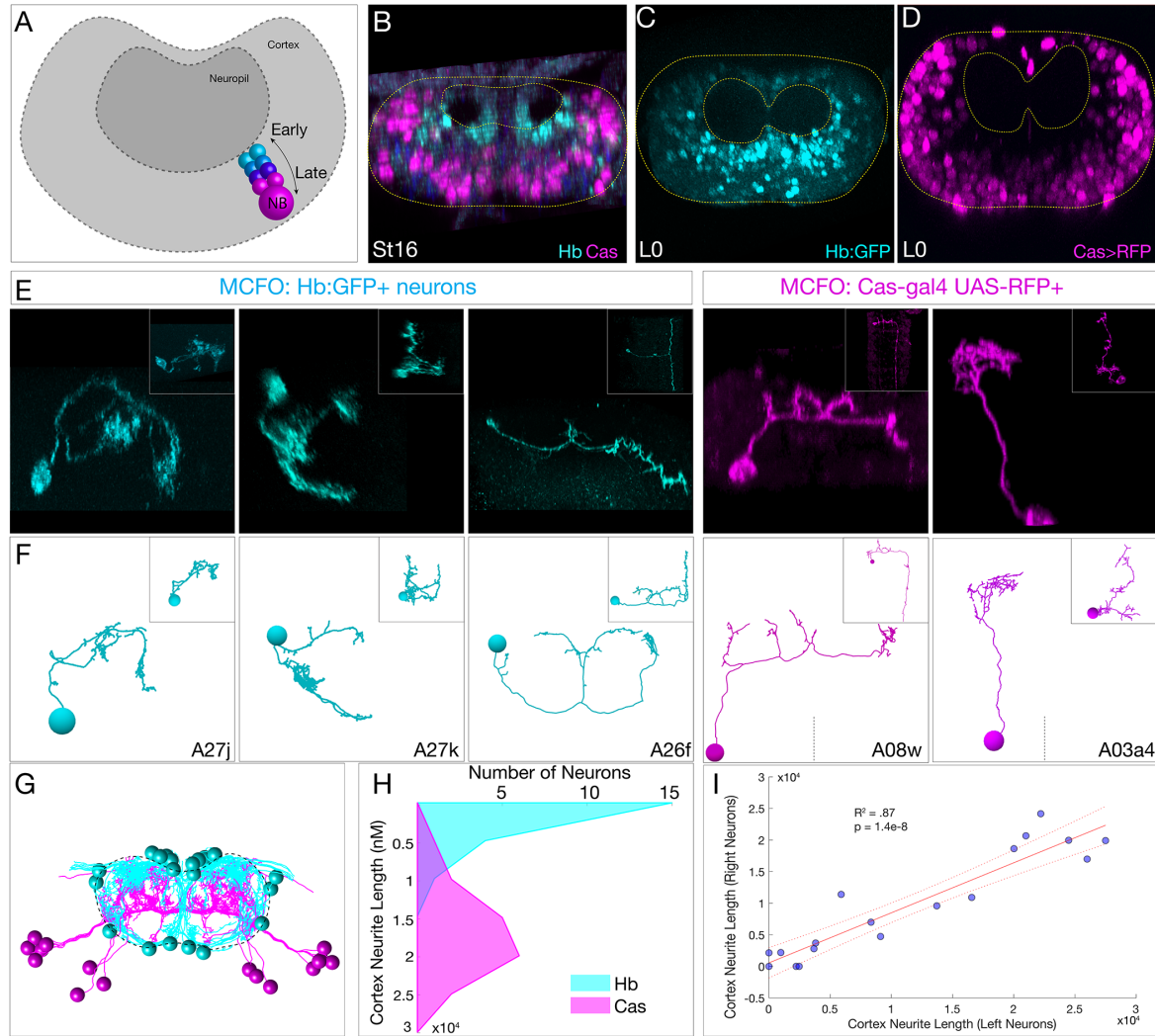


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675

Figure 5



676

Figure 6

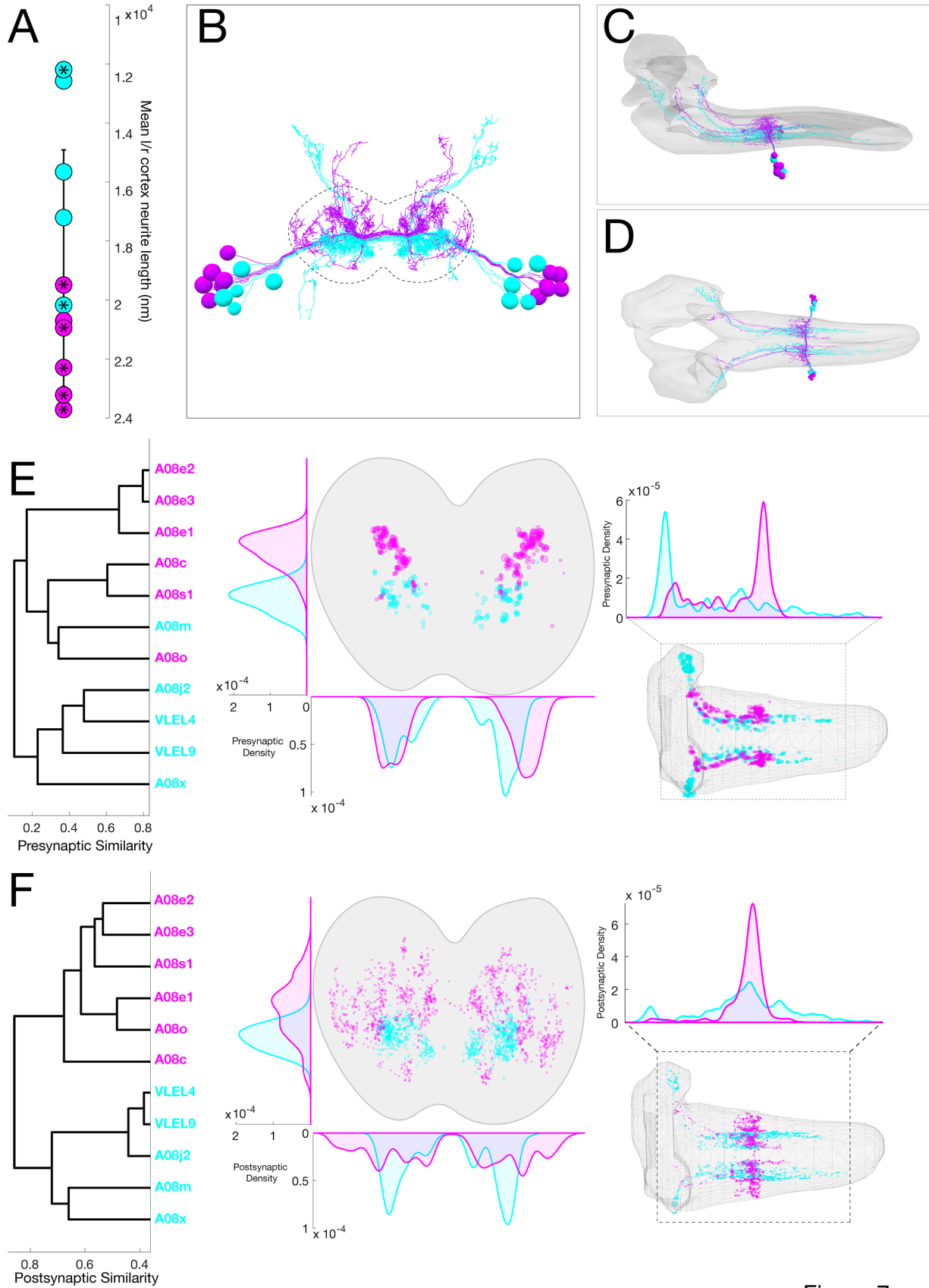
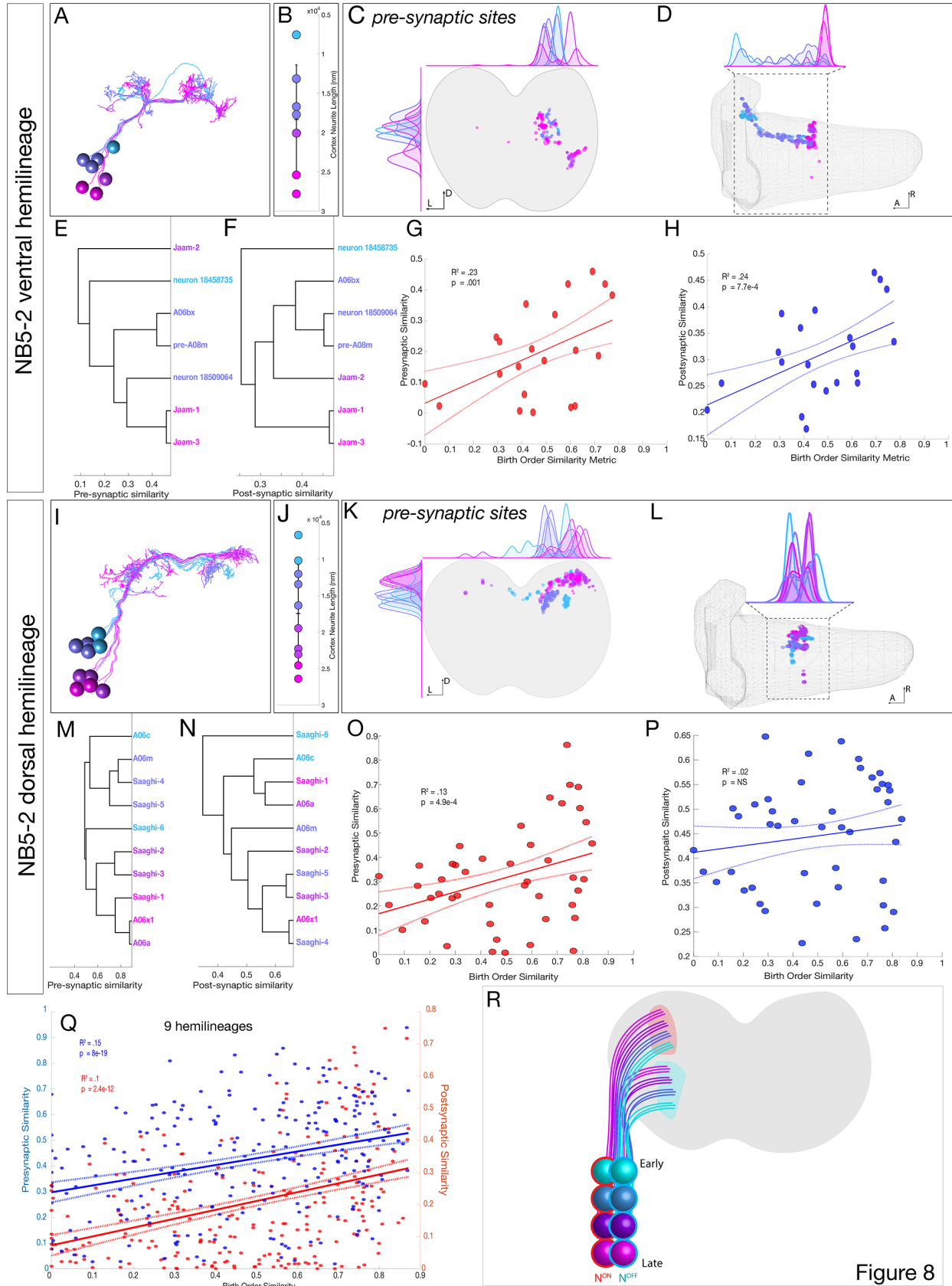


Figure 7





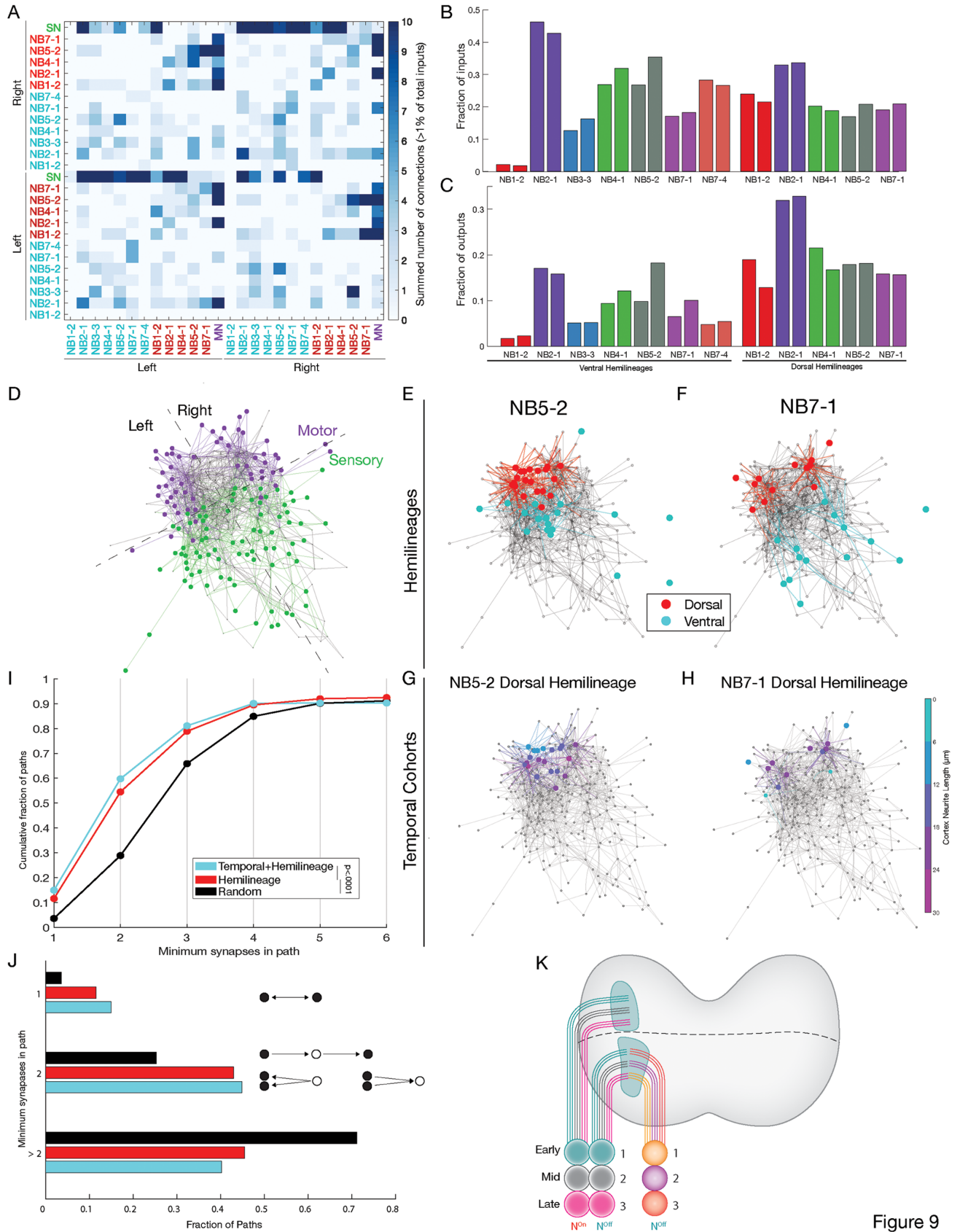
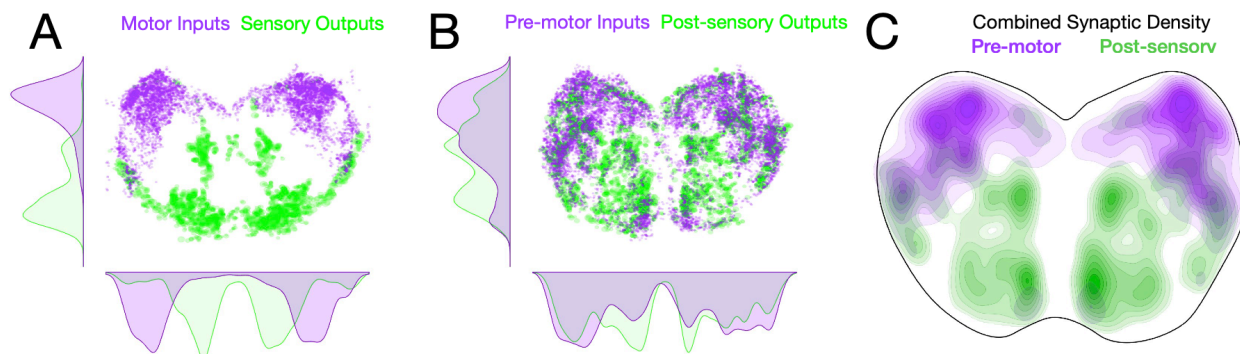


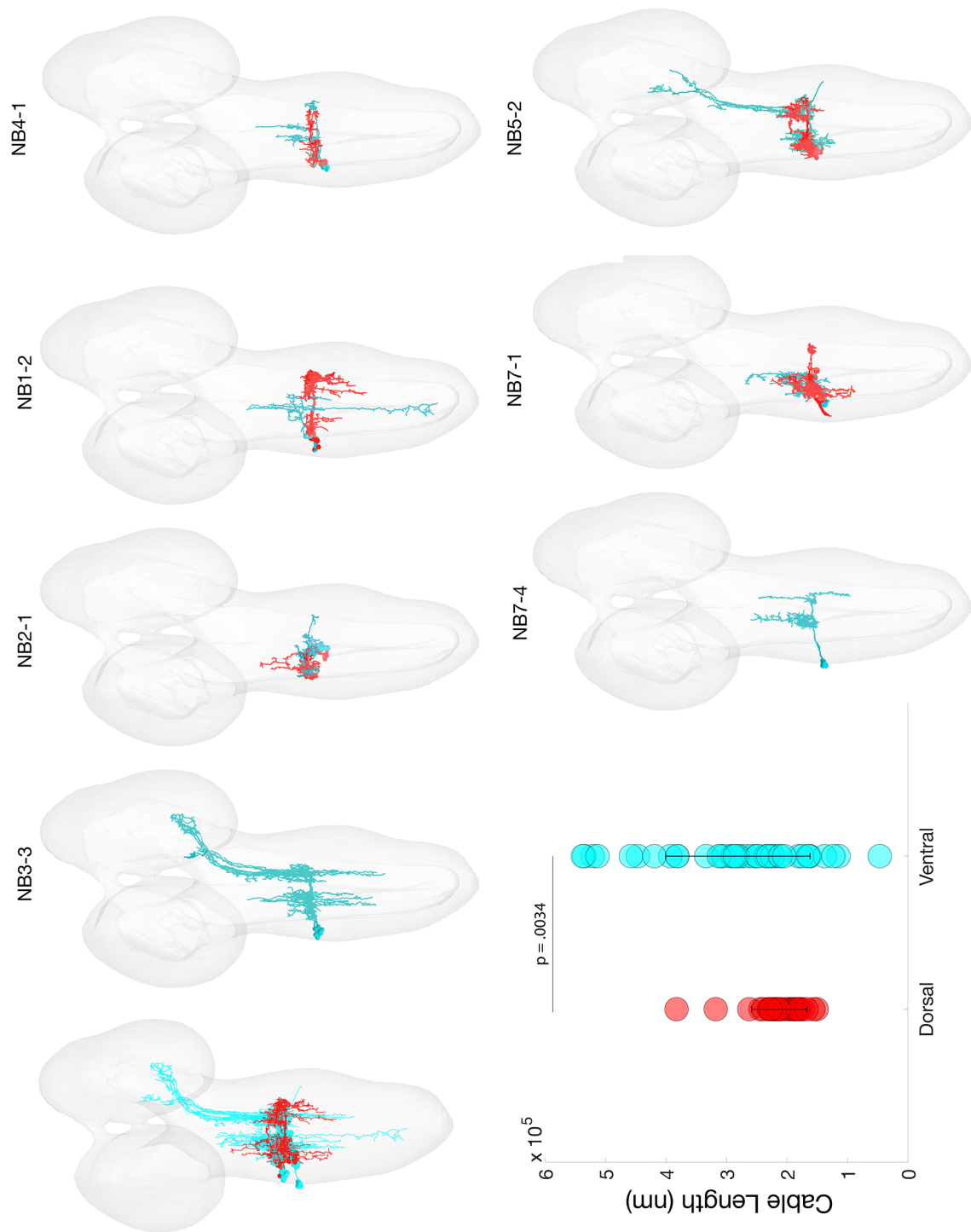
Figure 9

Pre-motor and post-sensory neuronal synapses localize to dorsal/ventral neuropil respectively



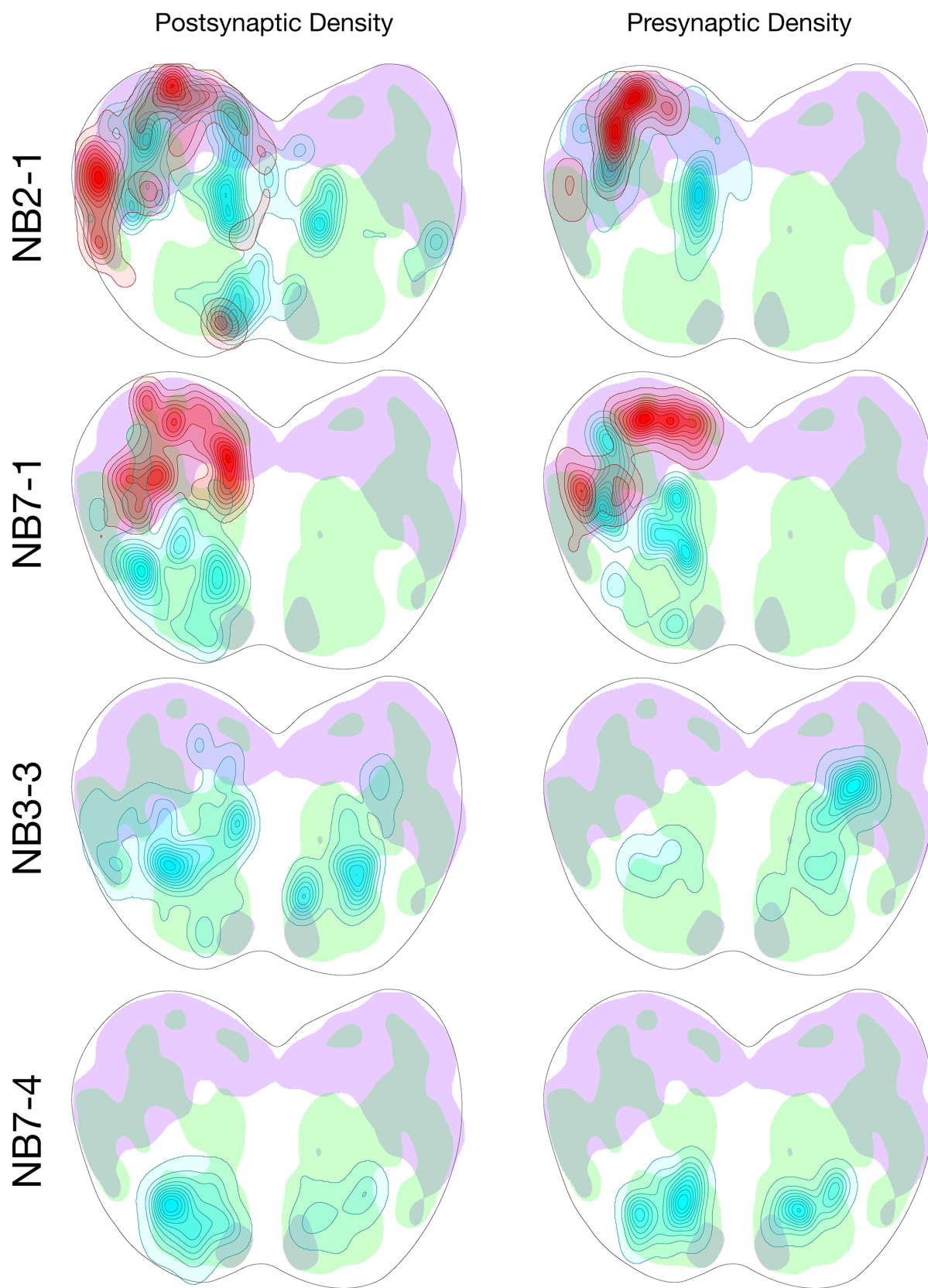
Supplemental Figure 1

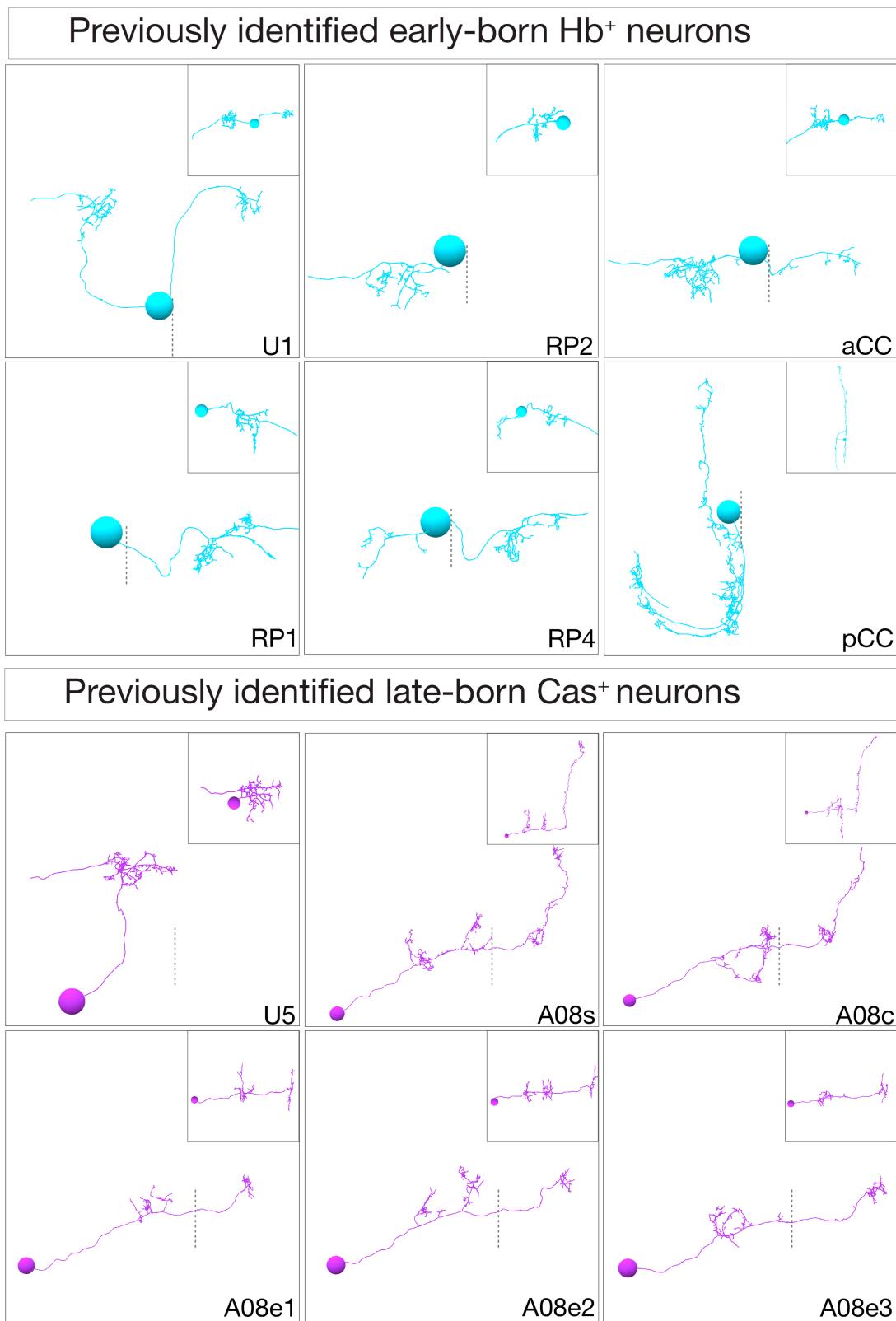
680



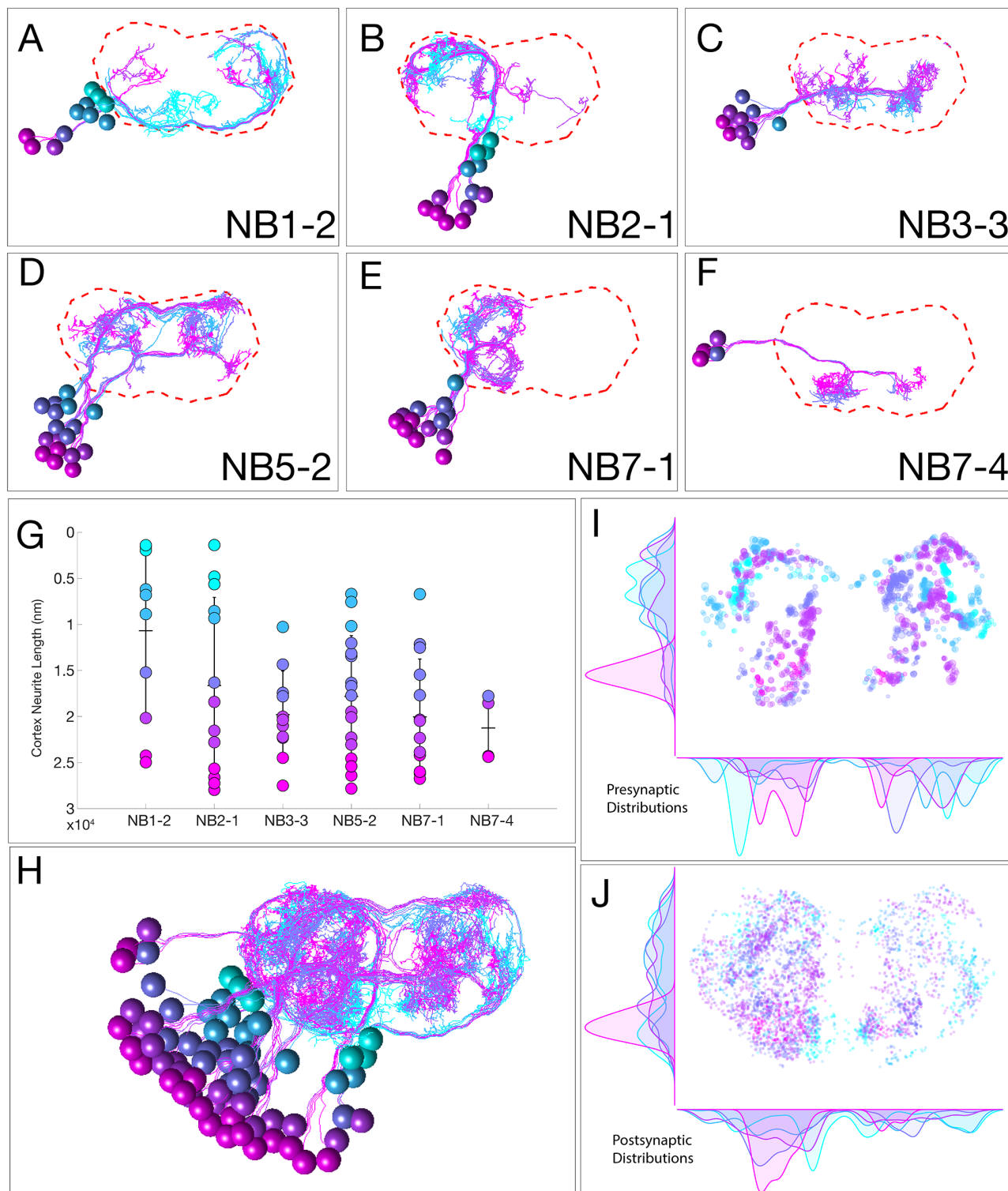
681

Supplemental Figure 2





Supplemental Figure 4



Supplemental Figure 5