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1 **A complete temporal transcription factor series in the fly**  
2 **visual system**

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34

35

36 The brain consists of thousands of neuronal types that are generated by stem cells  
37 producing different neuronal types as they age. In *Drosophila*, this temporal  
38 patterning is driven by the successive expression of temporal transcription factors  
39 (tTFs)<sup>1-3</sup>. We used single-cell mRNA sequencing to identify the complete series of  
40 tTFs that specify most *Drosophila* optic lobe neurons. We verify that tTFs regulate  
41 the progression of the series by activating the next tTF(s) and repressing the  
42 previous one(s), and also identify more complex regulations. Moreover, we  
43 establish the temporal window of origin and birth order of each neuronal type in the  
44 medulla and provide evidence that these tTFs are sufficient to explain the  
45 generation of all the neuronal diversity in this brain region. Finally, we describe the  
46 first steps of neuronal differentiation. We find that terminal differentiation genes,  
47 such as neurotransmitter-related genes, are present as transcripts, but not as  
48 proteins, in immature larval neurons; we show that these steps are conserved in  
49 humans. This comprehensive analysis of a temporal series of tTFs in the optic lobe  
50 offers mechanistic insights into how tTF series are regulated, and how they can  
51 lead to the generation of a complete set of neurons.

52

53

54 The brain is the most complex organ of the animal body: the human brain consists of over  
55 80 billion neurons<sup>4</sup> that belong to thousands of neuronal types. As neural stem cells age,  
56 temporal patterning allows them to generate different neuronal types in the correct order  
57 and stoichiometry<sup>1-3,5-7</sup>. Temporal patterning in neuronal systems was first described in  
58 the *Drosophila* ventral nerve cord (VNC), where a cascade of temporal transcription  
59 factors (tTFs) is expressed in embryonic neural stem cells (neuroblasts) as they divide  
60 and age<sup>8-10</sup>. This concept was later expanded to the *Drosophila* optic lobe, with a different  
61 tTF series. It was later suggested that tTFs also contribute to the generation of neuronal  
62 diversity in different mammalian neuronal tissues, such as the retina<sup>11-14</sup> and the cortex<sup>15</sup>.  
63 However, series of tTFs are incomplete, as they were discovered by relying on existing  
64 antibodies. To generate a comprehensive description of the tTFs patterning a neural  
65 structure we have used single-cell mRNA sequencing (scRNASeq) of the larval fly optic  
66 lobe.

67 The *Drosophila* optic lobe is an ideal system to address how neuronal diversity is  
68 generated and how neurons proceed to differentiate. It is an experimentally manageable,  
69 albeit complex structure, for which we have a very comprehensive catalogue of neuronal  
70 cell types. Meticulous work from the last decades has identified multiple cell types in the  
71 optic lobes based solely on morphological characters<sup>16</sup>. Recent work took advantage of  
72 elaborate molecular genetic tools, as well as scRNASeq, to expand the number of  
73 neuronal cell types to ~200, based on both morphology and molecular identity<sup>17-19</sup>.  
74 Importantly, the neuroblasts that generate the medulla, which is the largest optic lobe  
75 neuropil containing ~100 neuronal types, are formed by a wave of neurogenesis over a  
76 period of days<sup>20,21</sup> and progress through the same tTF temporal series<sup>22,23</sup>. This means  
77 that at any given developmental stage from mid third larval stage (L3) to early pupal stages  
78 (P15) the neurogenic region contains neuroblasts at all developmental stages (Figure 1a).

79 **Medulla neuroblast temporal series**

80 To study neuroblast and neuronal trajectories, we performed scRNASeq on the optic  
81 lobes. We obtained 49,893 single-cell transcriptomes from 40 L3 optic lobes (Extended  
82 Data Figure 1). The Outer Proliferation Center (OPC) neuroepithelium generates two optic  
83 lobe neuropils: the medulla from the medial side and the lamina from the lateral side<sup>20</sup>  
84 (Figure 1a). Medulla neuroepithelium, neuroblasts, intermediate precursors (called GMCs)  
85 and neurons were arranged in the UMAP<sup>24</sup> following a progression that resembled their  
86 differentiation *in vivo* (Figure 1b – Extended Data Figure 2a). Similarly, lamina  
87 neuroepithelium, precursor cells, and neurons were also arranged following a similar  
88 differentiation trajectory but in the opposite orientation of that of the medulla. The  
89 neuroblasts and the neurons that are generated from the Inner Proliferation Center (IPC)  
90 followed a different trajectory in the UMAP plot (Figure 1b).

91 We then merged the larval single-cell dataset with the annotated early pupal stage 15  
92 (P15) single-cell dataset<sup>18</sup>. The P15 neurons mapped at the tip of each of the neuronal  
93 trajectories (Figure 1c), which allowed us to identify the corresponding neuronal types. We  
94 identified neurons from all the neuropils of the optic lobe (lamina, medulla, lobula, and  
95 lobula plate), as well as a small number of neuroblasts and neurons from the central brain  
96 that were likely retained when microdissecting the optic lobe (Extended Data Figure 2b-  
97 c).

98 We then looked at the expression of the known spatial TFs in the OPC neuroepithelium  
99 and tTFs in the neuroblasts:

- 100 - The spatial TFs *Vsx1*, *Optix*, and *Rx*<sup>25</sup>, were expressed in largely non-overlapping  
101 subsets of neuroepithelial cells (Extended Data Figure 2d-e).

102 - The tTFs Homothorax (Hth), Eyeless (Ey), Sloppy-paired (Slp), D, and Tll<sup>22</sup> were  
103 expressed in neuroblast subsets that were temporally organized in the UMAP plot  
104 (Figure 1d).

105 Thus, the UMAP plot recapitulated both proliferation and differentiation axes in the  
106 developing tissue: the UMAP horizontal axis represents differentiation status, while the  
107 vertical axis represents neuroblasts progressing through their tTF series.

108 The larval scRNASeq dataset gave us the opportunity to look for all potential tTFs in an  
109 unbiased way. We isolated the medulla neuroblast cluster from the scRNASeq data and  
110 used Monocle<sup>26</sup> to reconstruct their developmental trajectory. Hth, Ey, Slp1/2, D and Tll  
111 were expressed in the previously described temporal order along the trajectory (Figure  
112 1e). We therefore examined the expression dynamics of all TFs and identified 14  
113 candidate tTFs whose expression was restricted to a specific pseudotime window,  
114 including the 6 previously known tTFs (Extended Data Figure 3). Using antibodies or *in*  
115 *situ* hybridization for the eight newly discovered candidate tTFs and those already known  
116 in medulla neuroblasts, we showed that their expression was indeed limited to restricted  
117 temporal windows (Figure 1f-l and Extended Data Figure 4), thus defining new temporal  
118 windows as the neuroblasts progress through divisions (Figure 1e).

### 119 **tTFs assume different roles in the series**

120 The previously known tTFs (except Hth) contribute to the progression of the series by  
121 activating the next tTF in the cascade and repressing the previous one<sup>22</sup>. To test which of  
122 the newly identified tTFs were involved in the progression of the temporal series (Figure  
123 2a), we generated tTF mutant neuroblast MARCM clones<sup>27</sup> or tTF RNAi knockdowns  
124 using the MZVUM-Gal4 line that is expressed in the Vsx1 domain<sup>28</sup> of the OPC (Figure 2,  
125 Extended Data Figures 5-6).

126 Early unit (Hth, Erm, Opa, Oaz):

127 Hth is expressed in the neuroepithelium and young neuroblasts, and is not required for Ey  
128 activation<sup>22</sup>. We identified two factors that regulate the expression of Ey in different  
129 manners: Erm is required to activate Ey and to inhibit Hth (Figure 2b), while Opa is  
130 required for the correct timing of Ey activation (Figure 2c). Opa also activates the  
131 expression of Oaz (Extended Data Figure 6b), which does not regulate the expression of  
132 any of the tTFs (Extended Data Figure 5f-j). Opa expression is repressed by Erm (Figure  
133 2d). Once Ey expression is initiated at the correct time by the combined action of Erm and  
134 Opa, Ey represses the expression of its activators (Figure 2e-f). Therefore, Erm is  
135 essential for the progression of the cascade, while Opa contributes to the correct timing  
136 of expression of the next tTFs.

137 Middle unit (Ey, Hbn, Slp, Scro, Opa)

138 We had previously shown that Ey activates Slp, which in turn inhibits Ey<sup>22</sup>. However, the  
139 developmental trajectory of neuroblasts uncovered a more complex situation. First, Ey  
140 activates Hbn (Figure 2g). Hbn then represses Ey and activates Slp (Figure 2h). Hbn also  
141 activates Scro and a second wave of Opa expression (Figure 2i-j). Hbn then inhibits the  
142 expression of Erm (Figure 2i) and Scro inhibits the expression of Ey (Figure 2k). Finally,  
143 Slp inhibits Hbn, Opa, and Oaz (Figure 2l-m, Extended Data Figure 6c).

144 Late unit: (D, BarH1, Tll)

145 D expression requires both Slp and Scro. We had previously shown that in *slp* mutant  
146 clones, D is not expressed<sup>22</sup>. Similarly, when Scro was knocked down by RNAi, D was not  
147 activated (Figure 2n). Scro is therefore important for the progression of the series, as it  
148 inhibits Ey and activates the expression of D. It remains expressed until the end of the  
149 neuroblast life. Once D is activated, it inhibits Slp<sup>22</sup> and activates BarH1 (Figure 2o), which  
150 in turn activates Tll (Figure 2p). Finally, similar to the inhibitory interaction between Tll and

151 D previously described<sup>22</sup>, Tll is sufficient but not necessary to inhibit BarH1 (Extended  
152 Data Figure 5n, Extended Data Figure 6j).

153 We have thus identified most, if not all, temporally expressed TFs in a developing neuronal  
154 system and show that these tTFs participate in the progression of the temporal series. We  
155 also confirmed many of these interactions by analyzing the effect of tTF mis-expression  
156 on the temporal cascade (Extended Data Figure 6d-j).

157 Besides their participation in the progression of the temporal series, tTFs regulate  
158 neuronal identity. Some tTFs are maintained in the neuronal subsets that are generated  
159 during their temporal window (Extended Data Figure 7a-a'), while others are only  
160 maintained in newly born neurons (Extended Data Figure 7a''-a'''-b). tTFs activate the  
161 expression of downstream neuronal transcription factors<sup>22,23</sup> that regulate effector genes  
162 in the absence of the tTF. To test how tTFs regulate neuronal identity, we asked whether  
163 knocking down the expression of the tTFs in neuroblasts affects the expression of  
164 neuronal transcription factors. The loss of *hth*, *ey*, and *slp* in neuroblasts leads to the loss  
165 of Bsh-, Vvl-, and Toy-positive neurons, respectively<sup>22</sup>. We show that Hbn is required for  
166 the specification of Toy, Traffic-jam (Tj) and Orthodenticle (Otd)-positive neurons  
167 (Extended Data Figure 7c-c') and Opa is required for the generation of TfAP-2-positive  
168 neurons (Extended Data Figure 7d). Therefore, Hbn and Opa, as well as Hth, Ey, and  
169 Slp<sup>22</sup>, regulate neuronal diversity not only by allowing the temporal series to progress, but  
170 also by regulating neuronal transcription factor expression.

### 171 **Temporal window of origin of medulla neurons**

172 The identified tTFs define at least 11 temporal windows, in which different neurons (and  
173 glia) are generated (Figure 3a). As they are generated, newly born neurons displace  
174 earlier born neurons away from the parent neuroblast<sup>29</sup>, creating a columnar arrangement



175 of neuronal cell bodies in the medulla cortex that represent birth order: Early born neurons  
176 are located close to the emerging medulla neuropil, while late born neurons are closer to  
177 the surface of the brain (Figure 3a')<sup>29,30</sup>. Neurons born in each temporal window express  
178 downstream effectors of tTFs (e.g., Bsh, Runt (Run) and Vvl) that were termed “concentric  
179 genes” due to their pattern of expression<sup>22,29</sup> (Figure 3a'). We used the expression of tTFs  
180 in GMCs, and previously described<sup>22,29</sup> and new concentric genes (this work) in  
181 scRNASeq neuronal clusters, together with their relative proximity in the UMAP plot  
182 (Figure 3b, Extended Data Figure 8), to assign the 105 neuronal clusters that comprise  
183 the medulla dataset (Extended Data Figure 8d) to their predicted temporal window of origin  
184 (Figure 3c and Supplementary Table 1). Proximal medulla (Pm) neurons are generated in  
185 the Hth and Hth/Opa temporal windows, while distal medulla (Dm) neurons are generated  
186 starting from the Ey temporal window. On the other hand, transmedullary (Tm) neurons  
187 are generated throughout most of the neuroblast life (Opa, Ey/Hbn and Slp temporal  
188 windows) (Figure 3c and Supplementary Table 1). Importantly, co-expression of some  
189 concentric genes is restricted to subregions of the medulla cortex, which allowed us to  
190 assign the spatial origin to several medulla neuron clusters (e.g. Extended Data Figure 8a  
191 arrowheads and Supplementary Table 1).

192 To assess the Notch status of all neuronal types, we also looked at the expression of  
193 Apterous (Ap), which is expressed in the Notch<sup>ON</sup> progeny of each GMC<sup>22</sup>. Among the 105  
194 neuronal types, 64 were Notch<sup>OFF</sup> and 41 Notch<sup>ON</sup> (Extended Data Figure 8c and  
195 Supplementary Table 1). Since a given GMC division generates one Notch<sup>ON</sup> and one  
196 Notch<sup>OFF</sup> neuron, Ap<sup>+</sup> and Ap<sup>-</sup> neurons are intermingled in the medulla cortex<sup>22</sup>.  
197 Therefore, the position in the medulla cortex of concentric TFs expressed in Notch<sup>ON</sup> and  
198 Notch<sup>OFF</sup> neurons allows us to infer sister neurons, for instance Run neurons are likely

199 sisters of TfAP-2 neurons, while early born Vvl neurons are likely sisters of Knot (Kn)  
200 neurons (Extended Data Figure 8a vii,xi).

201 Finally, we assigned neurotransmitter identity to all the medulla clusters at L3 and P15  
202 stages (Supplementary Table 1). Ap expression is highly correlated with cholinergic  
203 identity<sup>17</sup>, as nearly all Ap+, *i.e.* Notch<sup>ON</sup>, clusters in our dataset express ChAT and thus  
204 have cholinergic identity, while most of the Notch<sup>OFF</sup> clusters are either GABAergic (most  
205 of them express Lim3)<sup>17</sup> or glutamatergic (most of them express Tj or Fd59A)<sup>17</sup>.  
206 Interestingly, all the Notch<sup>OFF</sup> neurons from the same temporal window express the same  
207 neurotransmitter, independently of their spatial origin (Figure 3d and Supplementary Table  
208 1). This suggests that the temporal origin of medulla neurons and their Notch status  
209 instructs shared TF expression and neurotransmitter identity, and hence function. In  
210 summary, we defined the temporal (and spatial) origin, birth order, and Notch identity of  
211 all medulla cell types and highlight the role of tTFs in regulating the generation of neural  
212 diversity.

### 213 **Early commitment to neuronal identity**

214 To study the first steps of neuronal differentiation after specification, we merged the  
215 clusters from pupal stages (P15, P30, P40, P50, and P70) corresponding to the Mi1 cells  
216 with the L3 scRNASeq cluster and the GMCs most closely linked to them in the UMAP  
217 plot (Extended Data Figure 9a). We reconstructed their differentiation trajectory (Extended  
218 Data Figure 9b-c), identified groups of genes (modules) that co-vary along the entire  
219 trajectory from L3 to P70 and searched for the Gene Ontology (GO) terms enriched in  
220 each gene module (Figure 4a). The timing of differentiation appears to follow a specific  
221 path: At L3, cell cycle genes and DNA replication genes are first expressed, as expected  
222 from the division of GMCs. This is closely followed by genes involved in translation. Then,  
223 genes related to dendrite development and axon-guidance are upregulated from late L3

224 until P30, stages when the neurons direct their neurites to the appropriate neuropils.  
225 Genes important for neuronal function, such as neurotransmitter-related genes, synaptic  
226 transmission proteins, as well as ion channels start to be expressed as early as L3,  
227 reaching a plateau that is maintained until P15. Their expression then increases again  
228 until adulthood, when their products support neuronal function (Figure 4a). This timing of  
229 differentiation was observed not only for Mi1 but could be generalized to all optic lobe  
230 neurons (Figure 4c). These results indicate that not only is neuronal identity specified  
231 during the first hours of neuronal development, but their neuronal function (as indicated  
232 by the upregulation of chemical synaptic transmission terms) is implemented very early,  
233 although it will only be required much later. As this was unexpected, we asked whether  
234 neurotransmitter mRNA expression observed as early as late L3 was also translated into  
235 protein. Neurotransmitter-related genes, ChAT, VGluT, and Gad1 mRNA are all expressed  
236 in the scRNASeq data in non-overlapping neuronal sets (Figure 4d) and are maintained  
237 in the adult<sup>18</sup> (Supplementary Table 1). However, we did not observe protein expression  
238 at L3 (Figure 4e). This suggests that their transcription represents a commitment to a  
239 specific neurotransmitter identity early, but that other factors prevent premature translation  
240 of these mRNAs until they are needed at later stages of development.

#### 241 **Common trajectory of *Drosophila* and human neurons**

242 We then asked whether the *Drosophila* optic lobe neuronal differentiation trajectory was  
243 similar to human neuronal differentiation. We generated single-nuclear RNAseq data from  
244 the human fetal cortical plate at gestational week 19. We used Monocle3 to reconstruct  
245 their developmental trajectory (Extended Data Figure 9d-e) from apical progenitors to  
246 intermediate progenitors and postmitotic neurons (Extended Data Figure 9f) and identified  
247 gene modules that were co-regulated along the trajectory. GO analysis uncovered a  
248 remarkable similarity to *Drosophila* (Extended Data Figure 9g). We then plotted the

249 expression of the GO terms that were expressed at different stages of the differentiation  
250 trajectory in *Drosophila* on the human cortical differentiation trajectory (Figure 4b). We  
251 observed very similar dynamics; the main difference was the absence of enrichment for  
252 ribosome assembly and translation-related GO terms at early stages. This could  
253 potentially be explained by the slower development of human neurons compared to  
254 *Drosophila*, which leads to a slower increase in size and the fact that the divisions of the  
255 radial glia are more symmetric<sup>31</sup> than those of optic lobe neuroblasts. Despite this  
256 difference, these results show that neurons follow a similar differentiation trajectory in  
257 *Drosophila* and humans.

#### 258 ***Drosophila* tTFs in mouse progenitors**

259 Although temporal patterning is a universal neuronal specification mechanism<sup>32,33</sup>, it is  
260 unclear how it has evolved<sup>6,7</sup>. We asked whether the medulla tTFs were conserved in  
261 mouse cortical radial glia using a published scRNASeq dataset<sup>34</sup>. None of the medulla  
262 neuroblast tTFs were expressed in strict temporal windows in ageing radial glia, with the  
263 exception of Pax6, which was enriched in older progenitors (Extended Data Figure 10a).  
264 Reciprocally, the *Drosophila* orthologs of the mouse temporally expressed TFs<sup>33</sup> were not  
265 expressed temporally in the developing optic lobe.

266 The mouse orthologs of *Drosophila* VNC tTFs Ikzf1, Pou2f1/Pou2f2, and Casz1 are  
267 expressed temporally in mouse retinal progenitors<sup>11,12,14</sup>. We looked at the expression of  
268 the optic lobe tTFs in the mouse retina in a published scRNASeq dataset<sup>35</sup>. Pax6/Ey was  
269 constitutively expressed, Meis2/Hth, Zic5/Opa, and Sox12/D were expressed at  
270 embryonic stage 12, while Nr2e1, the ortholog of Tll (which is expressed when neuroblasts  
271 become gliogenic), was expressed late, when retinal progenitors become gliogenic and  
272 start generating Müller glia<sup>36</sup> (Extended Data Figure 10d). The lack of a strict conservation

273 of tTFs between flies and mice indicates that the acquisition of the specific temporal series  
274 occurred independently in each phylum.

## 275 **Conclusions**

276 The comprehensive series of transcription factors described in this work and their  
277 regulatory interactions temporally pattern a developing neural structure. We show that  
278 most tTFs are expressed in overlapping windows, creating combinatorial codes that  
279 differentiate neural stem cells of different ages and therefore provide them with the ability  
280 to generate diverse neurons after every division. We conservatively assigned them into  
281 11 distinct temporal windows (10 of which generate neurons), which, when integrated with  
282 spatial patterning (6 spatial domains) and the Notch binary cell fate decision, can explain  
283 the generation of ~120 cell types, which is close to the entire neuronal type diversity of the  
284 *Drosophila* medulla. Moreover, we determined the downstream TFs that were expressed  
285 in neurons produced temporally, which allowed us to establish the birth order of all medulla  
286 neurons. Additionally, we provide a detailed transcriptomic description of the first steps in  
287 the differentiation trajectory of a neuron. Terminal differentiation genes are expressed  
288 within the first 20 hours of neuronal life, approximately 2-4 days before their protein  
289 products can fulfil their function. Why these genes are expressed so early remains  
290 unknown, but we hypothesize that this reflects the commitment of neurons to a specific  
291 function. We also show that all neurons follow the same route for differentiation and that  
292 this is similar to the differentiation process in developing human cortical neurons. Hence,  
293 understanding the mechanisms of neuronal differentiation in flies can generate insight for  
294 the equivalent process in humans (see also Supplementary Discussion).

295

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### 310 **Author contributions**

311 N.K., I.H., A.M.R., and C.D. designed the project. N.K., I.H., A.M.R., A.E., and T.T  
312 performed the genetic experiments. N.K., I.H., A.M.R., A.E., L.D., T.T, A.M.J., and Y.C.C.,  
313 analyzed the data. N.K., M.N.O., and F.S. acquired the fly scRNA-seq data. N.M.T., J.F.F.,  
314 Z.S, and P.R. acquired the human scRNA-seq data. U.W. generated the *hbn* mutant. N.K.,  
315 L.D., A.M.J., and Y.C.C. performed scRNA-seq data analysis. N.K., I.H., A.M.R., and C.D.  
316 wrote the manuscript. All authors edited the manuscript.

### 317 **Declaration of Interests**

318 Authors declare no conflicts of interest.

319

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418 **Figure 1. Single-cell sequencing of the developing *Drosophila* larval optic lobe.**

419 (a) Schematic of the *Drosophila* L3 brain. The neuroepithelium is converted to lamina  
420 precursor cells (LPCs) laterally, and into medulla neuroblasts medially, in a wave of  
421 neurogenesis (youngest neuroblasts are closer to the neuroepithelium). As neuroblasts  
422 age, they produce different neuronal types (filled circles: Notch<sup>ON</sup>, outlined circles:  
423 Notch<sup>OFF</sup> neurons). The dashed arrow points from young to old neurons.

424 (b) UMAP plot of ~70,000 single-cell transcriptomes from the integrated L3 and P15 optic  
425 lobe dataset. The arrows depict differentiation trajectories (medulla: black solid arrow,  
426 lamina: dashed arrow, lobula plate: brown arrow). GMCs form an hourglass (left red arrow)  
427 with reduced transcriptomic diversity. A similar hourglass is observed in the transition from  
428 neuroepithelium to neuroblasts (right red arrow). The dashed box contains neuroblasts  
429 and GMCs shown in d.

430 (c) Same UMAP plot as in b with L3 (pink) and P15 datasets (cyan) labeled.

431 (d) Close-up of the UMAP plot showing neuroblasts and GMCs. The previously described  
432 temporal factors are expressed in partially overlapping sets of neuroblasts organized in  
433 the plot from bottom to top (dashed arrow). A row of neuroblasts that do not express a  
434 known tTF (arrowhead), succeeds the *hth*-positive neuroblasts.

435 (e) Temporal expression of previously known (purple) and candidate (green) tTFs along  
436 the neuroblast developmental trajectory. Heatmap colors represent scaled expression  
437 along the trajectory.

438 (f-l) Immunostainings of newly identified (green) and previously known (purple) tTFs. (f)  
439 Opa:V5<sup>37</sup> is expressed in two waves, one after Hth (arrowhead) and one before Slp  
440 (arrow). (g) Oaz is expressed after Hth (arrowhead) and until Slp (arrow). (h) Erm is

441 expressed after Hth. (i) *Esg* is expressed in a salt-and-pepper manner within the *Ey*  
442 window. (j) *Hbn* is expressed before *Slp*. (k) *Scro* is expressed after *Ey* (arrowhead). (l)  
443 *BarH1* is expressed after *D* (arrowheads). Arrows and arrowheads point to overlapping  
444 expression. Scale bars: 10  $\mu$ m

445 **Figure 2. Complex genetic interactions between tTFs control the progression of the**  
446 **temporal series.**

447 (a) The temporal series is subdivided into three units (early: green, middle: red and late:  
448 blue) by previously identified (purple) and new (green) tTFs.

449 (b) In *erm* MARCM mutant clones (GFP: green), *Hth* is extended and *Ey* is lost.

450 (c) In *opa* MARCM mutant clones (GFP: green), *Erm* is unaffected and *Ey* expression is  
451 delayed.

452 (d) In *erm* MARCM mutant clones (GFP: green), *Opa* is extended.

453 (e) Negatively labeled *ey* MARCM mutant clones (GFP-negative) express *Erm* beyond its  
454 temporal window.

455 (f) *ey* MARCM mutant clones (GFP-negative) express *Opa* beyond its temporal window.

456 (g) In *ey* MARCM mutant clones (GFP-negative), *Hbn* expression is lost.

457 (h) In *hbn* MARCM mutant clones (GFP: green), *Ey* expression is extended, while *Slp*  
458 expression is lost.

459 (i) In *hbn* MARCM mutant clones (GFP: green), *Erm* is expanded and *Scro* expression is  
460 lost.

461 (j) The second *Opa* expression window is lost in *hbn* mutant clones (GFP: green).

462 (k) Cells expressing *scro* RNAi (GFP+: green; *scro*-) continue to express Ey into later  
463 temporal windows.

464 (l) In *slp* MARCM mutant clones (GFP: green) Hbn expression is extended without  
465 affecting *Scro*.

466 (m) In *slp* MARCM mutant clones (GFP: green), *Opa* extends to later temporal windows.

467 (n) Left: In neuroblasts expressing *scro* RNAi (GFP+: green), *D* expression is lost. Right:  
468 Neurons coming from the *D* temporal window express *D* (green bracket). In the absence  
469 of *Scro*, *D* is no longer expressed (red bracket). Wild-type (green asterisk) and *scro*-RNAi  
470 expressing neuroblasts (*D*-, red asterisk) can be found in the surface.

471 (o) In negatively marked *D* mutant clones (GFP-negative), *BarH1* expression is lost and  
472 *Scro* expression is unaffected.

473 (p) In comparison to wild-type NBs (red asterisk), NBs expressing *BarH1* RNAi (GFP:  
474 green) (green asterisk) do not express *Tll*.

475 Genetic interactions uncovered in each experiment are shown for each panel. Scale bars:  
476 10  $\mu$ m

### 477 **Figure 3. Temporal window of origin and birth order of medulla neurons**

478 (a) Left: Frontal view of L3 medulla NBs sequentially expressing the indicated tTFs. Right:  
479 Schematic of tTF expression as medulla neuroblasts age, based on immunostaining.  
480 Dashed lines represent predicted temporal windows.

481 (a') Left: Sagittal section of L3 medulla cortex showing the expression of the concentric  
482 genes *Bsh*, *Run*, *Vvl* and *Toy*, and the tTF *Slp*. Early born neurons are located closer to  
483 the medulla neuropil, while later born neurons are farther. Right: Schematic of concentric

484 gene expression in medulla neurons activated and maintained through different temporal  
485 windows.

486 (b) Sagittal sections of optic lobes showing the expression of concentric genes at L3 stage.  
487 Anterior is to the right and posterior to the left. Earlier born neurons are located closer to  
488 the medulla neuropil (dashed crescent). Early, middle and late subdivisions are based on  
489 the predicted temporal windows when a given concentric gene starts to be expressed.  
490 Medulla neurons that express the corresponding concentric gene are indicated below  
491 each panel. Tj (ix) and Fork head (Fkh:GFP) (x) expression is restricted to specific regions  
492 of the medulla cortex. Hbn (xii) is also expressed in lamina wide-field neurons (Lawf1-2,  
493 arrowheads) that originate from the Dpp regions of the lamina neuroepithelium and  
494 migrate along the medulla neuropil<sup>38</sup>. Dll (xiv) is also expressed in medulla neuropil glia  
495 (mng, arrowhead)<sup>22</sup>.

496 (c) UMAP plot showing differently colored medulla clusters from the P15 dataset indicating  
497 groups that share concentric gene expression within a given temporal window. Cluster  
498 identity is indicated when known.

499 (d) Schematic representing tTF expression in medulla NBs and GMCs and downstream  
500 concentric gene expression in neurons. Neurons and glia predicted to be generated in  
501 each temporal window are indicated. Neurons sharing the same Notch status generated  
502 in the same temporal window express the same neurotransmitter (NT).

503 Scale bar: 10  $\mu$ m

#### 504 **Figure 4. Neuronal differentiation in flies and humans**

505 (a) Average expression of genes belonging to different Gene Ontology (GO) terms during  
506 the differentiation trajectory of Mi1 neurons. DNA replication is enriched at the beginning

507 of the trajectory, followed by ribosome assembly and translation-related terms. Then,  
508 terms involved in neurite development and neuropil targeting, such as dendrite  
509 development and axon guidance, are enriched and peak around P15 and P30. Neuronal  
510 function terms, such as regulation of membrane potential and neurotransmission start to  
511 be upregulated as early as L3, reach a plateau around P15, and increase drastically from  
512 P30.

513 (b) Average expression of genes belonging to the *Drosophila* GO terms that were  
514 described in “a”, over pseudotime in human cortical neurons. The differentiation path is  
515 similar to *Drosophila* neurons, with the exception of ribosome assembly terms, which are  
516 not enriched in human cortical neurons.

517 (c) UMAP plots containing L3 and P15 single cells showing the developmental progression  
518 in Mi1s neurons (top – dashed arrows), which is similar for all optic lobe neurons (bottom),  
519 including lamina and lobula plate neurons. Ribosome assembly, axon guidance and  
520 regulation of membrane potential terms are sequentially upregulated.

521 (d) UMAP plot showing the expression of *ChAT* (green), *VGlut* (blue), and *Gad1* (red), as  
522 markers for cholinergic, glutamatergic, and GABAergic cells, respectively, in medulla  
523 (brown), lamina (purple) and lobula plate (orange) neurons. These transcripts are already  
524 detected in cells at L3 stage.

525 (e) Immunostaining of VGlut (purple), ChAT (white) and Elav (green), in the developing  
526 *Drosophila* central nervous system at L3 stage. Neurons in the medulla (dashed line, m)  
527 express Elav (green) but do not express VGLuT or ChAT protein in the neuropil (n),  
528 although the transcripts are present at this stage. However, VGlut and ChAT are  
529 expressed in the projections of mature larval neurons (also marked by Elav) of the ventral  
530 nerve cord (arrow). Scale bar:20  $\mu$ m.

531 **Methods**

532 **Genetics**

533 To generate MARCM clones, crosses were kept at 25 °C and were heat-shocked for one  
534 hour at 37 °C four days before dissecting wandering L3 larvae. For RNAi experiments,  
535 MzVUM-Gal4 (Vsx-Gal4) flies were crossed to flies carrying the RNAi construct; the  
536 crosses were kept at 25 °C before dissecting wandering L3 larvae. The crosses are  
537 indicated below:

538 hth RNAi: *MzVUM-Gal4; UAS-CD8.GFP*; flies were crossed with *;hth-RNAi*; flies

539 Oaz RNAi: *MzVUM-Gal4; UAS-CD8.GFP*; flies were crossed with *yscvsev;; Oaz-RNAi*  
540 flies

541 scro RNAi: *MzVUM-Gal4; UAS-CD8.GFP*; flies were crossed with *yv;; scro-RNAi* flies

542 *BarH1* RNAi: *MzVUM-Gal4; UAS-CD8.GFP*; flies were crossed with *;BarH1-RNAi*; flies

543 erm- MARCM clones: *;erm1, FRT40A/CyO,act-GFP*; flies were crossed with *UAS-*  
544 *CD8GFP, hs-flp; FRT40A, tub-Gal80; tub-Gal4/TM6B* flies.

545 opa- MARCM clones: *;;FRT82B - opa(null)/TM6B* flies were crossed with *yw, hs-flp, UAS-*  
546 *GFP;; tub-Gal4, FRT82B, tub-Gal80/TM6C* flies.

547 ey- MARCM clones: *yw, hs-flp122; +/(CyO); FRT80B/TM6B; ey[j5.71]/In(4)* flies were  
548 crossed with *yw, hs-flp122; +/cyo; FRT80B ey-rescue (y+) ubiGFP/TM6B; ey [J5.71]/In(4)*  
549 flies.

550 D- MARCM clones: *yw; If/CyO; D[87],FRT2A/TM6B* flies were crossed with *yw, hs-flp;*  
551 *if/cyo; FRT2A, ubi-nlsGFP/TM6B* flies.



552 hbn- MARCM clones: *FRT42B(G13)*, *hbn15227* flies were crossed with *yw*, *hs-flp*;  
553 *FRT42B(G13)*, *tub-Gal80/CyO*, *act-GFP*; *tub-Gal4*, *UAS-CD8GFP/TM6*, *Tb,Hu* flies.

554 slp- MARCM clones: *yw*, *hs-flp122*; *slp[s37a]*, *FRT40A/SM6~TM6B* flies were crossed with  
555 *UAS-CD8GFP*, *hs-flp*; *FRT40A*, *tub-Gal80*; *tub-Gal4/TM6B* flies.

556 tll- MARCM clones: *w;;* *FRT82B*, *tll[149]/TM3,GFP*, *Ser* flies were crossed with *yw*, *hs-flp*,  
557 *UAS-GFP;;* *tub-Gal4*, *FRT82B*, *tub-Gal80/TM6C* flies.

558 Origin of all individual stocks is detailed in Supplementary Table 2.

### 559 **Antibody generation**

560 Polyclonal antibodies were generated by Genscript (<https://www.genscript.com/>). The  
561 epitopes used for each immunization are listed below.

#### 562 Erm

563 KTFSCLECGKVFNAHYNLTRHMPVHTGARPVCKVCGKGFQRQASTLCRHKIIHTSEKP  
564 HKCQTCGKAFNRSSTLNTHSRIHAGYKPFVCEYCGKGFHQKGNYNHKLTHSGEKAY  
565 KCNICKAFHQVYNLTFHMHTHNDKKPYTCRVCAKGFERNFDLKKHMRKLEIGGDLD  
566 DLDMPPTYDRRREYTRREPLASGYGQASGQLTPDSSSGSMSPPINVTTPPLSSGETSN  
567 PAWPRSAVSQYPPGGFHHQLGVAPPHDYPSSGSAFLQLQPQQPHPQSQQHHQQQR  
568 LSETFIAKVF

#### 569 Ey

570 MFTLQPTPTAIGTVVPPWSAGTLIERLPSLEDMAHKDNVIAMRNLPCLGTAGGSGLGGI  
571 AGKPSPTMEAVEASTASHPHSTSSYFATTYYHLTDDECHSGVNQLGGVFVGGRRPLPD  
572 STRQKIVELAHSGARPCDISRILQVSNCGVSKILGRYYETGSIRPRAIGGSKPRVATAEV  
573 VSKISQYKRECPSIFAWEIRDRLQENVCTNDNIPSVSSINRVLRLNLAQKEQQSTGSG

574 SSSTSAGNSISAKVSVSIGGNVSNVASGSRGTLSSSTDLMQTATPLNSSES GGASNSG  
575 EGSEQEAIYEKLRLLNTQHAAGPGPLEPARAAPLVGQSPNHLGTRSSHPQLVHGNHQA  
576 LQQHQQQSWPPRHYSGSWYPTSLSEIPISSAPNIASVTAYASGPSLAHSLSPNDIESL  
577 ASIGHQRNCPVATEDIHLKKELDGHQSDETGSGEGENSNGGASNIGNTEDDQARLILK  
578 RKLQRNRTSFTNDQIDSLEKEFER THYPDVFA

579 Esg

580 MHTVEDMLVEKNYSKCPLKKRPVNYQFEAPQNHSNTPNEPQDLCVKKMEILEENPSEE  
581 LINVSDCCEDGVDVDHTDDEHIEEDEDVDVDVSDPNQTQAAALAAAAAVAAAAA  
582 SVVPTPTYPKYPWNNFHMSPYTAEFYRTINQQGHQILPLRGDLIAPSSPSDSLGLSP  
583 PPHHYLHGRASSVSPMRSEIHRPIGVRQHRFLPYPQMPGYPSLGGYTHTHHHH

584 Hbn

585 MMTTTSQHHQHHPIMPPAMRPAPVQESVSRPRAVYSIDQILGNQHQIKRSDTPSEV  
586 LITHPHHGHPHHIHLHSSNSNGSNHLSHQQQQHSQQQHHSQQQQQQQLQVQAK  
587 REDSPTNTDGGLDVDNDELSSSLNNGHDLSDMERPRKVRRSRTTFTTFQLHQLERA  
588 FEKTQYPDVFTREDLAMRLDLSEARVQVWFQNRRAKWRKREKFMNQDKAGYLLPEQ  
589 GLPEFPLGIPLPPHGLPGHPGSMQSEFWPPHFALHQHFNPAAAAAAGLLPQHLMAPHY  
590 KLPNFHTLLSQYMGLSNLNGIFGAGAAAAAAAASAGYPQNLSLHAGLSAMSQVSPPCS  
591 NSSPREPKLVPHPTPPHATPPAGGNGGGLLTGGLISTAAQSPNSAAGASSNASTPV  
592 SVVTKGED

593 Scro

594 MSSHGLAYTTTRIERKSYRELQINRDQYFVTAPNEEDLVMSLSPKDTLIHTAISQHHQVDT  
595 STKLNTNETSTQNTVSTAAAAVAHHHHNLSSIHHLQNLHSQHQSTLFNSNH

596 Slp2

597 MVKIEEGLPSSEISAHSLHFQHHHHPLPPTTHHSALQSPHPVGLNLTNLMKMARTPHLK  
598 SSFSINSILPETVEHHDEDEEEDVEKKSPAKFPPNHNHNNLNTTNWGSPEDEHEAESDP  
599 ESDLDVTSMSAPVANPNESDPDEVDEEFVEEDIECDGETTDGDAENKSNKGKPVKD  
600 KKGNE

601 Vvl (rat)

602 EEDTPTSDDLEAFKQFKQRRIKLGFTQADVGLALGTLYGNVFSQTTICRFEALQLSFK  
603 NMCKLKPLLQKWLEEADSTTGSPTSIDKIAAQGRKRKRKRTSIEVSVKGALEQHFHKQPK  
604 PSAQEITSLADSLQLEKEVVRVWFCNRRQKEKRMTPPNTLGG

605 Tll

606 MQSSEGSPDMMDQKYNSVRLSPAASSRILYHVPCKVCRDHSSGKHYGIYACDGCAGF  
607 FKRSIRRSRQYVCKSQKQGLCVVDKTHRNQCACRLRKCFEVGMNKDAVQHERGPR  
608 NSTLRRHMAMYKDAMMGAGEMPQIPAEILMNTAALTGFPGVPMMPGLPQRAGHHP  
609 AHMAAFQPPPSAAAVALDLSVPRVPHHPVHQGHGFFSPTAAYMNALATRALPPTPPL  
610 MAAEHIKETAAEHLFKNVNWIKSVRAFTELMPDQLLLLLEESWKEFFILAMAQYLMPMN  
611 FAQLLFVYESENANREIMGMVTREVHAFQEVLNQLCHLNIDSTEYECLRAISLFRKSPPS  
612 ASSTEDLANSSILTGSGSPNSSASAESRGLLESKVAAMHNDARSALHNYIQRTHPSQ  
613 PMRFQTLGQVQLMHKVSSFTIEELFFRKTIGDITIVRLISDMYSQRKI

614 Otd

615 MAAGFLKSGDLGPHPHSYGGPHPHHSVPHGPLPPGMPMPSLGPFGLPHGLEAVGFS  
616 QGMWGVNTRKQRRERTTFFTRAQLDVLEALFGKTRYPDIFMREEVALKINLPESRVQVW  
617 FKNRRAKCRQQLQQQQSNSLSKSNASGGGSGNSCSCSSSANSRSNSNNNGSSSN  
618 NNTQSSGGNNSNKSSQKQNSQSSQQGGGSSGGNNSNNNSAAAAASAAAAVAAAQ  
619 SIKTHHSSFLSAAAAAASGGTNQSANNNSNNNNQGNSTPNSSSSGGGGGSQAGGHL

620 SAAAAAALNVTAAHQNSSPLLPTPATSVSPVSIVCKKEHLSGGYGSSVGGGGGGGG  
621 ASSGGLNLGVGVGVGVGVGVVSQDLLRSPYDQLKDAGGDIGAGVHHHHSIYGSAAG  
622 SNPRLQLPQGNITPMDSSSSITTPSPITPMSPQSAAAAAHAAQSAQSAHHSAAHSAAY  
623 MSNHDSYNFWHNQYQQYPNNYAQAPSYYSQMEYFSNQNQVNYNMGHSGYTASNFG  
624 LSPSPSFTGTVSAQAFSQNSLDYMSPODKYANMV

625 Run

626 MHLPAAGPTMVANNTQVLAAAAAAAAAAAAVAQGPQPQSSNATTASAIINPAQSLA  
627 NTSTHSASSTGSSTPDLSTNNTSSSSNATTSPQNSAKMPSSMTDMFASLHEMLQEYH  
628 GELAQTGSPSILCSALPNHWRSNKSLPGAFKVIALDDVPDGLVSIKCGNDENYCGELR  
629 NCTTTMKNQVAKFNDLRFVGRSGRGKSFTLTITITATYPVQIASYSKAIKVTVDGPREPRS  
630 KQSYGYPHPGAFNPFMLNPAWLDAAYMTYGYADYFRHQAAAQAAQVHHPALAKSSA  
631 SSVSPNPNPSVATSSSSAVQPSEYPHPAAAVAAAAGQPAAMMPSPPGAAPATPYAIPQ  
632 FPFNHVAAAAAACAATPHAFHPYNFAAAAAGLRARNAALHHQSEPVHVSPASSRPSSSS  
633 PTQQHVLLKLNTSIETSSIHEQSASDGSDDEQIDVVKSEFDLKDSLDVAPLRMRCDLK  
634 APSAMKPLYHESGPGAVANSRQPSPETTTKIKSAAVQQKTVWRPY

635 Kn

636 TGNTSLSISGHPLAPDSTYDGLYPPLPVATPCIKAISPSEGWTTGGATVIIVGDNFFDGL  
637 QVVFMTMLVWSELITSHAIRVQTPPRHIPGVVEVTLVSYKSKQFCKGSPGRFVYVSALNE  
638 PTIDYGFQRLQKLIPRHPGDPEKLQKEIILKRAADLVEALYSMPRSPGGSTGFNSYAGQL  
639 AVSVQDGGSGQWTEDDYQRAQSSSVSPRGGYCSSASTPHSSGGSYGATAASAABAAT  
640 ANGYAPAPNMGTLSSSPGVSFNSTSRVSSLSFNPFALPTCNTQGYSTQLVTSTK

641 Toy

642 MRTQRRSADTVDGSGRTSTANNPSGTTASSSVATSNNSTPGIVNSAINVAERTSSALV  
643 SNSLPEASNGPTVLGGEANTTHTSSESPPLQPAAPRLPLNSGFNTMYSSIPQPIATMAE  
644 NYNSSLGSMTPSCLQQRDAYPYMFHDPLSLGSPYVSAHRNTACNPSAAHQPPQH  
645 GVYTNSSPMPSSNTGVISAGVSPVQISTQNVSDLTGSNYWPRLQ

646 Sox102F

647 MKPPGEDQTNEKEHSDLGMIKQLQLIRNRILSQAHYDSMTDIDASAQQQQQLQNVQRL  
648 QHESCLQELHNHLSSQYGAVRFTAANPQHQNQQAVSVSSGNLMPFLPAFLQPPMPNA  
649 QQLLQIPGHENAQSVPTHHHSHPQSEAFSTHKMALAPMWSTA AVAAAHQAALAAAA  
650 VAAANNKNSSHFSNNTNIVGL

651 DII

652 MDAPDAPHTPKYMDGGNTAASVTPGINIPGKSAFVELQQHAAAGYGGIRSTYQHFGPQ  
653 GGQDSGFPSRSALGYPPPMHQNSYSGYHLGSYAPPCASPPKDDFSISDKCEDSGL  
654 RVNGKGGKMRKPRTIYSSLQLQQLNRRFQRTQYLALPERAELAASLGLTQTQVKIWFQ  
655 NRRSKYKMMKAAQPGTNSGMPLGGGGPNPGQHSPNQMHSGELANGRFLWAALE  
656 TNGTLALVHSTGGNNGGGSNSGSPSHYLPPGHSPTPSSTPVSELSPEFPPTGLSPPTQ  
657 APWDQKPHWIDHKPPPQMTQPPPHPAATLHPQTHHHNPPPQMGGYVPQYWYQPET  
658 NPSLVT

659 Oaz

660 MRTQRRSADTVDGSGRTSTANNPSGTTASSSVATSNNSTPGIVNSAINVAERTSSALV  
661 SNSLPEASNGPTVLGGEANTTHTSSESPPLQPAAPRLPLNSGFNTMYSSIPQPIATMAE  
662 NYNSSLGSMTPSCLQQRDAYPYMFHDPLSLGSPYVSAHRNTACNPSAAHQPPQH  
663 GVYTNSSPMPSSNTGVISAGVSPVQISTQNVSDLTGSNYWPRLQ

664 Ap

665 MRARNLVFHVNCFCCTVCHTPLTKGDQYGIIDALIYCRTHYSIAREGDTASSSMSATYP  
666 YSAQFGSPHNDSSSPHSDPSRSIVPTGIFVPASHVINGLPQPARQKGRPRKRKPKDIEA  
667 FTANIDLNTEYVDFGRGSHLSSSSRTRMRTSFKHHQLRTMKSYFAINHNPDADKDLKQL  
668 SQKTGLPKRVLQVWFQNARAKWRRMMMKGQDGSGLLEKGEALDLDSSISVHSPTSFIL  
669 GGNSTPPLNLD

670 TfAP-2

671 CLDKSKIDNEKK

672 Dpn

673 HTKLEKADILEMTVKHLQSVQRQQLNMAIQSDPSVVQKFKTGFVECAEEVNRVVSQMD  
674 GIDTGVRQRLSAHLNQCANSLEQIGSMSNFNGYRGGLFPATAVTAAPTPLFPSLPQD  
675 LNNNSRTESSAPAIQMGGGLQLIPSRLPSGEFALIMPNTGSAAPPPGPFAPGSAAGVA  
676 AGTASAALASIANPHTLNDYTQSFRMSAFSKPVNTSVPANLPENLIHTLPGQTQLPVKN  
677 STSPPLSPISSISSHCEESRAASPTVDVMSKHSFAGVFSTPPPTSAETSFNTSGSLNLSA  
678 GSHDSSGCSRPLAHLQQQQVSSTSGIAKRDREAEAESSDCSLDEPSSKKFLAGAIEKS  
679 SS

## 680 **Immunohistochemistry**

681 Wandering third instar larval *Drosophila* optic lobes were fixed in 4% formaldehyde for 15-  
682 20 minutes at room temperature (with the exception of immunostainings using mouse anti-  
683 eyeless, rat anti-Oaz and rabbit anti-Opa antibodies, for which fixation was on ice for 30  
684 minutes). After washing, they were incubated for 2 days with primary antibodies at 4 °C.  
685 After washing the primary antibody, the brains were incubated with the secondary  
686 antibodies overnight at 4 °C. The secondary antibodies were washed, and the brains were

687 mounted in Slowfade and imaged at a confocal microscope (Leica SP8) using a 63x  
688 glycerol objective. Images were processed in Fiji and Illustrator.

689 Origin of all individual antibodies is detailed in Supplementary Table 2.

## 690 **Statistics and reproducibility**

691 Male and female larvae (stage L3) were selected randomly from the fly vials for all  
692 experiments. Blinding across different genotypes was not performed, as the genotype can  
693 be distinguished by the experimenter. All immunohistochemistry experiments were  
694 performed in at least 3 different biological replicates (brains of different animals) for each  
695 genotype, which is sufficient as it is well-established that the structure and composition of  
696 the *Drosophila* brain is very stereotypical. Moreover, all mutant phenotypes were very  
697 penetrant. In particular, n = :

698 Figure 1: (f) 4, (g) 4, (h) 4, (i) 4, (j) 3, (k) 4, (l) 4

699 Figure 2: (b) 7, (c) 6, (d) 5, (e) 7, (f) 4, (g) 4, (h) 6, (i) 5, (j) 3, (k) 6, (l) 3, (m) 5, (n) 7, (o) 5,  
700 (p) 5

701 Figure 3b: (i) 4, (ii) 7, (iii) 7, (iv) 4, (v) 6, (vi) 24, (vii) 3, (viii) 10, (ix) 3, (x) 4, (xi) 4, (xii) 9,  
702 (xiii) 11, (xiv) 6

703 Figure 4e: 3

704 Extended Data Figure 3: (c) 5, (d) 2

705 Extended Data Figure 4: (a) 8, (b) 3, (c) 4, (d) 6, (e) 4, (f) 4, (g) 3, (h) 4, (i) 3

706 Extended Data Figure 5: (b) 5, (c) 3, (d) 6, (e) 6, (f) 4, (g) 4, (h) 4, (i) 3, (j) 4, (k) 10, (l) 3,  
707 (m) 3, (n) 4, (o) 4, (p) 4, (q) 3

708 Extended Data Figure 6: (b) 6, (c) 7, (d) 7, (e) 4, (f) 4, (g) 4, (h) 4, (i) 4, (j) 6

709 Extended Data Figure 7: (b) 4, (c) 4, (c') 4, (d) 6

710 Extended Data Figure 8a: (i) 4, (ii) 3, (iii) 3, (iv) 6, (v) 4, (vi) 7, (vii) 7, (viii) 4, (ix) 4, (x) 4,  
711 (xi) 5, (xii) 3, (xiii) 3, (xiv) 6

712 Extended Data Figure 8b: (i) 6, (ii) 11, (iii) 11

713 Extended Data Figure 8c: (i) 5, (ii) 5, (ii') 3, (ii'') 3, (ii''') 3, (iii) 4, (iii') 4, (iii'') 4, (iii''') 4, (iii''''')  
714 4

## 715 **Hybridization Chain Reaction-RNA FISH**

716 To perform HCR-RNA FISH, custom probes were designed for *BarH1*, *BarH2*, *Oaz* and  
717 *dpn* coding sequences and sourced from Molecular Instruments. Wash, amplification and  
718 hybridization buffers, and fluorophore-labelled amplification hairpins, were obtained from  
719 Molecular Instruments. The HCR protocol for *Drosophila* larval brains used was as  
720 specified in: [dx.doi.org/10.17504/protocols.io.bzh5p386](https://doi.org/10.17504/protocols.io.bzh5p386). Amplification hairpins used were  
721 labelled with Alexa Fluor 488, 546, 594 and 647.

## 722 **Birth order of medulla neurons and temporal window assignment**

723 The current L3-P15 scRNASeq dataset contains some neurons that do not originate from  
724 the main OPC neuroepithelium that generates medulla neurons. We removed from the  
725 analysis Low Quality (LQ) clusters, clusters containing more than one cell type, glial  
726 clusters, clusters with a different origin from the medulla and clusters that were  
727 transcriptionally more similar to these than to medulla neurons (see Extended Data Figure  
728 8). Additionally, clusters that express a combination of concentric genes that we do not  
729 observe in the medulla cortex by immunostaining were removed, such as 13 (Run+TfAP-



730 2) (Extended Data Figure 8a vii), 114 (Hbn+Ap) (Extended Data Figure 8c ii'''), and  
731 clusters 51 and 169b (Toy+Hbn) (Extended Data Figure 8a xiv). To establish the birth  
732 order of all the clusters in the medulla dataset and its predicted temporal window of origin  
733 (Figure 3c and Supplementary Table 1), we used the mRNA expression of tTFs in GMCs,  
734 and tTFs and concentric genes in medulla neuronal clusters at L3 and P15, together with  
735 their relative position in UMAP plot (Extended Data Figure 7a-a''' and 3c). To identify  
736 medulla clusters expressing a given concentric gene and/or tTF (Supplementary Table 1),  
737 we used Mixture Modelling<sup>39</sup> at P15 stage from Ozel *et al.*, 2020<sup>18</sup>, and confirmed the  
738 results using violin plots for each concentric gene. To define the relative order of concentric  
739 gene expression in medulla neurons, we immunostained late L3 brains with these TFs  
740 (see Figure 3b) and analyzed the most medial part of the medulla cortex, where neurons  
741 are more mature and hence more similar to the P15 dataset. Several criteria were  
742 considered to assign a cluster to a given temporal window: tTF expression in GMCs and  
743 neuronal clusters, concentric gene expression in neuronal clusters, UMAP position,  
744 transcriptional similarity based on hierarchical cluster tree (Extended Data Figure 8) and  
745 experimental data from MARCM mutant clones in NB tTFs where a given concentric gene  
746 is lost in neurons (Extended Data Figure 7c-d and <sup>22,23,29,40</sup>).

## 747 **Single-cell RNA seq**

### 748 *Drosophila* optic lobes sample preparation

749 The developing central nervous system from male and female flies was dissected from  
750 Canton-S wandering third instar larvae in PBS. The optic lobes were separated from the  
751 central brain using Vannas Spring Scissors with a 2mm cutting edge (Fine Science Tools  
752 Cat no. 15000-04). The optic lobes were dissociated into single cell suspension by  
753 incubating in 2mg/mL collagenase and 2mg/mL dispase in PBS for 15 minutes at 25 °C.

754 The enzymes were then carefully removed and replaced with PBS + 0.1% BSA. The brains  
755 are soft but remain intact if pipetted slowly. The brains were pipetted up and down many  
756 times (> 100) until most large chunks of tissue were dissociated. The cells/tissue were  
757 kept cold by putting the tubes on ice. The cells were then filtered using 20 µm cell strainers.  
758 The concentration of the cell suspension was then measured staining the cells with 1/2000  
759 Hoechst, using an epifluorescent microscope and a 0.02-mm deep cytometer.

#### 760 Library preparation and sequencing

761 Droplet-based purification, amplification and barcoding of single-cell transcriptomes were  
762 performed using Chromium Single Cell 3' Reagent Kit v2 (10x Genomics) as described  
763 in the manufacturer's manual (Chromium Single Cell 3' Reagent Kits v2 User Guide -  
764 Rev D), with a target recovery of 7,000 cells per experiment. We prepared 10 libraries  
765 (biological replicates), which were subjected to paired-end sequencing (26 × 8 × 98) with  
766 NovaSeq 6000 (Genome Technology Center at NYU Langone Health) to an average  
767 50,000 reads per cell sequenced (that is, 350,000,000 reads for an experiment with 7,000  
768 cells).

#### 769 **Single-nucleus RNA seq**

##### 770 Human cortical plate sample preparation

771 Tissue was collected from de-identified prenatal autopsy specimens without  
772 neuropathological abnormalities. All autopsies were done with written consent from the  
773 legal next-of-kin. The Icahn School of Medicine Institution Review Board considers  
774 autopsies as non-human subjects. Utilization of fetal specimens was determined as non-  
775 human research by the Icahn School of Medicine Institution Review Board and exemption  
776 was provided to Dr. Nadejda Tsankova (HS#: 14-01007). The cortical plate was dissected

777 fresh from the anterior frontal lobe of anatomically intact brain specimens with postmortem  
778 time interval less than 24 hours, and immediately fresh-frozen on dry ice.

779 Isolation and fluorescence-activated nuclear sorting (FANS) with hashing.

780 All buffers were supplemented with RNase inhibitors (Takara). 25mg of frozen  
781 postmortem human brain tissue was homogenized in cold lysis buffer (0.32M Sucrose, 5  
782 mM CaCl<sub>2</sub>, 3 mM Magnesium acetate, 0.1 mM EDTA, 10mM Tris-HCl, pH8, 1 mM DTT,  
783 0.1% Triton X-100) and filtered through a 40 µm cell strainer. The flow-through was  
784 underlaid with sucrose solution (1.8 M Sucrose, 3 mM Magnesium acetate, 1 mM DTT, 10  
785 mM Tris-HCl, pH8) and centrifuged at 107,000 g for 1 hour at 4 °C. Pellets were re-  
786 suspended in PBS supplemented with 0.5% bovine serum albumin (BSA).

787 Four samples were processed in parallel. 2 million nuclei from each sample were pelleted  
788 at 500 g for 5 minutes at 4 °C. Following centrifugation, nuclei were re-suspended in 100  
789 µl staining buffer (2% BSA, 0.02% Tween-20 in PBS) and incubated with 1 µg of a unique  
790 TotalSeq-A nuclear hashing antibody (Biolegend) for 30 min at 4 °C. Prior to FANS,  
791 volumes were brought up to 250 µl with PBS and DAPI (Thermoscientific) added to a final  
792 concentration of 1 µg/ml. DAPI positive nuclei were sorted into tubes pre-coated with 5%  
793 BSA using a FACSAria flow cytometer (BD Biosciences).

794 snRNAseq and library preparation.

795 Following FANS, nuclei were subjected to 2 washes in 200 µl staining buffer, after which  
796 they were re-suspended in 15 µl PBS and quantified (Countess II, Life Technologies).  
797 Concentrations were normalized and equal amounts of differentially hash-tagged nuclei  
798 were pooled. A total of 40,000 (10,000 each) pooled nuclei were processed using 10x  
799 Genomics single cell 3' v3 reagents. At the cDNA amplification step (step 2.2), 1 µl of 2  
800 µM HTO cDNA PCR "additive" primer was added<sup>41</sup>. After cDNA amplification, supernatant

801 from 0.6x SPRI selection was retained for HTO library generation. cDNA library was  
802 prepared according to 10x Genomics protocol. HTO libraries were prepared as previously  
803 described<sup>41</sup>. cDNA and HTO libraries were sequenced at NYGC using the Novaseq  
804 platform (Illumina).

## 805 **Bioinformatic analyses**

806 Detailed scripts and related R objects can be found here:  
807 [https://drive.google.com/open?id=12260\\_PQkmEpIL1hNBFbQX9eEpzC4pySy&authuser](https://drive.google.com/open?id=12260_PQkmEpIL1hNBFbQX9eEpzC4pySy&authuser=nk1845%40nyu.edu&usp=drive_fs)  
808 [=nk1845%40nyu.edu&usp=drive\\_fs](https://drive.google.com/open?id=12260_PQkmEpIL1hNBFbQX9eEpzC4pySy&authuser=nk1845%40nyu.edu&usp=drive_fs)

### 809 Mapping and integration of larval (L3) and pupal (P15) datasets

810 We mapped the sequenced libraries to the *D. melanogaster* genome assembly BDGP6.88  
811 using CellRanger 3.0.1. We kept only genes that were expressed in at least 3 cells across  
812 all cells and cells with counts with at least 200 genes for further analysis. After processing,  
813 the dataset comprised 49,893 cells passing quality filters, with a median of 3,635 UMIs  
814 and 1,343 genes per cell.

815 We used the procedure implemented in Seurat v.3 to remove batch effects from our  
816 sequenced libraries. We used default parameters except for the dimensionality for which  
817 we tried the values 100, 150 and 200. We compared the results using the Seurat function  
818 LocalStruct with default parameters. The results obtained were 83.7%, 84.9% and 83.6%,  
819 respectively. We therefore chose a dimensionality of 150 for the larval dataset.

820 The dataset was then clustered with a resolution of 2. Notably, in this developing structure,  
821 cells are clustered both by identity and by differentiation stage. For example, Mi1 cells fall  
822 into 2 clusters, an immature (cluster 23) and a mature cluster (cluster 53).

823 Larval and pupal datasets were merged using default parameters. 150 PCs were used  
824 subsequently for generating the UMAP to remain consistent with the integration of the  
825 different larval libraries.

#### 826 Spatial patterning analysis

827 To focus on the heterogeneity within the neuroepithelial cells, the larval dataset was  
828 further subsetted using marker expression with Seurat v3. Expression of neuroepithelial  
829 markers *shg*, *tom*, and *brd* were examined for each cluster<sup>42</sup>. Clusters with average  
830 expression higher than 95<sup>th</sup> percentile of normalized expression of *tom* and *brd* were  
831 selected as neuroepithelial clusters. DE-Cadherin (Shg) is known to be enriched in  
832 neuroepithelial cells<sup>43</sup> and is enriched in the selected clusters (logFC = 0.75, adjusted p  
833 value = 0).

834 Principal components were calculated using variable features found in the subsetted  
835 neuroepithelial cells. Examination of PC1 revealed that *tll*, an early marker of lamina  
836 precursor cells<sup>44</sup>, is expressed in a near-mutually exclusive fashion with *hth* (enriched in  
837 neuroepithelium and young medulla neuroblasts), suggesting the subset contained both  
838 OPC neuroepithelium and lamina precursor cells. To keep only OPC neuroepithelial cells,  
839 we sub-clustered the cells and examined the average expression of *hth* and *tll* for each  
840 cluster. This process is performed iteratively to keep only *hth+/-tll-* clusters. The remaining  
841 cells were assigned as OPC neuroepithelium for further analysis of spatial temporal  
842 factors.

#### 843 Trajectory analysis: identification of candidate tTFs

844 To study temporal patterning in neuroblasts, we first identified the cluster that  
845 corresponded to the medulla neuroblasts (cluster 9) based on the expression of Dpn and

846 Ase, as well as the expression of the known temporal factors. We extracted the counts  
847 from these cells and inputted them into Monocle. We used default parameters to order the  
848 cells in pseudotime. We used the DDRTree method for dimensionality reduction. The cells  
849 were then ordered in pseudotime and the beginning and end of the trajectory were defined  
850 based on the expression of the known tTFs (i.e. Hth marked the beginning of the trajectory  
851 and Tll marked the end). We then looked at the expression along the pseudotime of 629  
852 genes annotated as transcription factors in FlyBase to identify the candidate tTFs.  
853 identified 39 candidate that exhibited temporally restricted expression. These fell into two  
854 distinct categories: 14 of them were expressed at relatively high levels and included the 6  
855 known tTFs (Extended Data Figure 3a), while 25 of them were expressed at lower levels  
856 along the trajectory (Extended Data Figure 3b). We tested the expression pattern of 4 of  
857 the 25 lowly expressed candidates, *apterous (ap)*, *cut*, *gcm*, and *gemini (gem)* in the  
858 developing optic lobes. *Ap* is expressed in neurons<sup>22</sup>, *gem* was not expressed in the optic  
859 lobe, *gcm* is expressed in glial cells coming from the Tll temporal window<sup>45</sup>, and *cut* was  
860 not expressed in a temporal manner (Extended Data Figure 3c-d). We therefore decided  
861 not to pursue these candidates further as their fluctuations likely represent noise.

#### 862 Merging of larval and pupal Mi1 and DE analysis over pseudotime

863 Larval and pupal (P15, P30, P40, P50, and P70) datasets were merged after cells were  
864 batch effect corrected for each stage separately. The standard Monocle workflow was  
865 followed to generate trajectories. The L3 and P15 trajectories were ordered manually.

866 Based on the way the optic lobe develops, there are cells at the same differentiation stage  
867 in the L3 and P15 datasets. We therefore decided to align these two datasets in order to  
868 get a continuum of expression. We tested different genes and ended up using  
869 “Ggamma30A” as a reference gene. Ggamma30A starts increasing in the middle of the

870 L3 trajectory and continues all the way to P15 in a linear manner. We adjusted the  
871 expression of Ggamma30A in P15 using linear regression, which was then applied to all  
872 genes of P15. This does not change the dynamics of expression, just the relative levels,  
873 and serves the purpose of aligning the trajectories over pseudotime of L3 and P15.

874 To identify differentially expressed genes along the differentiation trajectory from L3 to  
875 P70, we used two methods: “principal graph” and “knn”. We selected genes that were  
876 identified as differentially expressed with at least one of the two methods. We then used  
877 the `find_gene_modules` function to group the differentially expressed genes into modules  
878 of genes that co-vary. These genes were then used for GO analysis.

#### 879 GO enrichment analysis

880 We performed GO enrichment analysis and calculated enrichment for ‘Biological Process’  
881 using The Gene Ontology Resource (<http://geneontology.org/>) using a Fisher's exact test  
882 to calculate p-value. Multiple testing correction was performed by calculating the False  
883 Discovery Rate.

884 To find the expression of GO terms over time, we added and normalized the expression  
885 of all genes that belong to a specific GO term and plotted it over pseudotime or on the  
886 UMAP.

#### 887 Analysis of human data

888 We mapped the sequenced libraries to the *H. sapiens* genome assembly GRCh38 (hg38)  
889 using CellRanger 3.1.0. For the hashtag oligos (HTO), we used the CITE-seq-Count 1.4.2  
890 version to align HTO to 10x barcodes using the following command:

```
891 CITE-seq-Count -R1 reas1 -R2 read2 -T 1 -t tag -cbf 1 -cbl 16 -umif 17 -umil 26 -cells  
892 40000 -o output --sliding-window # --dense
```

893 After processing, the dataset comprised 3,363 cells passing quality filters, with a median  
894 of 4,736 UMIs and 2,414 genes per cell.

895 We selected the radial glia (expressing Pax6), intermediate progenitors (expressing  
896 Eomes), and neurons (expressing NeuroD2) that were forming a trajectory in UMAP and  
897 imported the data into Monocle and used default parameters to calculate the trajectories.  
898 We used the find\_gene\_modules function to group genes into 6 modules of genes that  
899 co-vary. These modules were then used for GO analysis.

#### 900 Analysis of mouse cortical data

901 The dataset that was generated by Telley *et al.*<sup>34</sup> was downloaded from GEO  
902 (GSE118953). The raw counts were inputted into Seurat and the standard workflow was  
903 followed (log-normalization, followed by clustering and UMAP using 25 PCs, and  
904 clustering was done with a resolution of 2). The radial glia clusters (clusters 2 and 3) were  
905 identified based on the expression of known radial glia markers, such as SOX2 and PAX6.  
906 Radial glia from different embryonic days 12, 13, 14, and 15 were used to generate the  
907 violin plots of Extended Data Figure 10a.

#### 908 Analysis of mouse retina data

909 We downloaded the dataset that was generated by Clark *et al.*<sup>35</sup>. We inputted the raw  
910 counts into Seurat and the standard workflow was followed (log-normalization, followed  
911 by clustering and UMAP using 50 PCs, and clustering was done with a resolution of 0.5).  
912 We used the annotation provided by the authors<sup>35</sup> to select early and late retinal progenitor  
913 cells (RPCs). RPCs from embryonic days 11, 12, 14, 16, and 18 and postnatal day 0 were  
914 used to generate the violin plots of Extended Data Figure 10c-d.

915



916

917

918 **Code availability**

919 All related code that was used in this manuscript can be found on Github:  
920 [https://github.com/NikosKonst/larva\\_scSeq2022](https://github.com/NikosKonst/larva_scSeq2022)

921 **Data Availability**

922 All *Drosophila* raw and processed data referenced were uploaded to GEO: accession  
923 number GSE167266.

924 The human source data described in this manuscript are available via the PsychENCODE  
925 Knowledge Portal (<https://psychencode.synapse.org/>). The PsychENCODE Knowledge  
926 Portal is a platform for accessing data, analyses, and tools generated through grants  
927 funded by the National Institute of Mental Health (NIMH) PsychENCODE program. Data  
928 is available for general research use according to the following requirements for data  
929 access and data attribution: (<https://psychencode.synapse.org/DataAccess>). For access  
930 to content described in this manuscript see: [www.doi.org/10.7303/syn24975927](http://www.doi.org/10.7303/syn24975927)

931 The publicly available single-cell sequencing datasets that were used can be found in  
932 GEO: accession numbers GSE142787 (*Drosophila* pupal development), GSE118953  
933 (mouse cortical radial glia), and GSE118614 (mouse retinal progenitors).

934

935 **Extended Data figure legends**

936 **Extended Data figure 1: Integration of libraries**

937 (a-a') Comparison of library distribution on a tSNE plot of datasets before (merged) and  
938 after library integration (batch effect correction). Before integration, there is a clear bias in  
939 the distribution of the libraries within clusters. After integration, this bias is largely  
940 eliminated.

941 (b-b') Comparison of the library contribution in each cluster before (merged) and after  
942 library integration (batch effect correction). With the exception of few clusters in each case,  
943 all clusters have a similar percentage of cells coming from each library.

944 (c) Comparison of cluster sizes per library before (merged - n = 71 clusters) and after  
945 library integration (batch effect correction - n = 93 clusters). The variance in the merged  
946 dataset is larger than the one in the integrated one, indicating noise that was potentially  
947 alleviated by the batch correction. Boxplots display the first, second and third quartiles.  
948 Whiskers extend from the box to the highest or lowest values in the 1.5 inter-quartile range,  
949 and outlying datapoints are represented by a dot.

950 **Extended Data figure 2: Annotation of the developing optic lobe UMAP plot**

951 (a) UMAP plots showing the expression of different cell type markers that allows for the  
952 annotation of the different clusters. *Shotgun/DE-Cad (shg)* is expressed in the  
953 neuroepithelium (NE) and young neuroblasts (NBs). *Deadpan (dpr)* is expressed in  
954 neuroepithelium and neuroblasts. *Asense (ase)* is expressed in the neuroblasts and  
955 GMCs. *Elav* is mostly expressed in the neurons, although the transcript can already be  
956 seen in the GMCs. Finally, *repo* is expressed in glial cells.

957 (b) UMAP plot of single-cells coming from larval (L3) and pupal (P15) developing optic  
958 lobes. Using cell type annotation, we identify cell types that belong to the four optic lobe  
959 neuropils (lamina, medulla, lobula, and lobula plate), as well as central brain cells that  
960 were not removed during the dissections, and glial cells.

961 (c) Expression of markers for the lamina and the lobula plate. Lamina is marked by the  
962 expression of *gcm*, *eya*, *sim*, *tll*, and *dac*, while lobula plate expresses strongly *acj6*, faintly  
963 *dac*, and the progenitors (neuroblasts and GMCs) express *tll*.

964 (d) (Top) UMAP: Spatial transcription factors (*Vsx*, *Optix*, and *Rx*) are not expressed in  
965 medulla neuroblasts (NBs), while only *Vsx* is expressed in some neuronal types (it is  
966 unknown whether this expression reflects their origin from the *Vsx* spatial domain). (Inset)  
967 sTFs are only expressed in the neuroepithelium (NE) in largely non-overlapping domains.  
968 (Bottom). UMAP plots with the expression of individual sTFs in the neuroepithelium.

969 (e) UMAP plot of the neuroepithelial cells. Top: Expression of the spatial transcription  
970 factors (*Vsx1*, *Optix*, and *Rx*) can be seen in largely non-overlapping clusters. Bottom:  
971 Semi-supervised clustering of the neuroepithelial cells and identification of the three  
972 spatial clusters (*Vsx*, *Optix*, and *Rx*).

### 973 **Extended Data figure 3: Candidate tTF expression in neuroblast trajectory**

974 Expression pattern over pseudotime of all TFs that were found to be expressed in a  
975 temporal manner along the medulla neuroblast trajectory.

976 (a) 14 transcription factors were found to be expressed temporally in high relative  
977 expression levels. These include the already known tTFs (*hth*, *ey*, *slp1*, *slp2*, *D*, and *tll* in  
978 purple), as well as eight new candidate tTFs (in green).

979 (b) Another 25 transcription factors were found to be expressed temporally in lower relative  
980 expression levels. *Ap*, *ct*, *gcm*, and *gem* expression were tested in developing optic lobes  
981 and they were not expressed temporally. Hence, these 25 transcription factors were  
982 excluded from downstream analysis.

983 (c) Gcm-Gal4 (green) is expressed in glial cells coming from the TII temporal window  
984 (magenta)<sup>45</sup>. Scale bar: 10  $\mu$ m.

985 (d) Cut-Gal4 (green) is expressed in neuroblasts of all ages found in the surface of the  
986 optic lobe (arrow). Scale bar: 10  $\mu$ m.

987 **Extended Data figure 4: Newly identified tTFs are expressed temporally**

988 (a) Antibody staining (frontal view) against five of the eight new temporal transcription  
989 factors, Opa (red), Erm (blue), Hbn (green), Scro (white), and BarH1 (green), shown in  
990 different combinations.

991 (b) Antibody staining against Opa, Hth, Ey, and Arm (which marks all cells). Opa is  
992 expressed in two waves, one succeeding and partially overlapping with the Hth window  
993 and one later partially overlapping with Ey. The expression of Opa covers the previous  
994 “gap” between Hth- and Ey-expressing neuroblasts.

995 (c) Antibody staining against Erm and Ey. Erm starts being expressed before Ey, partially  
996 overlapping with it (magenta arrowheads).

997 (d) Antibody staining against Hbn and Ey. Hbn expression begins slightly after Ey and  
998 overlaps almost completely with it. Ey-positive, Hbn-negative neuroblasts are indicated  
999 with arrowheads.

1000 (e) Antibody staining against Oaz and D. Oaz expression precedes the expression of D.

1001 (f) Antibody staining against Dpn, Oaz, BarH1, and Arm (which marks all cells). BarH1 is  
1002 expressed after Oaz.

1003 (g) Antibody staining against BarH1 and Tll. BarH1 is expressed before and overlaps with  
1004 Tll.

1005 (h) Fluorescent *in situ* hybridization against *dpn*, *Oaz*, *BarH1* and *BarH2*. BarH2 is  
1006 expressed in the same neuroblasts as BarH1, after Oaz has stopped being expressed.  
1007 Hoechst marks all cells.

1008 (i) Hbn is expressed between the two Opa temporal windows in neurons, while BarH1 is  
1009 expressed after the second Opa temporal window. Hbn is also expressed in later born  
1010 neurons, after the second Opa wave, and is lost from the first lamina (in between Opa) as  
1011 neurons mature.

1012 Scale bars: 10  $\mu$ m

### 1013 **Extended Data figure 5: Negative genetic interactions between tTFs**

1014 (a) Diagram of genetic interactions between tTFs in medulla neuroblasts. Red "X"s within  
1015 the diagram indicate no genetic interaction. Within the early unit (green box), we identified  
1016 three new tTFs: Opa, Erm and Oaz. Hth does not activate Erm or Opa. Furthermore, Opa  
1017 does not repress Hth or activate Erm. Within the middle unit (red box), we identified three  
1018 new temporal factors: Homeobrain (Hbn), Scarecrow (Scro) and Opa. Opa is not inhibited  
1019 by D or BarH1. Oaz is also not inhibited by D or BarH1. Within the late unit (blue box) we  
1020 identified one new temporal factor: BarH1. Tailless (Tll) is not necessary to inhibit BarH1.

1021 (b) In cells expressing *hth* RNAi driven by *Vsx-Gal4* (GFP: green), Opa expression is not  
1022 affected, indicating that Hth does not activate Opa.

1023 (c) Erm is unaffected in cells expressing *hth* RNAi driven by *Vsx-Gal4* (GFP: green).

1024 Hence, Hth does not activate Erm.

1025 (d) In *opa* mutant clones (GFP: green), Hth and Erm expression are unaffected, indicating

1026 that Opa does not inhibit Hth and does not activate Erm.

1027 (e) In *opa* mutant clones (GFP: green), Ey expression is delayed, indicating that Opa helps

1028 to time the expression of Ey.

1029 (f-j) In cells expressing *Oaz* RNAi driven by *Vsx-Gal4* (GFP: green), (f) Hth, Ey, Slp, (g)

1030 Opa, (h) Erm, Hbn, Tll, (i) Scro, D, and (j) BarH1 are unaffected, indicating that Oaz does

1031 not regulate their expression.

1032 (k) Scro knock-down upon expression of *scro* RNAi (GFP: green) does not affect the

1033 expression of Hbn or Slp, as was expected given that Scro is coexpressed with both (see

1034 Figure 2).

1035 (l) In negatively marked *D* mutant clones, the expression of Opa is unaffected, indicating

1036 that D does not inhibit Opa.

1037 (m) Similarly, in negatively marked *D* mutant clones, the expression of Oaz is unaffected,

1038 indicating that D does not inhibit of Oaz.

1039 (n) In *Tll* mutant clones (GFP: green), neither D nor BarH1 expression are affected,

1040 indicating that Tll is not necessary to inhibit either factor.

1041 (o-q) In cells expressing RNAi against *BarH1*, (o) Opa, (p) Oaz, and (q) D are unaffected,

1042 indicating that BarH1 does not regulate their expression.

1043 Scale bars: 10  $\mu$ m

1044 **Extended Data figure 6: Additional genetic interactions between tTFs**

1045 (a) Diagram of genetic interactions between tTFs in medulla neuroblasts. Magenta dashed  
1046 arrows within the diagram indicate genetic interactions determined with misexpression of  
1047 tTFs that confirm previous results from MARCM and knock-down experiments. Orange  
1048 dashed arrows indicate genetic interactions uncovered with misexpression experiments.  
1049 Blue dashed arrows indicate genetic interactions determined with misexpression  
1050 experiments that are opposite to previous MARCM or knock-down experiments.

1051 (b) Left: In *opa* mutant clones (GFP: green), *Oaz* is not expressed, suggesting that *Opa* is  
1052 necessary for the activation of *Oaz*. Right: Accordingly, *oaz* neurons are lost in *opa* mutant  
1053 clones.

1054 (c) *Slp* inhibits the expression of *Oaz*. In *slp* mutant clones (GFP: green), *Oaz* remains  
1055 expressed in older neuroblasts.

1056 (d) In cells misexpressing *Hth* driven by *Vsx-Gal4* (GFP: green), *Erm* expression is not  
1057 affected, indicating that *Hth* does not activate *Erm*.

1058 (e) In cells misexpressing *Hth* driven by *Vsx-Gal4* (GFP: green), *Opa* expression is not  
1059 affected, indicating that *Hth* does not activate *Opa*.

1060 (f) In cells misexpressing *Ey* driven by *Vsx-Gal4* (GFP: green), *Hbn* expression is activated  
1061 and *Scro* expression repressed.

1062 (g) In cells misexpressing *Hbn* driven by *Vsx-Gal4* (GFP: green), *Slp* expression is not  
1063 activated earlier. White arrowhead indicates low levels of *Hbn* protein being misexpressed  
1064 early compared to adjacent wildtype tissue.

1065 (h) In cells misexpressing *Scro* driven by *Vsx-Gal4* (GFP: green), *D* expression is not  
1066 activated earlier. White arrowhead indicates low levels of *Scro* protein being misexpressed  
1067 early compared to adjacent wildtype tissue.



1068 (i) In cells misexpressing D driven by *Vsx-Gal4* (GFP: green), both *Scro* and *BarH1*  
1069 expression are repressed.

1070 (j) In cells misexpressing Tll driven by *Vsx-Gal4* (GFP: green), both D and *BarH1*  
1071 expression are repressed.

1072 Scale bars: 10  $\mu$ m

1073 **Extended Data figure 7: Temporal transcription factors are inherited in neurons**  
1074 **and regulate neuronal diversity**

1075 (a) UMAP plots showing the expression of all temporal transcription factors in the  
1076 developing *Drosophila* optic lobe. (a) *Hth* maintains its expression in some of their  
1077 neuronal progeny, such as Mi1, Pm1, and Pm2. (a') On the other hand, *ey*, *D*, *erm* and  
1078 *Scro* not only maintain their expression in some neuronal progeny, but they are also  
1079 activated independently in neuronal types that come from different temporal windows.  
1080 Hence, most of them are expressed in both early- and late-born neuronal types (see also  
1081 Supplementary Table 1). (a'') *Slp1*, *opa*, *BarH1*, *Oaz*, and *tll* do not maintain their  
1082 transcription in neurons. However, using their expression in GMCs at the root of neuronal  
1083 branches, we can predict neuronal types that come from these temporal windows (such  
1084 Tm1, Tm2, and Tm4 for *Opa*). (a''') Finally, *hbn* is not maintained in neuronal progeny of  
1085 the Hbn+ neuroblasts, but it is activated independently in later-born neurons (such as T1).  
1086 Neuronal types that come from the relevant temporal windows are indicated.

1087 (b) Expression of temporal transcription factors in neuronal progeny shows that the tTFs  
1088 are expressed in the newly born progeny of their respective neuroblast temporal window.  
1089 Time is depicted by the arrow; neurons born from young neuroblasts are on the top of the  
1090 Figure. *Opa*-positive neurons are born from young neuroblasts (red), followed by *Erm*  
1091 neurons (green). Then, neurons expressing *Hbn* and *Opa* can be detected. (red-white).

1092 Finally, Scro-positive (blue) and Scro and BarH1-positive neurons (blue-white) are born  
1093 from older neuroblasts. Single-channel images can be seen in the right panels.

1094 (c) Hbn is involved in the generation of neuronal diversity by regulating the expression of  
1095 downstream transcription factors. In *Hbn*-mutant MARCM clones (green) neurons  
1096 expressing Toy, Otd (c) and Tj (c') are not found.

1097 (d) *Opa* is also involved in the generation of neuronal diversity by regulating the expression  
1098 of the downstream transcription factor TfAP-2 in some neurons. *Opa*-mutant MARCM  
1099 clones (green) have fewer TfAP-2 positive cells (magenta) compared to the adjacent wild-  
1100 type tissue.

1101 Scale bars: 10  $\mu$ m

1102 **Extended Data figure 8: Temporal expression of concentric genes in medulla**  
1103 **neurons**

1104 **(a) Temporal expression of concentric genes in the medulla cortex.**

1105 (i-i''') Expression (from early to late born) of Bsh, Run, Vvl, Toy and Slp in medulla neurons  
1106 at L3 stage. Bsh neurons are closer to the medulla neuropil (indicated with dashed line),  
1107 while Slp neurons (arrowhead) are closer to the surface of the brain (NB layer). Anterior  
1108 is shown to the right and posterior to the left throughout the Figure.

1109 (ii-ii''') Expression of Hth, Svp and Toy in medulla neurons at L3 stage. Hth is expressed  
1110 in the first-born neurons, located closer to the medulla neuropil. Svp is expressed in  
1111 several laminae along the medulla cortex. Toy is expressed in mid-late born neurons.

1112 (iii-iii''') Expression of Svp, Forkhead domain 59A (Fd59A) and Hbn in medulla neurons at  
1113 L3 stage. Svp is expressed in several laminae along the medulla cortex. Fd59A is

1114 expressed in mid-late born neurons, some of them (magenta arrowheads) generated  
1115 before Hbn neurons. Cyan arrowheads denote Lawf1-2 neurons, which have a different  
1116 origin<sup>38</sup>.

1117 (iv-iv''') Expression of Tup, Knot (Kn), and Toy in medulla neurons at L3 stage. Tup is  
1118 expressed in several laminae of early and late born neurons along the medulla cortex,  
1119 some of them being born before Kn neurons (iv'). Tup+Toy neurons are the first-born Toy  
1120 neurons in the ventral side of the medulla (iv'', arrowhead). Kn neurons are generated  
1121 before Kn+Toy neurons (iv''', arrowhead).

1122 (v-v''') Expression of Toy, Fkh:GFP and Otd in medulla neurons at L3 stage. Some Toy  
1123 neurons are generated before Fkh and Otd neurons (E'-E''). Fkh+Otd neurons  
1124 (arrowhead) are only found in the posterior tips of the main OPC (E'''), mainly in the Dpp  
1125 region, as previously described<sup>40</sup>.

1126 (vi-vi''') Expression of Toy, Fd59A and Ets65A:GFP in medulla neurons at L3 stage. Toy  
1127 and Fd59A expressing neurons are intermingled in the medulla cortex, being coexpressed  
1128 in specific subregions of the medulla cortex (vi', arrowheads). Many Toy and Fd59A  
1129 neurons are generated before Ets65A-expressing neurons (vi''-vi''').

1130 (vii) Expression of TfAP-2 and Run in medulla neurons at L3 stage. The first laminae of  
1131 TfAP-2+Ap+ neurons (Notch<sup>ON</sup>) are intermingled with Run neurons (Notch<sup>OFF</sup>)  
1132 (arrowheads), suggesting that they could be sister neurons.

1133 (viii) Expression of TfAP-2 and Vvl in medulla neurons at L3 stage. The first laminae of  
1134 TfAP-2 neurons are generated before Vvl neurons. The second laminae of TfAP-2  
1135 neurons are generated after some Vvl neurons.

1136 (ix) Expression of TfAP-2 and Toy in medulla neurons at L3 stage. The first laminae of  
1137 TfAP-2 neurons are generated before Toy neurons. The second laminae of TfAP-2  
1138 neurons are intermingled with some Toy neurons.

1139 (x) Expression of TfAP-2 and Ets65A:GFP in medulla neurons at L3 stage. All TfAP-2  
1140 neurons are born before Ets65A-expressing neurons, except in the cases where both TFs  
1141 are coexpressed (arrowhead). These neurons are very late born and are the only Notch<sup>ON</sup>  
1142 medulla neurons that are glutamatergic.

1143 (xi) Expression of Vvl and Kn in medulla neurons at L3 stage. Vvl+Ap+ (Notch<sup>ON</sup>) and Kn  
1144 neurons (Notch<sup>OFF</sup>) start to be generated at similar times, suggesting that they could be  
1145 sister neurons.

1146 (xii) Expression of Toy and Sox102F in medulla neurons at L3 stage. Toy and Sox102F  
1147 neurons are mid-late born neurons intermingled in the medulla cortex. Toy and Sox102F  
1148 are expressed individually or coexpressed in medulla neurons.

1149 (xiii) Expression of Toy and Tj in medulla neurons at L3 stage. Toy and Tj neurons are  
1150 expressed in mid-late born neurons intermingled in the medulla cortex. They are  
1151 coexpressed in specific subregions of the medulla cortex (arrowhead).

1152 (xiv) Expression of Toy and Hbn in medulla neurons at L3 stage. Hbn neurons are  
1153 generated after some Toy neurons.

1154 Scale bars: 10  $\mu$ m

1155 **(b) Concentric genes expressed both early and late in medulla neurons.**

1156 (i) TFs Seven-Up (Svp), (ii) TfAP-2 and (iii) Tailup (Tup) are expressed in several laminae  
1157 along the medulla cortex and are expressed in neurons generated in several temporal  
1158 windows.

1159 **(c) Notch status of medulla neurons and concentric gene expression.**

1160 (i) Expression of Bsh and Ap in medulla neurons. All Bsh neurons express Ap. Anterior is  
1161 to the right and posterior to the left in this and subsequent panels.

1162 (ii) Expression of Run and Ap in medulla neurons at L3 stage. None of the Run positive  
1163 neurons express Ap.

1164 (ii') Expression of Ap and Svp in medulla neurons at L3 stage. None of the Svp positive  
1165 neurons express Ap.

1166 (ii'') Expression of Ap and Fd59A in medulla neurons at L3 stage. None of the Fd59A  
1167 positive neurons express Ap.

1168 (ii''') Expression of Ap and Hbn in medulla neurons at L3 stage. None of the Hbn positive  
1169 neurons express Ap.

1170 (iii) Expression of Ap and Vvl in medulla neurons at L3 stage. Vvl neurons that are earlier  
1171 born, such as Tm9, express Ap (white arrowhead), while later born Vvl neurons, such as  
1172 Dm8 and Dm11, do not (magenta arrowhead).

1173 (iii') Expression of Ap and Toy in medulla neurons at L3 stage. Earlier born Toy neurons,  
1174 such as Dm10, do not express Ap (magenta arrowhead), while later born Toy neurons,  
1175 such as Tm20, express Ap (white arrowhead).

1176 (iii'') Expression of Ap and TfAP-2 in medulla neurons at L3 stage. First born TfAP-2  
1177 neurons, such as Pm1, Pm2 and Pm3, do not express Ap (magenta arrowheads), while  
1178 later born TfAP-2-expressing neurons, such as Tm1, express Ap (white arrowhead).

1179 (iii''') Expression of Ap and D in medulla neurons at L3 stage. Earlier born D neurons do  
1180 not express Ap (magenta arrowhead) and express D in the absence of this tTF in their  
1181 NBs of origin, while later born D neurons express Ap (white arrowhead) and are generated  
1182 in the D temporal window.

1183 (iii''''') Expression of Ap and Ets65A:GFP in medulla neurons at L3 stage. Earlier born  
1184 Ets65A neurons do not express Ap (magenta arrowhead), while later born Ets65A neurons  
1185 express Ap (white arrowhead).

1186 Scale bars: 10  $\mu$ m

1187 **(d) Medulla clusters.** Hierarchical tree relating the average variable gene expression from  
1188 each cluster of the L3-P15 dataset. Low quality (LQ) clusters or clusters that have a  
1189 different origin from the medulla side of the OPC neuroepithelium, such as PRs, L neurons,  
1190 Lawf1-2, LCNs and LPLCs neurons are shown in red.

### 1191 **Extended Data figure 9: Neuronal differentiation in flies and humans**

1192 (a) *Bsh* is expressed almost exclusively in Mi1s and was used to identify the Mi1 clusters.  
1193 Cluster Mi1 represents the pupal annotated cluster. Cluster L3\_23 consists of GMCs that  
1194 give rise to Mi1s and newly born Mi1s, while cluster L3\_53 corresponds to more mature  
1195 Mi1 cells, as assessed by their proximity to the P15 Mi1 cells.

1196 (b) UMAP plot of Mi1 cells at different stages of differentiation from L3 to P70. The  
1197 expression of *ase* (b) and *bsh* (b') were used to find the beginning and end, respectively,  
1198 of the L3 trajectory.

1199 (c) UMAP plot of Mi1 cells at different stages of differentiation from L3 to P70. L3 and P15  
1200 trajectories are elongated, depicting Mi1 cells of different ages. Transcriptomes are then  
1201 synchronized (compact group of clusters) around P30.

1202 (d) UMAP plot showing the trajectory of 3,363 single cell transcriptomes of the developing  
1203 human cortex (gestational week 19), as generated by Monocle3. The orientation of the  
1204 trajectory was identified by looking at the expression of marker genes for progenitors,  
1205 intermediate progenitors and neurons (see panel f). Colors indicate different clusters along  
1206 the trajectory.

1207 (e) UMAP plot focusing on the PAX6-positive single-cell transcriptomes of the developing  
1208 human cortex (gestational week 19). The radial glia population contains both ventricular  
1209 radial glia (FBXO32-positive cells) and outer radial glia (HOPX-positive cells).

1210 (f) UMAP plot of 3,363 single-cell transcriptomes of the developing human cortex  
1211 (gestational week 19). The trajectory from progenitors to neurons can be observed by the  
1212 expression of Pax6 (apical progenitors), Eomes (intermediate progenitors), and NeuroD2  
1213 (neurons). The dashed arrow depicts the differentiation trajectory.

1214 (g) Differential expression analysis along the trajectory of the cortical neurons identified  
1215 six modules of genes. Gene Ontology enrichment analysis found the first two modules to  
1216 be enriched in terms such as cell proliferation (FDR= $10^{-3}$ ) and DNA replication (FDR= $10^{-44}$ );  
1217 they likely correspond to the progenitor cells. Then, the third module is enriched in  
1218 neurite development terms, such as axon guidance (FDR= $10^{-7}$ ), while the fourth one is  
1219 enriched in terms related with synapse organization (FDR= $10^{-11}$ ). The fifth one contains  
1220 “functional genes”, such as calcium-dependent exocytosis (FDR= $10^{-2}$ ). The sixth module  
1221 does not show a clear peak of expression and no GO terms were found to be enriched.

1222 **Extended Data Figure 10: Expression of *Drosophila* tTFs in mouse neural**  
1223 **progenitors**

1224 (a) The mouse orthologs of the *Drosophila* optic lobe temporal transcription factors are not  
1225 expressed in specific temporal windows in mouse cortical radial glia during embryonic  
1226 stages E12-E15, which span their neurogenic period, except for Pax6, which is enriched  
1227 in young progenitors (two-sided Wilcoxon rank sum test, adjusted p-value = 1.969240e-  
1228 08).

1229 (b) Heatmap of expression of Igf2bp1 and Bach2 (orthologs of *Drosophila* Imp and chinmo,  
1230 respectively) in radial glia and neuronal progeny. Igf2bp1 is expressed in young apical  
1231 progenitors, while Bach2 is expressed in young apical progenitors and neurons that are  
1232 born by these young progenitors. Source: <http://genebrowser.unige.ch/telagirdon/>

1233 (c) Violin plots showing the expression of the known temporal factors of the mouse retinal  
1234 progenitor cells, *Ikzf1*, *Pou2f2* and *Casz1*, orthologs of the *Drosophila* VNC temporal  
1235 transcription factors, *hb*, *pdm* and *cas*, respectively. *Pou2f2* is expressed in younger  
1236 progenitors and *Casz1* in older progenitors. We do not see expression of *Ikzf1* at these  
1237 stages, as has been reported before<sup>46</sup>.

1238 (d) Violin plots showing the expression of the orthologs of the *Drosophila* optic lobe tTFs  
1239 in the mouse retinal progenitor cells. The expression of the orthologs of *hth* (*Meis2*), *opa*  
1240 (*Zic5*), and *D* (*Sox12*) seem to be restricted to embryonic stage 12, while the ortholog of  
1241 *ey* (*Pax6*) is expressed constitutively, as expected. Interestingly, *Nr2e1* is expressed just  
1242 before birth, which is when the retinal progenitor cells start generating Müller glia ortholog;  
1243 similarly, its ortholog *tll* also starts to be expressed when the *Drosophila* medulla  
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1245



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