

A global molecular code for birth order and neuronal identity in *Drosophila*

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The assembly of functional neural circuits relies on the generation of diverse neural types with precise molecular identity and connectivity. Unlocking general principles of neuronal specification and wiring across the nervous system requires a systematic and high-resolution characterization of its diversity, recently enabled by advances in single-cell transcriptomics and connectomics. However, linking the molecular identity of neurons to circuit architecture remains a key challenge. Here we present a high-resolution developmental transcriptional atlas for the *Drosophila melanogaster* nerve cord, the central hub for sensory–motor circuits. With a considerable 38× aggregate coverage relative to its reference connectome^{1,2}, our atlas captures extensive molecular diversity and enables robust alignment to the adult connectome. We identified three developmental principles underlying neuronal diversity in the nerve cord. First, the timing of neurogenesis shapes diversification of molecular identity: embryonic-born neurons diverge faster than larval-born neurons, as also observed in the adult connectome. Second, 17 transcription factors common to neurons from all lineages provide a global molecular identity code for birth order. Lastly, by mapping sex-specific transcriptional profiles to the connectome, we identified female-specific apoptosis and transcriptional divergence as key global drivers of sex specification. By revealing key organizational axes of molecular identity, this atlas opens avenues to dissect the molecular mechanisms underpinning the development and evolution of neural circuits.

The brain's ability to process information and produce adaptive behaviours relies on its cellular composition and circuit organization. Throughout development, neurons adopt specific molecular identities that define their physiological function and guide their integration into precise circuit architectures. While spatial and temporal patterning mechanisms coordinating neuronal fate are starting to be elucidated^{3–5}, we cannot yet account for the vast cellular complexity of the brain. To gain a comprehensive understanding of how neurons are specified across the nervous system we need systematic, high-resolution characterization of this diversity. Over the past decade, this need has driven two types of large-scale atlas efforts: single-cell transcriptomics to define molecular identities^{6–10}, and connectomics to reveal anatomical and synaptic relationships of neurons^{1,11–14}.

The dimensionality of these large datasets can be reduced by grouping individual neurons into cell types, which represent the functional units of neuronal circuits^{14–16}. In vertebrates, recent mouse and human brain atlases have identified more than 5,000 and 3,000 transcriptional types, respectively^{6,9}. Eventually it will be essential to integrate such molecular taxonomies with other key modalities including neuroanatomy and connectivity^{15,16}. There is currently no clear path to establish a comprehensive correspondence between transcriptomic

and anatomical types in vertebrates. However, the situation is more promising for the highly stereotyped and numerically smaller brain of *D. melanogaster*. In this case, the systematic analysis and annotation of the combined datasets of the central nervous system, the largest connectomes yet completed, revealed over 11,000 neuronal types across 160,000 neurons^{2,14,17}. This complete description of the texture of a complex nervous system provides an exciting opportunity to better understand both the developmental and organizational logic of neural circuits. Nevertheless, comprehensive cross-identification of anatomical and molecular cell types remains challenging.

The most complete matching to date has been obtained in sensory systems: for olfactory projection neurons¹⁸ and in the visual system (optic lobes)^{19–22}, where around 800 repeated ommatidia result in hundreds of copies for a relatively small number of cell types. Outside the optic lobes, where most cell types are represented by around 2 neurons per hemisphere, transcriptional coverage becomes a limiting factor: empirical observations and simulation suggest that a minimum of 20–30 cells is needed to identify a cell type in single-cell RNA-sequencing (scRNA-seq) datasets^{15,23}. As a consequence, low-coverage atlases are inherently unsuited to resolve the full connectomic diversity, explaining why large-scale atlases outside the optic

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lobes^{24–26} reported ten times fewer neuronal types^{2,14,15}. However, high coverage alone might not be sufficient to resolve connectomic types, as work in the olfactory and visual system has shown that transcriptional diversity peaks during development and decreases in adults^{5,18,20,27}, making developmental atlases better suited to capture the diversity in the connectome.

Stimulated by these considerations, we have generated a developmental transcriptional atlas of the *D. melanogaster* ventral nerve cord (VNC), which houses the sensory–motor circuits that form the core interface between the nervous system and the body. The atlas is available for browsing at Scope (https://scope.aertslab.org/#/Hundred-Drills*/welcome) and at a dedicated website (<https://flyem.mrc-lmb.cam.ac.uk/VNCatlas>). Its deep coverage (38× aggregate coverage) enabled high-resolution mapping of neuronal diversity as well as emergent organizational features of molecular identity; comprehensive annotation allowed most cells to be linked to the connectome. Together, these properties of the atlas enabled the identification of a global molecular temporal coordinate system: a sequence of 17 transcription factors (TFs) that define a persistent lineage-independent correlate of neuronal birth order. Comparison of male and female data identified apoptosis and transcriptional divergence as key developmental mechanisms of sex differences and also enabled precise matching with connectomic cell types. This atlas provides a resource and intellectual framework for identifying principles of circuit assembly.

Developmental atlas of the nerve cord

To reveal the transcriptional diversity of the 16,000 intrinsic neurons of the VNC, we built an atlas using scRNA-seq (Extended Data Fig. 1). After quality control, we obtained 459,091 cells from four developmental stages (6, 24, 36 and 48 h after puparium formation (h.a.p.f.)), spanning the period when neuronal morphology and connectivity are established²⁸ (Fig. 1a). The atlas includes a balanced number of male and female cells, making it suitable for studying the development and function of sexually dimorphic circuits (Extended Data Fig. 1f).

While the atlas contains neurons and glia (see Supplementary Fig. 1 for glia), we focused our attention on the neuronal subset. One of the main drivers of transcriptional diversity is maturation, which results in the layered organization of neurons in uniform manifold approximation and projection (UMAP) space according to the developmental stage at which they were sampled (Fig. 1b). This pattern is mirrored by graded expression of nSyb, a synaptic protein associated with neuronal maturation (Fig. 1b (i and ii)).

This embedding captures the molecular dynamics of maturation but it hinders matching cell types across development (Extended Data Fig. 6c). By regressing out maturation-related variability, we integrated transcriptomes from the same cell types across different stages. One exception is the stem region, which contains neuronal precursors and less differentiated neurons from samples from the 6 h.a.p.f. and 24 h.a.p.f. that do not integrate with those from the 36 h.a.p.f. and 48 h.a.p.f. (Extended Data Fig. 2), as those stages lack the corresponding immature cells (Fig. 1b). Our neuronal atlas contains 302,765 cells representing the 7,880 neurons per lateral half of the male VNC connectome (MANC)², yielding 38× aggregate coverage (per-stage coverage is shown in Extended Data Fig. 1a,f).

Two modes of neuronal diversification

In insects, there are two waves of neurogenesis. Primary neurons are born from stem cell progenitors (neuroblasts) in the embryo where they build the larval brain and are later remodelled through pruning and regrowth of arbourous during metamorphosis. The same neuroblasts produce secondary neurons during larval stages, which integrate into adult circuits only in the pupal stage²⁹ (Fig. 1a). We identified primary and secondary populations in the atlas by the mutually

exclusive expression of *Imp* and *dati* (Fig. 1c) and found primaries segregate into a region of punctate small groupings (clusters) and secondaries into a region of elongated low-dimensional manifolds (trajectories), typically radiating from the stem region (Extended Data Fig. 2). Outside small clusters, we found *Imp* expression at the tips of trajectories, distal to the stem. These are likely to be the first born neurons produced when the neuroblasts resume division after larval hatching and were annotated as ‘early secondaries’. Neurogenesis wave annotation is supported by the observation that secondary neurons make up 59% of the transcriptional atlas, similar to the 60% reported in a recent scRNA-seq larval atlas³⁰ and to the 66% observed in MANC (Fig. 1d). Moreover, there is a similar trend in cell size, where primary > early secondary > secondary, as measured by number of RNA molecules per cell (transcriptome) and soma volume (connectome) (Fig. 1e).

Spatial segregation in UMAP space of primary and secondary neurons highlights neurogenesis wave as a strong driver of transcriptional divergence. However, local organization is also different (puncta versus trajectories) suggesting that these two populations differ in the degree of separation between one cell type and its closest neighbour, with primary neurons diverging more rapidly than secondary neurons. This distinct organization is not an artifact of the joint analysis of primary and secondary neurons, as it is preserved when the two subpopulations are analysed independently (Fig. 1c). Notably, after adjusting for coverage, many primary clusters have sizes compatible with one cell, which is the median number of cells per type in the connectome (Fig. 1f,g), while the median cluster size is three cells. By contrast, secondary neurons yielded a median of 13 cells per cluster at the same clustering resolution, compared to 2 in the connectome (Fig. 1f,g). To better compare primary and secondary neuron clusters, we quantified their degree of similarity using an overlap metric defined as the fraction of neurons of which the nearest neighbours belong to a different cluster. Using this measure, secondary neuron clusters showed greater intermixing than primary clusters, with inter-cluster relationships forming ordered, trajectory-like structures, in contrast to the more heterogeneous organization of primary clusters (Fig. 1g). This observation aligns with the MANC connectome, in which we find that primary neurons are statistically more distinct from their most similar neighbour than secondary ones (Fig. 1h) and that the nearest neighbour of a secondary neuron is more likely to have the same developmental origin (that is, hemilineage identity) than for primary neurons (Fig. 1i). Together, these results strongly support the idea that secondary neurons diverge more gradually than primary neurons.

Hemilineages link the atlas to the connectome

Secondary neurons are born from a defined set of larval neuroblasts (on average 25 per thoracic segment) each defining a stereotyped neuronal lineage. Sibling neurons from each lineage form two hemilineages based on Notch signalling during asymmetric cell division: A (Notch on) and B (Notch off)^{28,31,32} (Fig. 2a). Crucially, neurons of the same hemilineage bundle together to enter the neuropile, leaving a structural footprint that has been exploited to group them in the connectome achieving over 90% hemilineage annotation coverage².

As hemilineage identity is one of the main sources of transcriptional heterogeneity in the VNC²⁶, we examined whether the trajectories in UMAP space reflected hemilineage identity. First, we assigned neurotransmitter identity, as neurons from the same hemilineage share neurotransmitter use^{2,33,34}. We found that cells in each trajectory expressed primarily just one fast-acting neurotransmitter (Fig. 2b and Supplementary Fig. 2), supporting the idea that trajectories represent hemilineages.

To provide direct experimental evidence for hemilineage identity, we included samples from four GFP reporter lines that label specific

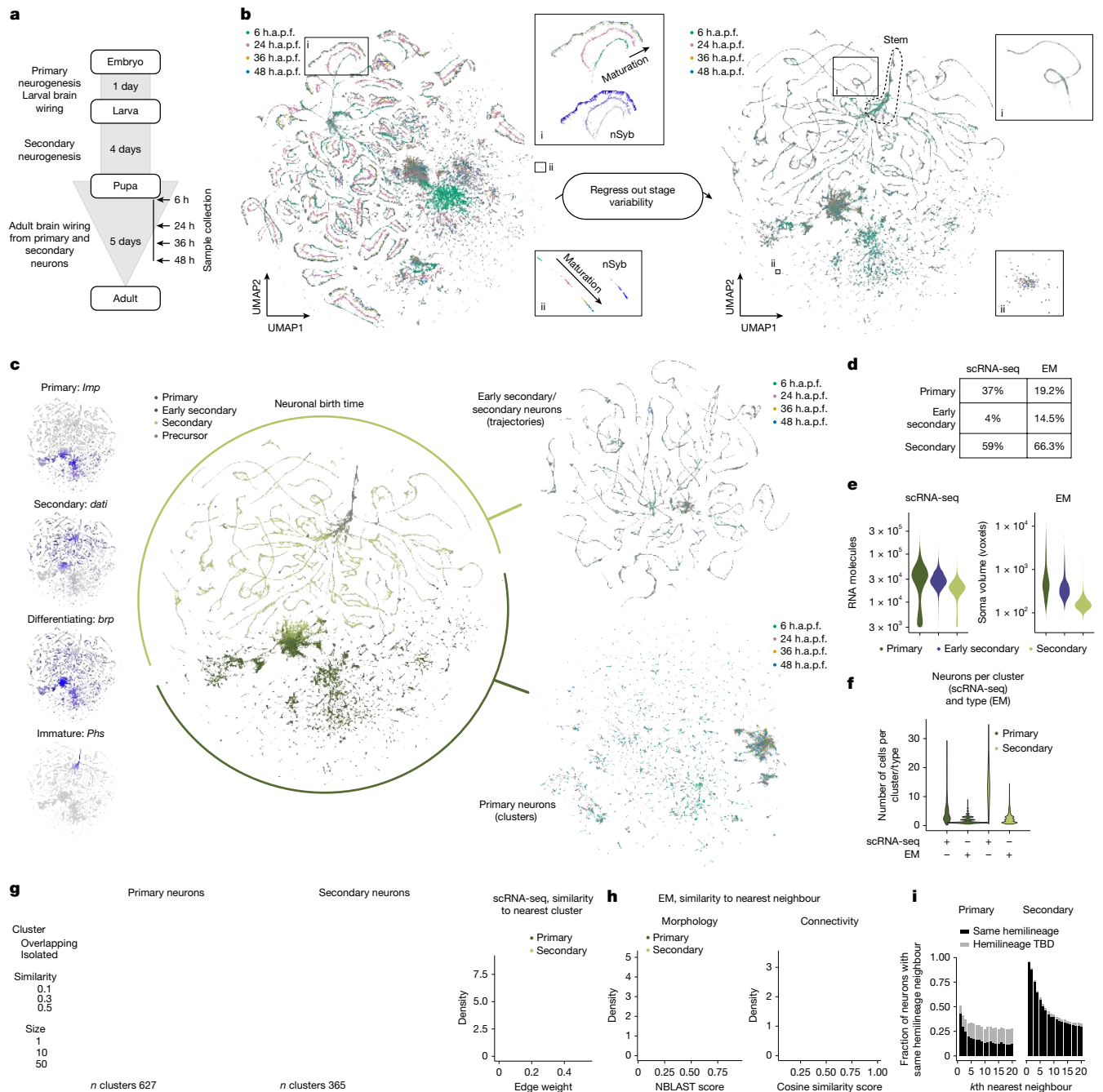


Fig. 1 | A developmental atlas of the *Drosophila* VNC identifies two modes of neuronal diversification. **a, Two neurogenic waves in *Drosophila* development. **b**, UMAPs showing neurons coloured by stage before (left) and after (right) regression of maturation-driven variability. Insets: the same subsets of cells (i and ii) in either embedding. *nSyb* expression increases with stage. The dotted line indicates the immature neuron stem region. **c**, UMAPs for markers of neurogenic wave and maturation status (left). Middle, neurogenic wave and maturation status annotation. Right, UMAPs of primary and secondary neuronal subsets. **d**, Fractions of primary, secondary and early secondary neurons in the atlas and the connectome. EM, electron microscopy. **e**, RNA abundance in the atlas (unique molecular identifiers, left) and soma volume in the connectome (right) by neurogenesis wave. **f**, Neurons per scRNA-seq cluster after normalizing for coverage (24 + 36 + 48 h.a.p.f., 28×) and per connectomic type. **g**, Constellation plots showing intercluster overlaps. Nodes represent the cluster centroids, and the size is proportional to the cluster size. Brown indicates isolated clusters after weight filtering < 0.1.**

Edge widths are proportional to the summed bidirectional fraction shared between the two clusters. Given clusters A and B, the edge weight is the fraction of A's nearest neighbours belonging to B, plus the fraction of B's nearest neighbours belonging to A. Density plot of the strongest edge for each cluster. $n = 627$ (primary), $n = 365$ (secondary) clusters. Statistical analysis was performed using two-sided Wilcoxon rank-sum test; $P < 1.3 \times 10^{-14}$. **h**, Distribution of NBLAST morphology scores to the top-match neuron (left). $n = 1,386$, median = 0.45 (primary); $n = 9,919$, median = 0.55 (secondary). Right, distribution of cosine similarity connectivity scores to the nearest neighbour. $n = 1,356$, median = 0.55 (primary); $n = 9,726$, median = 0.74 (secondary). For both comparisons, secondary neurons are more similar than primary neurons. Statistical analysis was performed using two-sided Wilcoxon rank-sum test; $P < 2.2 \times 10^{-16}$. **i**, Fraction of neurons sharing hemilineage with the k th neighbour at increasing neighbour rank. Black, the same hemilineage; grey, hemilineage identity of neighbour to be determined (TBD).

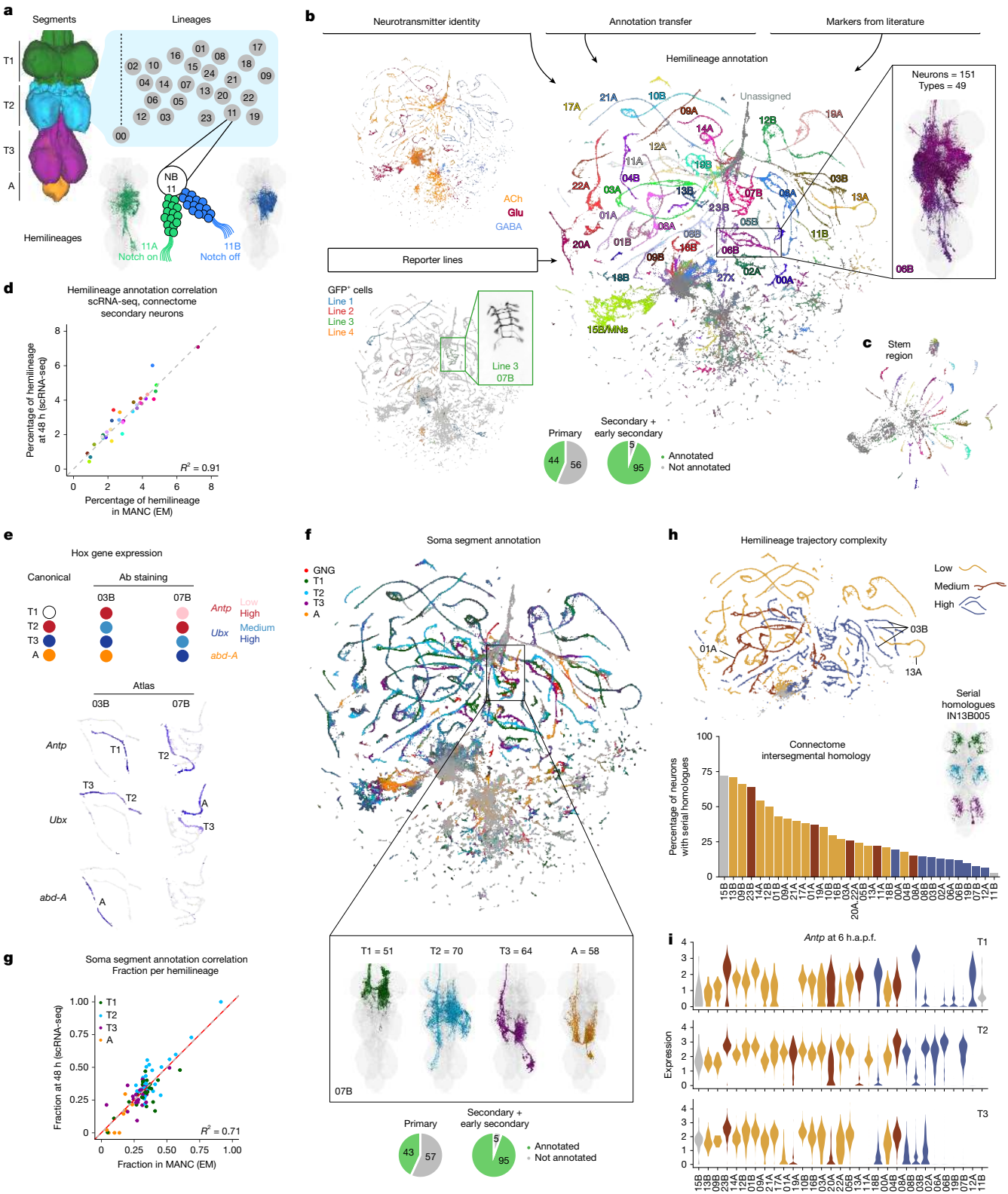


Fig. 2 | See next page for caption.

hemilineages (09A, 01+10B, 07B, 08B+09B)³⁵ throughout development and into adulthood; the distribution of GFP-positive cells along specific trajectories confirms that trajectories correspond to hemilineages

(Fig. 2b). We next used multiple sources of evidence (Fig 2b and Extended Data Fig. 3a) to annotate hemilineage identity for over 95% of secondary and 44% of primary neurons, linking them to groups of,

Fig. 2 | Hemilineage and segment annotations link the transcriptome to the connectome. **a**, Adult VNC showing thoracic (T1, T2, T3) and abdominal (A) segments. 25 stem cells (neuroblasts, NB) per segment generate secondary lineages, their progeny splitting into A and B hemilineages determined by Notch signalling. Male connectome (MANC) renderings for hemilineages 11A and 11B. **b**, The central UMAP shows hemilineage identity assignments based on cumulative evidence (Extended Data Fig. 4): neurotransmitter predictions, adult VNC scRNA-seq atlas²⁶ annotation, markers from the literature and GFP⁺ cells from reporter lines in the atlas. Cross-matching between transcriptomic and connectomics atlases is shown for hemilineage 06B (MANC neurons in purple). The fraction of annotated neurons is shown. MNs, motor neurons. **c**, Precursor cell UMAP with manual annotation based on markers. **d**, Hemilineage annotation correlation (secondary neurons) between MANC and 48 h.a.p.f. atlas neurons. The dotted grey line indicates identity. **e**, Expression of Hox genes across segments: canonical (top left) and observed by larval stainings in hemilineages 03B and 07B (top right). Bottom, expression in 03B and 07B

secondary neurons at 48 h.a.p.f. The labels show predicted segmental identities. **f**, Soma segment annotation. GNG neurons are only at 6 h.a.p.f. Inset: MANC 07B secondary/early secondary neurons (left side). The fraction of annotated neurons is shown. **g**, Segment annotation correlation showing the fraction of secondary neurons per segment per hemilineage annotated in MANC and 48 h.a.p.f. secondary neurons. The dotted grey line indicates identity. The solid red line is the fitted linear model. **h**, UMAP of secondary neurons colour-coded by hemilineage trajectory complexity (top). Bottom, the per-hemilineage proportion of secondary neurons with serial homologues in MANC coloured by trajectory complexity. Trajectory complexity in the atlas is significantly correlated with intersegmental homology in the connectome. Statistical analysis was performed using the one-sided Jonckheere–Terpstra test; $P = 2 \times 10^{-4}$. A neuronal type with serial homologues across thoracic segments is shown as an example. **i**, *Antp* expression at 6 h.a.p.f. per hemilineage across segments and coloured by trajectory complexity. Hemilineage order is as in **h**.

on average, 153 neurons and 60 neuronal types in the connectome (for example, 151 neurons and 49 types in 06B). We next extracted markers for hemilineage identity, of which 88% were TFs (Extended Data Fig. 3b), and Notch status (Supplementary Fig. 3). We found that 15 out of 34 hemilineages can be distinguished by the expression of a single marker, while the rest are identified by marker combinations; these markers provide genetic access to hemilineages and helped extend hemilineage annotation to neurons in the stem region, poorly predicted by our annotation pipeline (Fig. 2c).

The accuracy of our atlas annotation is supported by a very strong correlation between the observed sizes of hemilineages in the atlas and the connectome ($R^2 = 0.9$; Fig. 2d).

Segmental identity links the atlas to the connectome

Like the vertebrate spinal cord, the VNC has a repeated segmental organization: homologous lineages are generated across segments, acquiring segment-specific characteristics through anteroposterior patterning specified by gradients of Hox gene expression^{36,37} (Fig. 2a). To assess the impact of segmental organization on the transcriptome while refining our transcriptome–connectome matching, we combined Hox gene expression in the atlas with Hox protein expression in larvae to annotate segments across hemilineages (Fig. 2e and Extended Data Fig. 4a–c). In total, we provide segment identity predictions for 94% of secondary neurons and 43% of primary neurons (Fig. 2f; annotation validation is shown in Supplementary Fig. 4).

The intersection of hemilineage and segment predictions subsets the atlas into 122 groups of secondary neurons, each mapping onto a mean of 51 neurons or 26 types in the connectome. The quality of the matching is supported by a strong correlation between the fraction assigned to each group in either dataset ($R^2 = 0.71$; Fig. 2g).

For some hemilineages, intersegmental differences are large, resulting in independent trajectories each displaying specific marker genes (such as 03B), while others overlap partially (01A) or completely (13A) (Fig. 2h, Extended Data Fig. 5 and Supplementary Fig. 4). This difference in trajectory complexity could underlie the different degree of neuronal intersegmental (serial) homology observed in the connectome (for example, IN13B005 type has serially homologous neurons in all three thoracic segments; Fig. 2h). To test this, we classified hemilineages into three categories of complexity: low (fully overlapping), medium (partially overlapping) and high (non-overlapping). We found that the transcriptional categories are significantly linked to the degree of intersegmental homology reported in the connectome^{2,38} (Fig. 2h). Notably, *Antp* is expressed across the three segments in low-complexity hemilineages, instead of the expected restriction to T2 (Fig. 2i and Extended Data Fig. 4b), suggesting that *Antp* may contribute to reinforcing neuronal homology across thoracic segments. This might be needed to obtain neuronal identities

implementing segmentally repeated sensory–motor circuits (for example, leg movement).

A global temporal code for birth order

Sequential generation of different neuronal types from shared progenitors is a core developmental feature of the insect central nervous system^{39,40} and other systems, such as the mammalian retina⁴¹. Notably, the expression patterns of *Imp* (confined to the tip of trajectories opposite to the stem; Fig. 1c) and of known genetic markers of birth-order identity^{42,43} (*mamo*, *jim* and *br*) (Fig. 3a and Extended Data Fig. 6a) provide strong evidence that neurons are arranged along trajectories according to their birth order. Neuronal diversity within lineages should therefore result from changes in gene expression along each trajectory (Fig. 3a). Using trajectory inference analysis, we identified a total of 1,508 genes differentially expressed along trajectory pseudotimes for all hemilineages, of which 149 were TFs (Fig. 3b,c). Notably, 40 of those TFs were common to more than 50% of the hemilineages, raising the possibility of a shared temporal program across hemilineages. This was supported by the observation that some TFs were expressed in a conserved order across lineages and invariant through developmental stages when assessed relative to bona fide cell-type-specific markers such as neuropeptides (Fig. 3d and Extended Data Fig. 6b,c).

To facilitate a direct comparison of gene expression dynamics across hemilineages and segments, we aligned all trajectories to a single reference trajectory—03A-T1, one of the longest continuous trajectories in the atlas (Fig. 3e and Extended Data Fig. 6d,e). This shared space enabled us to identify expression peaks that are aligned in pseudotime across hemilineages and segments (Fig. 3e,f). As a result, we found 17 TFs that exhibit a conserved pattern of expression along pseudotime and across hemilineages (*hth*, *chinmo*, *pdm3*, *pros*, *ab*, *mamo*, *CG7368*, *rn*, *CG3726*, *jim*, *br*, *dati*, *Eip93F*, *bab1*, *bab2*, *danr* and *dan*) (Fig. 3g and Supplementary Fig. 5), hereafter referred to as shared TFs (shTFs). Several shTFs are expressed multiple times along trajectories, expanding the number of conserved expression peaks to 33 (Fig. 3g). Most shTFs show persistent expression throughout developmental stages (Extended Data Fig. 6f) and we confirmed protein expression in adults for nine of them (*Hth*, *Bab1*, *Bab2*, *Dan*, *Br*, *Eip93F*, *Mamo*, *Pdm3* and *Pros*), in both the VNC and central brain (Extended Data Fig. 7). Thus, the combinatorial action of shTFs offers a possible mechanism to generate neuronal diversity. Moreover, the intersection of shTFs expression with hemilineage-specific markers could be turned into a modular genetic approach to access cell types. We confirmed this possibility by labelling small populations of neurons born during the *danr* temporal window from hemilineages 04B and 13A in adults using the split-GAL4 system (Fig. 3h).

To validate that shTF expression is locked to neuronal birth time, we performed pulse–chase labelling, in which larvae were fed EdU during non-overlapping time windows (Fig. 4a). We found that neurons

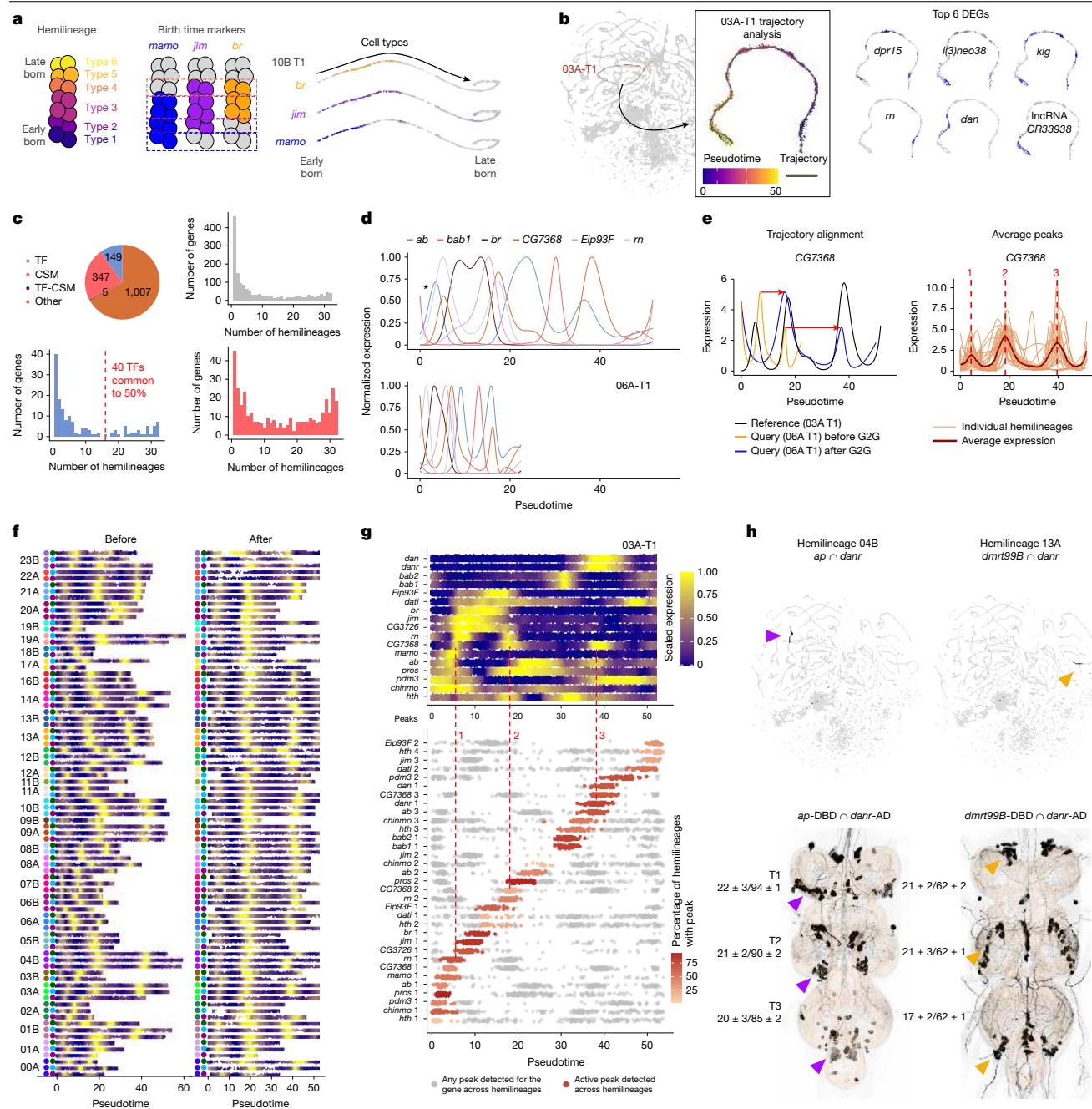


Fig. 3 | 17 TFs define a global temporal code of neuronal birth order.

a, Within hemilineages, cell types are born sequentially (left). Right, hemilineage 10B-T1 UMAPs showing three consecutive birth-order markers. **b**, Fitted trajectory and pseudotimes for 03A-T1 and 6 DEGs. **c**, DEGs with $q < 0.01$ and Moran's value > 0.3 along trajectories. The distribution of DEGs across gene categories (pie chart) and hemilineages (histograms) are shown. Each bar represents genes shared by that number of hemilineages. Grey, DEGs; blue, TFs; red, cell surface molecules (CSM); brown, dual function. **d**, Normalized expression profile along pseudotime for 6 TFs that are differentially expressed in 95% of hemilineages. Dashed lines link expression peaks. The asterisk indicates the peak that is absent in 06A-T1. **e**, Expression profiles of *CG7368* along pseudotime for 06A-T1 before and after alignment (left) and across trajectories after alignment (right). The average expression profile and average peaks are shown in brown. **f**, *CG7368* normalized expression along pseudotime in trajectories before (left) and after (right) alignment. The y-axis dot colours represent hemilineages from Fig. 2 (left) and segments (right; green, T1; blue, T2;

and purple, T3). **g**, TFs expressed in a shared temporal order across lineages (shTFs). Top, for 03A-T1, the normalized expression along pseudotime of shTFs ordered by the first peak in the consensus sequence. Bottom, aligned gene expression peaks positions arranged by consensus order. Each dot is a peak in a single trajectory. The coloured peaks match the average active peak (the intensity represents the detection frequency across trajectories) and the grey peaks represent other peaks. **h**, Specific adult neuronal populations labelled by *danr*–hemilineage-specific marker intersection. The UMAPs represent cells co-expressing hemilineage markers (*ap* for 04B and *dmrt99B* for 13A) and *danr*. Maximum-intensity projection of confocal stacks registered to the VNCIS2 template: split-GAL4 intersection patterns in adult VNCs. The numbers indicate the average hemilineage neurons labelled per side $\pm$ s.d./average MANC hemilineage neurons per side. The arrowheads indicate neurons belonging to the expected hemilineage. In agreement with atlas predictions, clusters not belonging to 04B or 13A are visible, likely primary neurons.

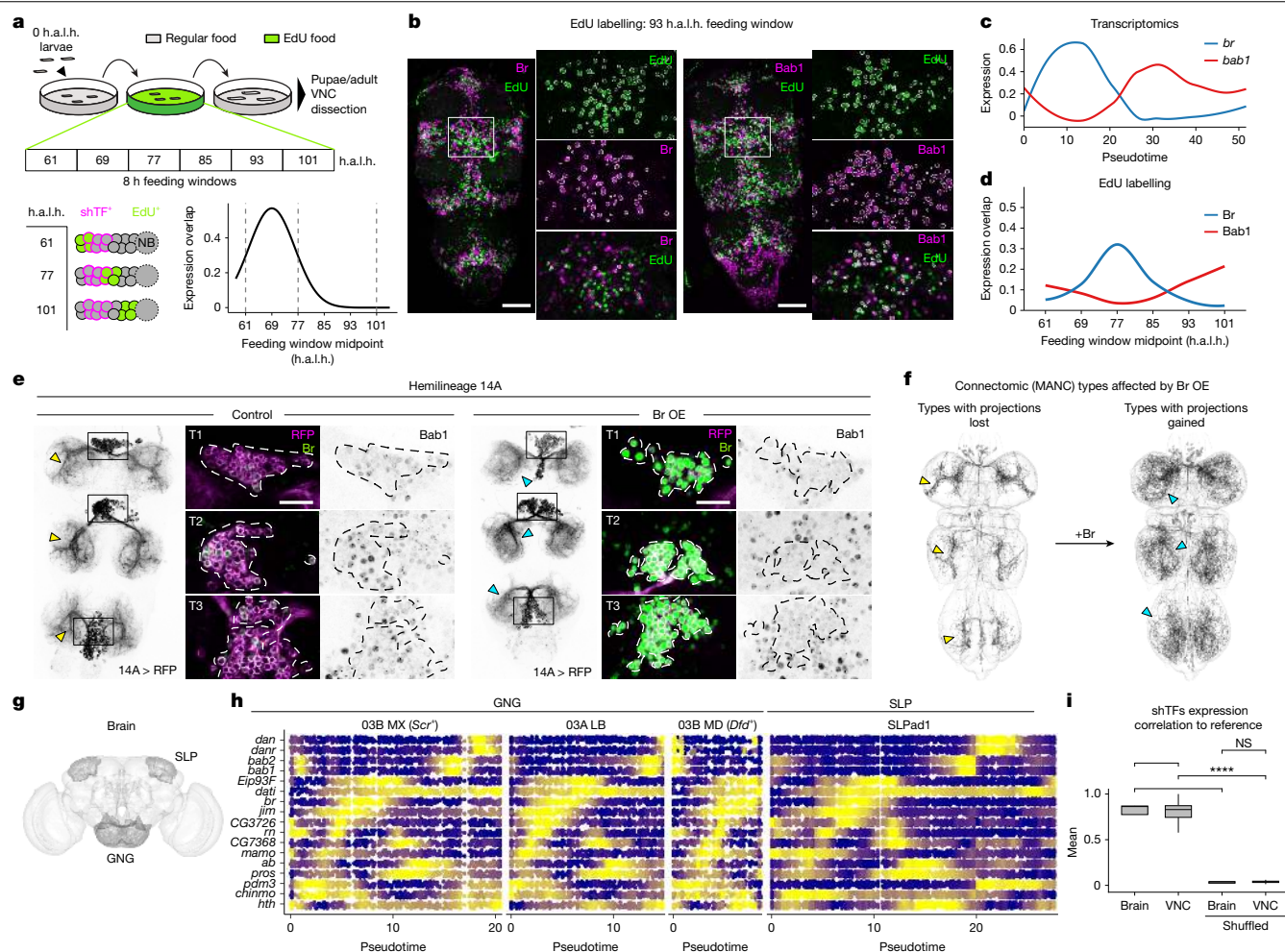


Fig. 4 | The VNC's global temporal transcription code is conserved in the brain. **a**, EdU pulse-chase experiments. h.a.l.h., hours after larval hatching. **b**, EdU-labelled (green) VNCs from larvae fed during the 93 h.a.l.h. window, together with *Br* (magenta, left) or *Bab1* (magenta, right). Whole VNCs are displayed as maximum-intensity projections. Insets: single z plane and the corresponding segmentation results for *EdU* (top), *shTF* (middle) and their intersection (bottom). Scale bars, 50 μ m. **c**, Expression along pseudotime for *br* (blue) and *bab1* (red) in hemilineage 03A-T1. **d**, Fraction of *shTF*⁺ cells colocalizing with *EdU* across feeding windows (see the 'EdU labelling' section of the Methods). Each dot represents one VNC ($n = 2-4$ per condition). **e**, Overexpression (OE) of *Br-Z4* isoform in hemilineage 14A causes the disappearance of *Bab1*⁺ cells within 14A and a change in projections morphology. Projections lost (yellow) or gained (cyan) in the *Br-Z4*-overexpression condition are marked by arrowheads. Whole VNCs are displayed as maximum-intensity

projections. Insets: a single z plane. Scale bars, 20 μ m. Images are representative of $n = 4$ per condition. **f**, Connectomic (MANC) types lost (left, yellow arrowheads) or gained (right, cyan arrowheads) by *Br-Z4* overexpression. **g**, Rendering of the brain highlighting regions of origin for the hemilineages examined in **h**. **h**, Expression of shTFs for hemilineages in the brain as in Fig. 3g. GNG data from 6 h.a.p.f. and SLP data from 6–48 h.a.p.f. LB, labial; MX, maxillary; MD, mandibular. **i**, shTFs expression correlation between the reference trajectory (03A-T1) and aligned trajectories from brain (**h**) or VNC. Each dot represents the mean correlation across 17 shTFs for a hemilineage-segment combination ($n = 4$ CB, 89 VNC). The plot shows the median of the mean correlation $\pm$ interquartile range (IQR); the whiskers extend to the most extreme datapoints within $1.5 \times$ IQR. Shuffled, correlations were calculated by shuffling shTF identity 200 $\times$ per hemilineage. Statistical analysis was performed using two-sided Wilcoxon rank-sum tests; * $P = 0.029$, **** $P < 2 \times 10^{-16}$; NS, $P > 0.5$.

expressing shTFs from non-overlapping temporal domains in the atlas (*br* and *bab1*; Fig. 4b,c) are born in distinct temporal windows in the larva: *Bab1*⁺ cells are born in early and late windows, while *Br*⁺ cells are born in between (Fig. 4d), consistent with their expression in the atlas.

Broad defines neuronal identity

The persistent expression of shTFs suggests they may act as terminal selector genes⁴⁴. We tested this possibility by looking for changes in neuronal identity when ectopically expressing *Br* outside of its temporal window in hemilineage 14A. We used *Bab1* expression as a readout of genetic interaction between shTFs expressed in non-overlapping temporal windows (Fig. 4c) and found that, while it is readily detected in control animals, ectopic *Br* expression eliminates *Bab1* expression

within 14A neurons (Fig. 4e). Furthermore, some neuronal processes are substantially reduced, while others are enhanced (Fig. 4e), with no significant changes in the number of neurons (mean $\pm$ s.e.m. in thoracic segment 1 (T1), overexpression, 152 ± 1.5 , $n = 4$; control, 155.3 ± 2.0 , $n = 3$; two-tailed Student's *t*-test, $P = 0.23$). Notably, in the MANC connectome, we identified 14A neuronal types compatible with the lost or gained processes (Fig. 4f). We conclude that ectopic *Br* expression is sufficient to shift neuronal identity within the 14A hemilineage, supporting a role as a terminal selector in the VNC.

The shTF code is conserved in the brain

We examined whether the shTF code extends to secondary neurons in the brain by analysing a small subset of hemilineages in two brain

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regions: the gnathal ganglia (GNG), evolutionarily linked to the VNC, and the superior lateral protocerebrum (SLP), a more derived region without clear transcriptional, morphological or functional homology to the VNC⁴⁵ (Fig. 4g). For the GNG, we used trajectories present in 6 h.a.p.f. samples due to imprecise separation of brain and VNC tissues (Extended Data Fig. 4d,e), while the SLP hemilineage was isolated from an independent brain dataset (Methods).

In both regions and across all four hemilineages, shTFs exhibited expression patterns consistent with those observed in the VNC (Fig. 4h). We quantified this observation by calculating expression correlation between each hemilineage and the O3A-T1 reference. We did not observe a statistically significant difference between brain and VNC scores (Fig. 4i), supporting the notion that shTFs influence the development of all secondary neurons in the central nervous system, excluding the optic lobes.

Sex differences across the nerve cord

Many aspects of sexually dimorphic behaviours are implemented by neural circuits in the VNC, such as courtship song production in male flies or post-mating behaviour in female flies⁴⁶. VNC connectomes for both sexes enable comparative analysis and the identification of dimorphisms at scale¹⁷, of which the origin and nature could be elucidated by adding developmental molecular information.

The balanced number of cells per sex in the atlas enabled us to identify regions displaying differential abundance, containing 4.3% of the neurons at 48 h.a.p.f. (Fig. 5a,b and Extended Data Fig. 8a). Male-enriched cells are more likely to be secondary neurons (81%), while female-enriched cells are biased towards primary neurons (78%) (Fig. 5b) and these differences correlated with the expression of the key sex-determination genes *fruitless* (*fru*) and/or *doublesex* (*dsx*)⁴⁶ (Fig. 5b).

Among the well-annotated secondary neurons, sexual dimorphisms map to a small number of hemilineage–segment combinations (Fig. 5c and Extended Data Fig. 8b,c). We validated our predictions in the male (MANC) and female (FANC) connectomes, focussing on six hemilineage–segment combinations: O8B-T1 and O8B-T2 (male enriched), O1A-T1 (reported sexually dimorphic⁴⁷) and O1A-T2, O6A-T1 and O6B-T1 (isomorphic) (Fig. 5c). We established an automated pipeline to obtain one-to-one matching between MANC and FANC neurons and transfer hemilineage labels from the well-annotated MANC to FANC (Fig. 5d and Methods). As predicted by the atlas, we found the greatest numerical differences in O8B-T1 and O8B-T2, which also had the largest number of MANC-specific neuronal types (Fig. 5e).

While not all *fru*⁺ neurons belong to numerically dimorphic groups (Extended Data Fig. 8d), nearly all sex-biased secondary neurons expressed *fru* (Fig. 5c). We mapped these neurons in the MANC connectome using published light-level images of *fru*⁺ lineage-related neurons (MARCM clones)⁴⁷ (Fig. 5f and Supplementary Table 2). By linking gene expression, hemilineage identity and neuronal morphology, we matched small groups of *fru*⁺ neurons across the atlas and the connectome (Extended Data Fig. 9). To validate our annotations we focused on *fru*⁺ neurons from hemilineage O8B-T2, which we mapped to the vPr-I clone. In the atlas, vPr-I neurons are born at three pseudotime windows: most are early born and male specific, while two later-born small groups have neurons from both sexes (Fig. 5g). Consistent with this, in the connectome, most vPr-I types are male specific, with only two types (three neurons per side) shared between sexes (Fig. 5g). Furthermore, MANC–FANC matching and intersectional labelling experiments confirmed atlas predictions of male-specific hemilineage O4B neurons in all thoracic segments, despite a corresponding clone (vPr-g) reported only in T1⁴⁷ (Extended Data Fig. 8e,f). Notably, O4B *fru*⁺ neurons are late born in the atlas, consistent with the external position of male-specific vPr-g cell bodies in MANC (Extended Data Fig. 8e). These results showcase the atlas's power for studying known and previously unknown sexual dimorphisms.

Molecular mechanisms shaping dimorphism

We next examined how sex differences emerge during development. The atlas shows that dimorphisms develop progressively throughout metamorphosis (compare Extended Data Fig. 10a and Fig. 5c). Accordingly, neurons within the vPr-I male-specific temporal window are gradually lost in females through pupal development. This correlates with increased expression of the pro-apoptotic gene *rpr* at 24 h.a.p.f. only in females (Fig. 5h and Extended Data Fig. 10b), suggesting that female-specific apoptosis causes the imbalanced cell number. Regions of female-specific apoptosis are present across the atlas (Extended Data Fig. 10c), pointing to its widespread role in shaping male-specific domains, extending earlier results in the brain^{48,49}.

While some neurons are sex specific, others only show morphological differences⁴⁷. To investigate the molecular signatures underlying morphological dimorphisms we focused on hemilineage O1A-T1, in which we mapped previously described sex differences in the dPr-a *fru*⁺ clone⁴⁷ to both MANC and FANC connectomes (Fig. 5i). The atlas shows that *fru*⁺ neurons display a clear separation between males and females in O1A-T1 UMAP space (Fig. 5j). We calculated gene expression differences between sexes in clusters of the O1A-T1 lineage and identified the dimorphic cluster to have the highest number (Fig. 5j). Notably, among the most significant genes, we found several with synaptic partner matching functions, such as members of the *beat*, *side* and *dpr* families⁵⁰ (Extended Data Fig. 10d), that might have an instructive role in dimorphic wiring. Finally, we quantified the emergence of dimorphism through metamorphosis, by defining a per-cell male–female molecular distance metric (Fig. 5j and Methods). This shows that molecular distance, and the number of differentially expressed genes (DEGs), increase with developmental time specifically in *fru*⁺ neurons (Fig. 5k).

Overall, these results show that sexual dimorphisms emerge through development from neurons with the same temporal origin by using two alternative strategies: sex specific apoptosis and transcriptional divergence. Two recent independent studies reached similar conclusions that sexual dimorphisms reflect sex-specific developmental programs acting on neurons born in the same lineage and birth order, rather than de novo generation of sex-specific cell types^{51,52}.

Discussion

Here we report a high-resolution developmental transcriptional atlas of the *Drosophila* nerve cord. This provides a platform to investigate the molecular logic of the thousands of cell types that wire up to form the sensory–motor circuits in the nerve cord, similarly to how more focussed atlases within the brain have been used to understand wiring specificity of *Drosophila* olfactory³³ and visual circuits^{54–56}. To facilitate exploration, our data are available at Scope (https://scope.aertslab.org/#/HundredDrills*/welcome) for gene expression browsing, and on a dedicated website (<https://flyem.mrc-lmb.cam.ac.uk/VNCatlas>), which integrates transcriptomic and connectome metadata. We linked the atlas and the connectome by sequentially annotating primary and secondary neurons, hemilineages, segments and *fru* expression. Our description of molecular identity of consecutively born secondary neurons opens the possibility for alignment of transcriptomic and connectomic types. Comparative analysis of sexual dimorphisms in *fru*⁺ neurons refined cross-matching with the connectome to as few as three neurons (Fig. 5g). While exhaustive cross-annotation is a future goal, this correspondence for *fru*⁺ neurons shows that our atlas contains sufficient information for identification of precise connectomic cell types. Stochastic labelling of neuronal morphology^{39,40}, or dense labelling of shTFs coupled with light-microscopy-based connectomics (LICONN)⁵⁷ are promising avenues for full-scale alignment with the connectome.

Our data show that the molecular profile of consecutively born neuronal types diverge much more gradually in the larva than the embryo. Increasing the coverage of the atlas will establish whether secondary

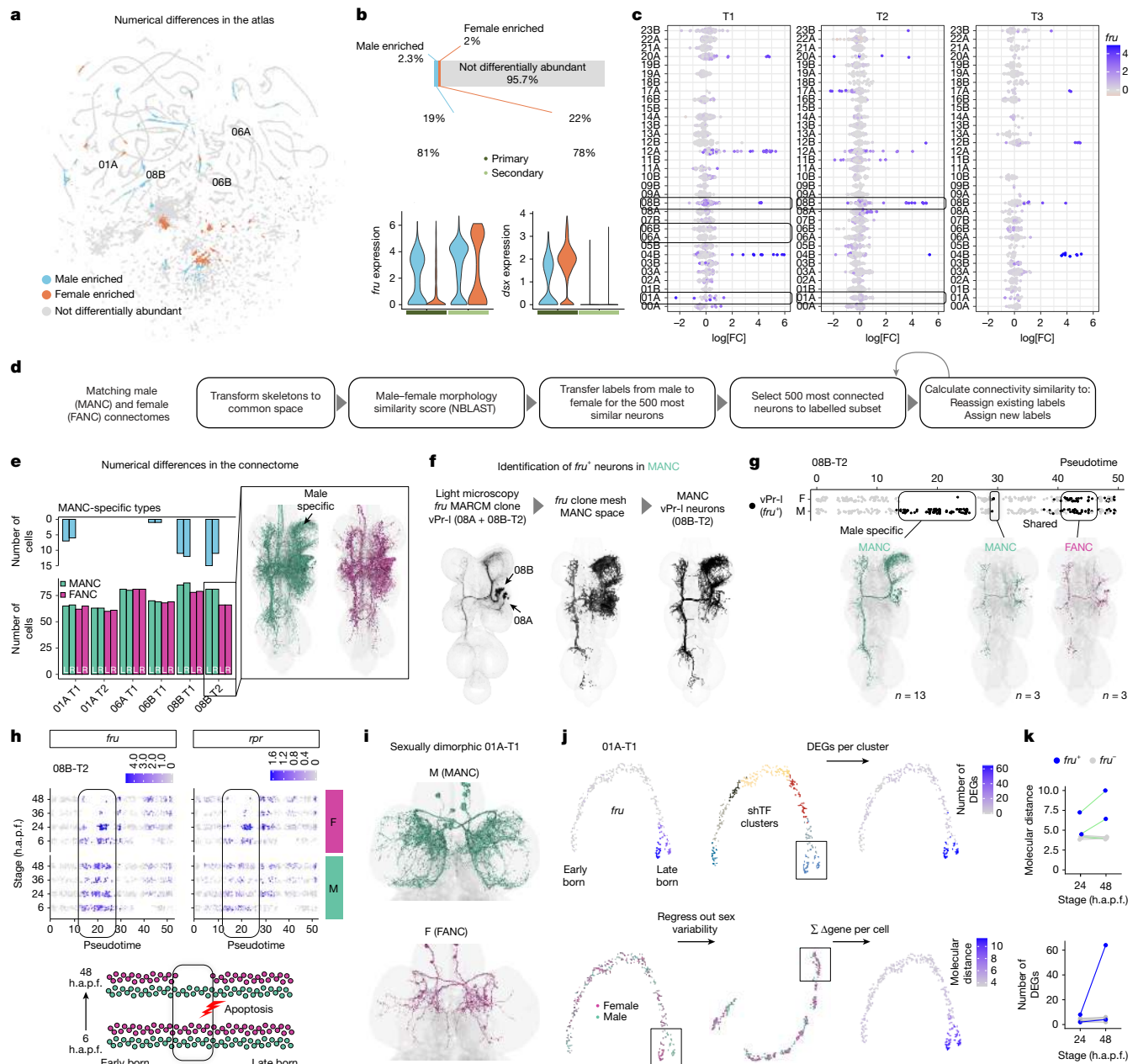


Fig. 5 | The transcriptional atlas predicts sex differences between male and female connectomes. a, UMAP of differentially abundant regions per sex at 48 h.a.p.f. (blue, male enriched; orange, female enriched). **b**, Percentage of neurons enriched per sex at 48 h.a.p.f.; percentage of primary and secondary neurons in sex-enriched regions; and *fru* and *dsx* expression in sex-specific neighbourhoods at 48 h. **c**, Beeswarm plots showing the secondary neuron differential abundance at 48 h.a.p.f., grouped by hemilineage and soma segment. Each dot corresponds to a neighbourhood in miloR analysis, coloured by mean *fru* expression. The boxes show hemilineages proofread in FANC. FC, fold change. **d**, Pipeline for matching the MANC and FANC connectomes. **e**, Proofread hemilineage annotation in FANC. Bottom, neurons per hemilineage, per side, per sex after proofreading (L, left; R, right). Top, the number of MANC neurons belonging to types for which FANC matches were not found by the automatic pipeline. Inset: O8B-T2 manually curated neurons in MANC (green, left) and FANC (magenta, right) in MANC space. **f**, Pipeline to

identify candidate *fru*⁺ neurons in MANC starting from MARCM clones. **g**, The atlas predicts sex-specific and shared *fru*⁺ neurons in the connectome. Top, 48 h.a.p.f. secondary O8B-T2 neurons arranged by pseudotime and coloured by vPr-I clone. Bottom, vPr-I neurons in the connectome. Green, MANC; magenta, FANC. Left, male-specific neurons. Right, shared neurons. *n* values indicate the number of neurons. **h**, *fru* (left) and *rpr* (right) expression in O8B-T2 secondary neurons along pseudotime, split by sex and stage. **i**, Male and female dimorphic *fru*⁺ neurons. **j**, UMAP showing O1A-T1 secondary neurons at 48 h.a.p.f. Colours represent *fru* expression, cluster identity calculated using shTFs, the number of DEGs per cluster, sex and per-cell correction score after sex regression. **k**, Molecular differences at 24 and 48 h.a.p.f. Average correction score per cluster (top). Number of DEGs between male and female per cluster (bottom). The lines connect corresponding clusters. Green or blue lines indicate $P < 0.01$, as calculated using two-sided Student's *t*-test with Bonferroni correction. $n > 26$.

types indeed resolve in fully isolated clusters. Notably, the temporal patterning mechanisms specifying embryonic-born versus larval-born neurons mirror this distinct organization: a discrete temporal cascade

of TFs in embryonic neuroblasts^{4,58,59} versus opposing gradients of the RNA binding proteins Imp (early) and Syp (late) in larval neuroblasts^{60,61}. The relationship between temporal origin and molecular identity has

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been characterized in embryonic and optic lobe neurons^{4,62}, but how *Imp/Syp* gradients translate to neuronal identities is only starting to be explored^{42,60,63}. We propose that, by encoding a persistent molecular timestamp of neuronal birth order, the 17 shTFs are perfectly poised to be key intermediaries in this process. Although the precise functional contributions of most shTFs in specifying and maintaining neuronal identity remain unresolved, instructive roles in fate specification have been identified for some^{21,52,64,65}. Our Br overexpression confirms this role in the VNC. From an evolutionary perspective, a gradual molecular identity system could offer several advantages. It is more evolvable, as small shifts in gene regulation can generate new neuron types without requiring new patterning modules, enabling gradual diversification and behavioural adaptation.

The shTFs that we identify are reminiscent of concentric genes demarcating successive cohorts of neurons in the *Drosophila* optic lobe^{22,62,66}. However, the shTF code operates in the central brain and VNC, in which there are over 10,000 cell types in contrast to a few hundred intrinsic cell types in the optic lobe¹³. Our atlas provides the strongest evidence to date for a persistent molecular correlate of birth order operating across secondary neurons, extending temporal logic beyond previously studied, region-specific systems^{22,27,42,62,66}. Consistent with our findings, nine shTFs had previously been identified as markers of specific projection neuron (PN) types²⁷. In this context, *br* and *Eip93F* label mid-born projection neurons, whereas *danr* marks late-born projection neurons. Moreover, two recent studies have identified most shTFs in a small subset of central brain hemilineages in pupae and adults^{52,67} (Supplementary Table 3). However, beyond the 17 shTFs, other TFs identified in those studies are not broadly shared across lineages and therefore do not meet our inclusion criteria for shTFs.

Notably, the temporal specification of neurons in flies has parallels in mammals. Homologues of *Imp* and *Syp* exist and have been implicated in neurogenesis^{68,69}. Furthermore, a shared transcriptional code has recently been described in maturing neurons of the mouse spinal cord, where early neurons express *Onecut* family TFs, intermediate neurons express *Pou2f2*, *Zfhx3* and *Zfhx4*, and late-born neurons express *Nfia* and *Nfib*, often in combination with *Neurod2* and *Neurod6*^{70,71}. This evidence points to an evolutionarily conserved temporal logic governing the generation and organization of neuronal diversity.

This shared code is likely to result from the common evolutionary origin of progenitors and its conservation suggests functional importance. Shared temporal modularity could ensure stepwise circuit assembly and wiring coordination in local neuronal domains, as shown for local premotor circuits⁷², or between distant brain and VNC regions. This opens paths to study the idea that “neurons that are born together wire together”⁷³. The recurrent expression of the same TF in neurons from different temporal windows could be responsible for reoccurring morphological features in temporally distant neurons within lineages^{39,40}. Notably, six shTFs have roles specifying distal structures (*hth*⁷⁴, *dan*, *danr*⁷⁵, *bab1*, *bab2*, *rn*⁷⁶). Proximal–distal patterning has a temporal component, with positional identity being the result of the timing of exposure to spatial patterning factors. This suggests that regulatory networks may be repurposed in different developmental contexts or a deep common evolutionary origin for neuronal and non-neuronal tissue patterning.

Many additional insights remain latent in the atlas, which will be a valuable resource not only for the *Drosophila* neuroscience community, but also for comparative studies exploring the evolutionary origins of neuronal diversity and function across phyla. It also provides a strong motivation to obtain a correspondingly high-coverage developmental brain atlas to complete the molecular description of the fly central nervous system.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information,

acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-026-10797-w>.

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