

Supplementary Figures

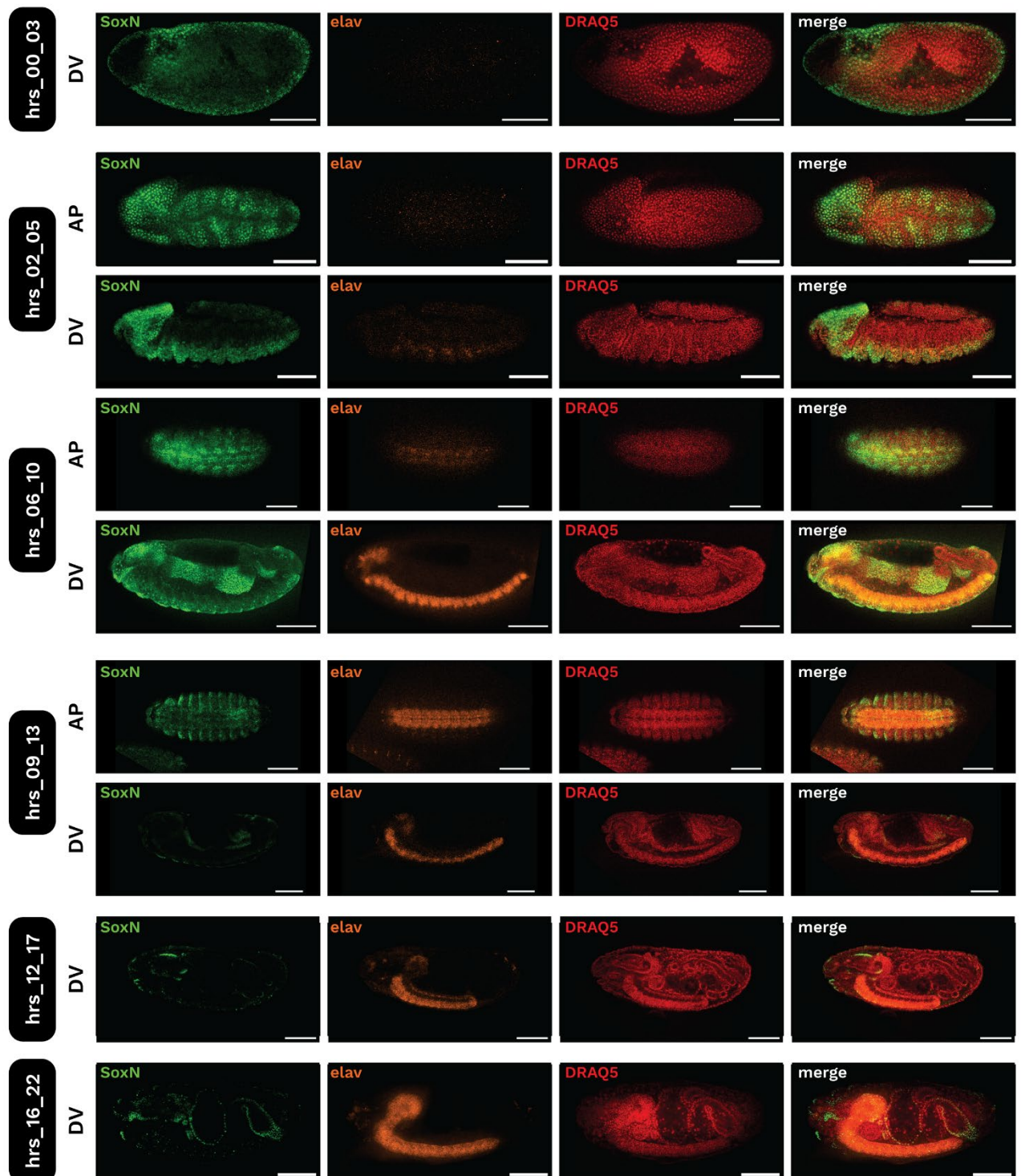
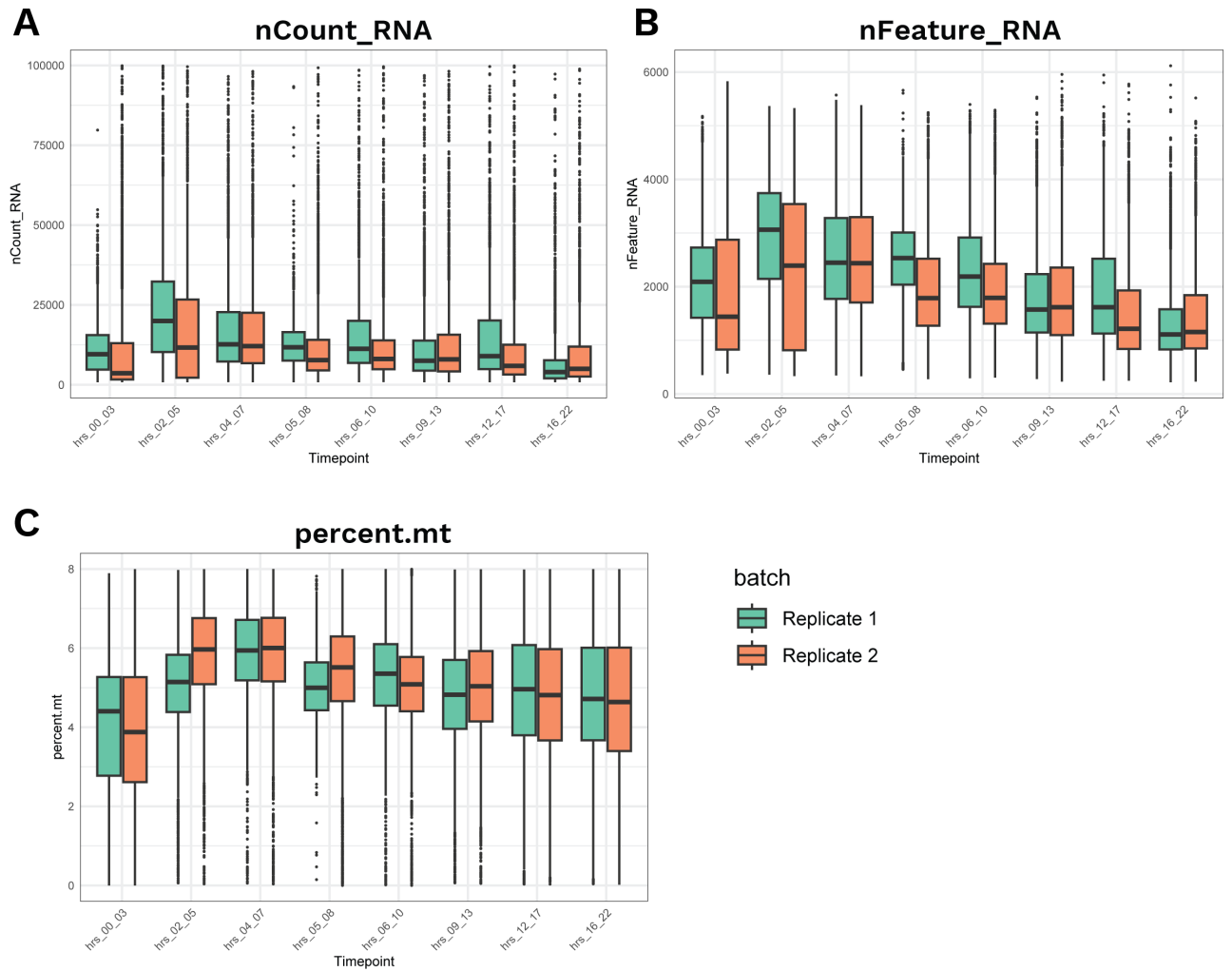


Figure S1. Temporal expression dynamics of SoxN and Elav in *Drosophila* across embryogenesis.

Confocal images showing immunofluorescence staining of *Drosophila* embryos at representative developmental windows using antibodies against SoxN (green, neural progenitor marker), Elav (orange, post-mitotic neuronal marker), and nuclear counterstain DRAQ5 (red). Embryos were imaged at six developmental stages: 0–3

9 hpf, 2–5 hpf, 6–10 hpf, 9–13 hpf, 12–17 hpf, and 16–22 hpf. Both anterior-posterior
10 (AP) and dorsal-ventral (DV) views are shown where applicable. Early-stage embryos
11 (0–5 hpf) display broad SoxN expression and minimal Elav signal. As development
12 progresses, SoxN expression reduces, while Elav expression is progressively
13 upregulated in differentiating neurons beginning ~6–10 hpf. By stage 13 (~12 hpf),
14 Elav+ neurons are abundantly distributed throughout the VNC and brain lobes, while
15 SoxN expression is minimal. Scale bars = 100 μ m.



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17 **Figure S2. Quality metrics by timepoint and replicate**

18 **A. UMI counts per cell (*nCount_RNA*), B. detected genes per cell (*nFeature_RNA*),**
 19 **and C. mitochondrial RNA fraction (*percent.mt*).** Boxplots show the median and IQR;
 20 whiskers extend 1.5×IQR; points are individual cells. Colors denote biological
 21 replicates (Replicate 1, Replicate 2).

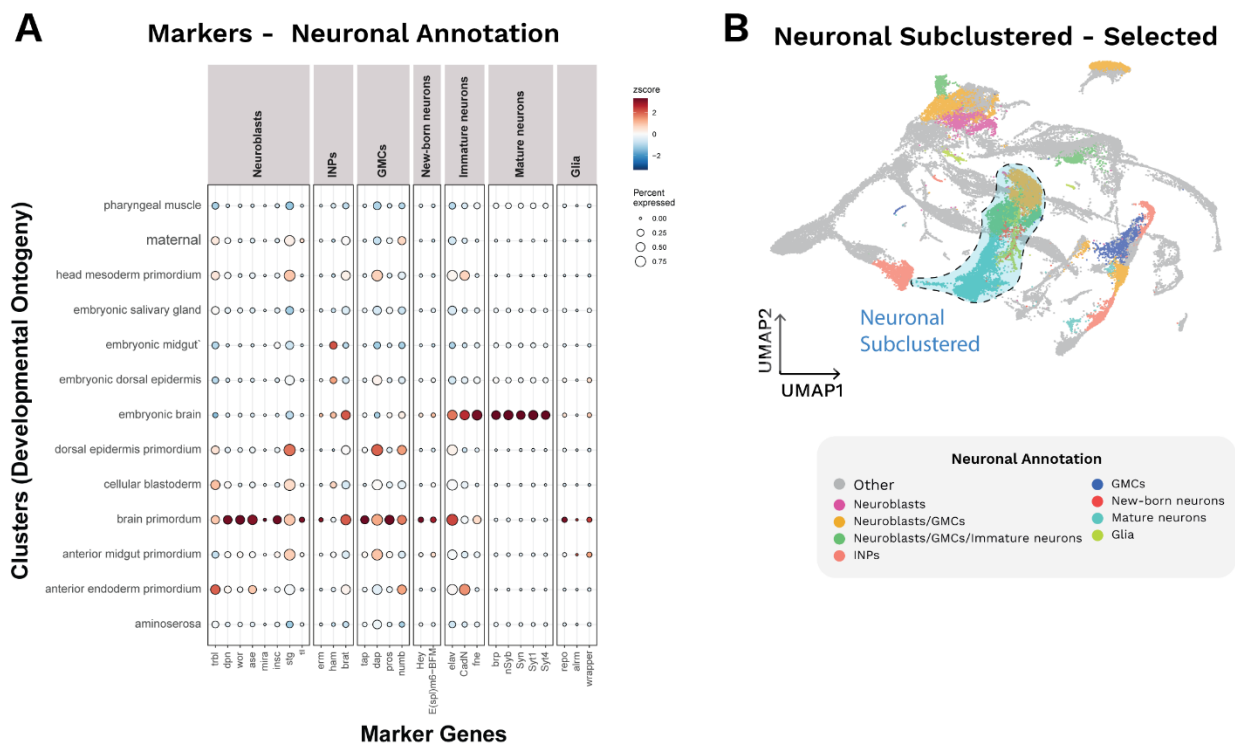


Figure S3. Congruence between neuronal-state annotations and developmental ontogeny, and selection of the maturing neuronal lineage for subclustering.

A. Dot plot comparing literature-curated neuronal markers with clusters annotated by developmental ontogeny. Rows list ontogeny-defined clusters (e.g., maternal, cellular blastoderm, brain primordium, embryonic brain, and non-neural tissues). Columns group genes by neuronal state: neuroblasts, intermediate neural progenitors (INPs), ganglion mother cells (GMCs), new-born neurons, immature neurons, mature neurons, and glia. Dot size indicates the fraction of cells in a cluster expressing a given gene; colour reflects z-scored average expression. **B.** UMAP of the neuronal atlas with the 'Neuronal subclustered' selection outlined in blue. These selected clusters correspond to the maturing neuronal lineage and were carried forward for downstream subclustering.

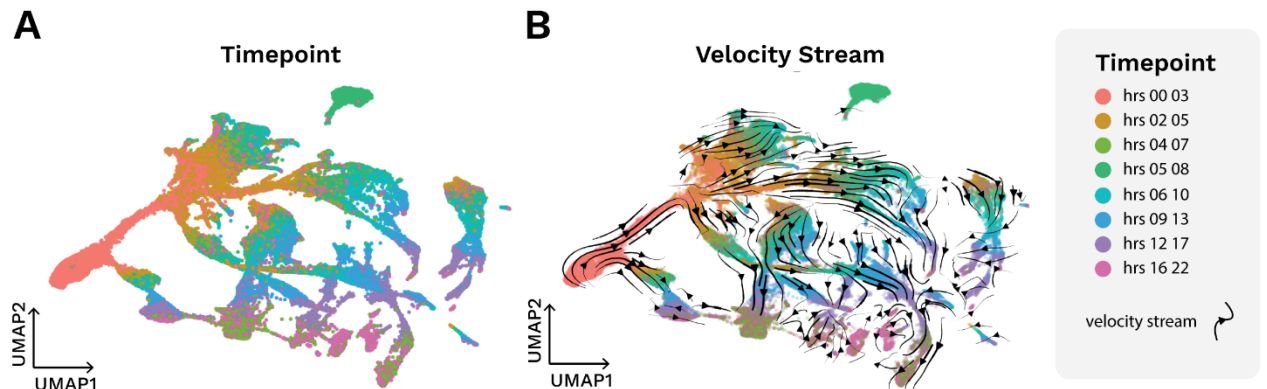


Figure S4. Concordance of timepoint annotations with RNA velocity across the full dataset

A. UMAP plot coloured by timepoint. **B.** Same UMAP embedding with velocity streamlines overlaid. Streamlines run along the same manifold and point from early to late timepoints, indicating that sampling times and inferred dynamical flow agree.

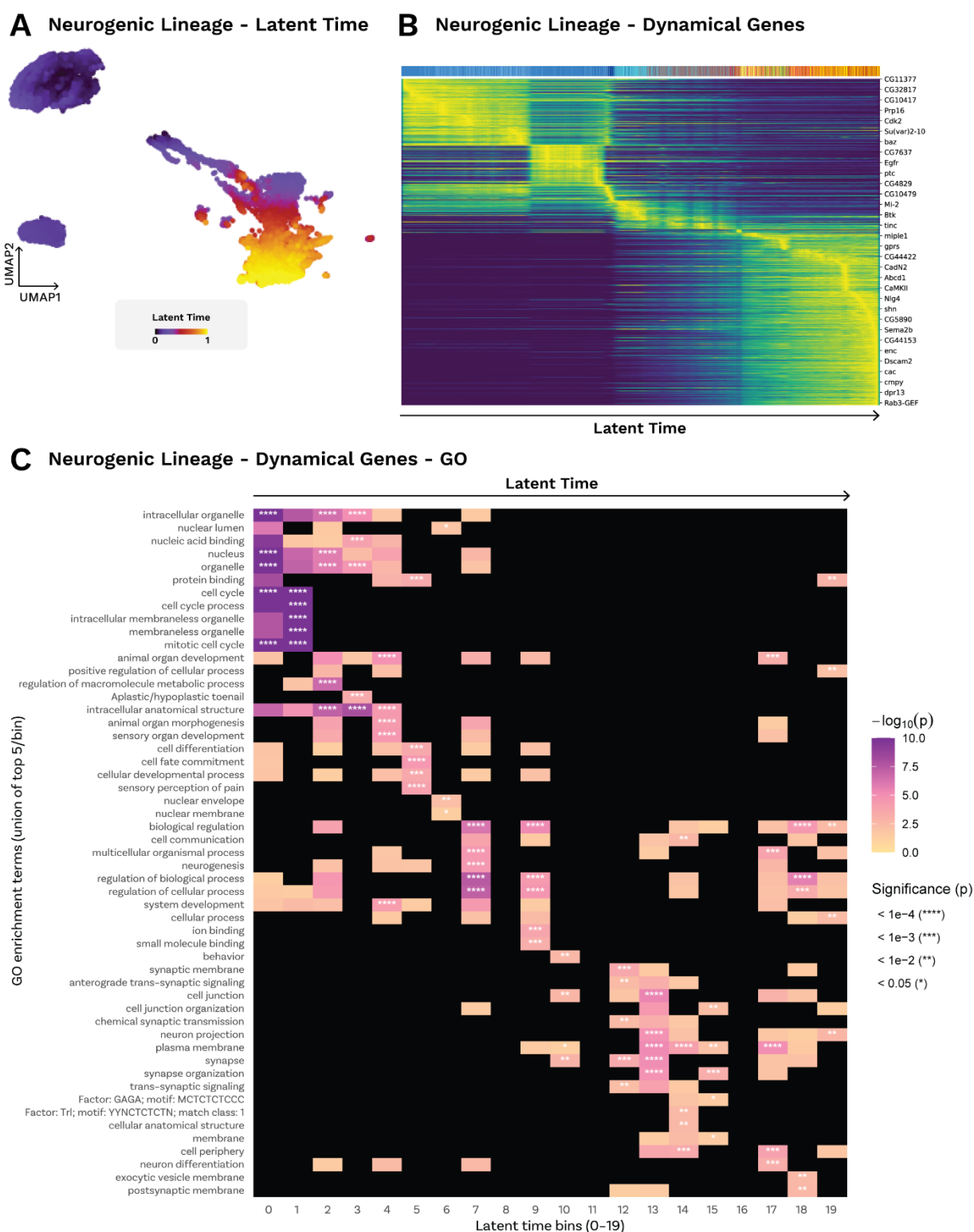


Figure S5. Neurogenic lineage – Latent time, dynamical genes, and gene ontology across the trajectory

A. Latent time. UMAP of the neurogenic lineage subset coloured by scVelo latent time (0→1). The arrow denotes the inferred developmental progression. **B. Dynamical genes.** Heatmap of top scVelo dynamical genes, with cells ordered by latent time and genes ordered by their peak latent-time of expression. Values are z-scored per gene;

49 brighter colours indicate higher expression. **C. Gene Ontology across latent time.**
50 Dynamical genes were assigned to one of 20 equal latent-time bins by their peak time
51 and tested for GO enrichment (g:Profiler; *D. melanogaster*; GO:BP and GO:MF). Tiles
52 show enrichment magnitude (fill = $-\log_{10} p$); white dots mark FDR-significant terms
53 (BH-adjusted $p \leq 0.05$). Terms are displayed at the bin where enrichment peaks,
54 revealing a trajectory from early organelle/biogenesis and cell-cycle programs to late
55 neuronal differentiation, synaptic specification, and axon/neurite functions.

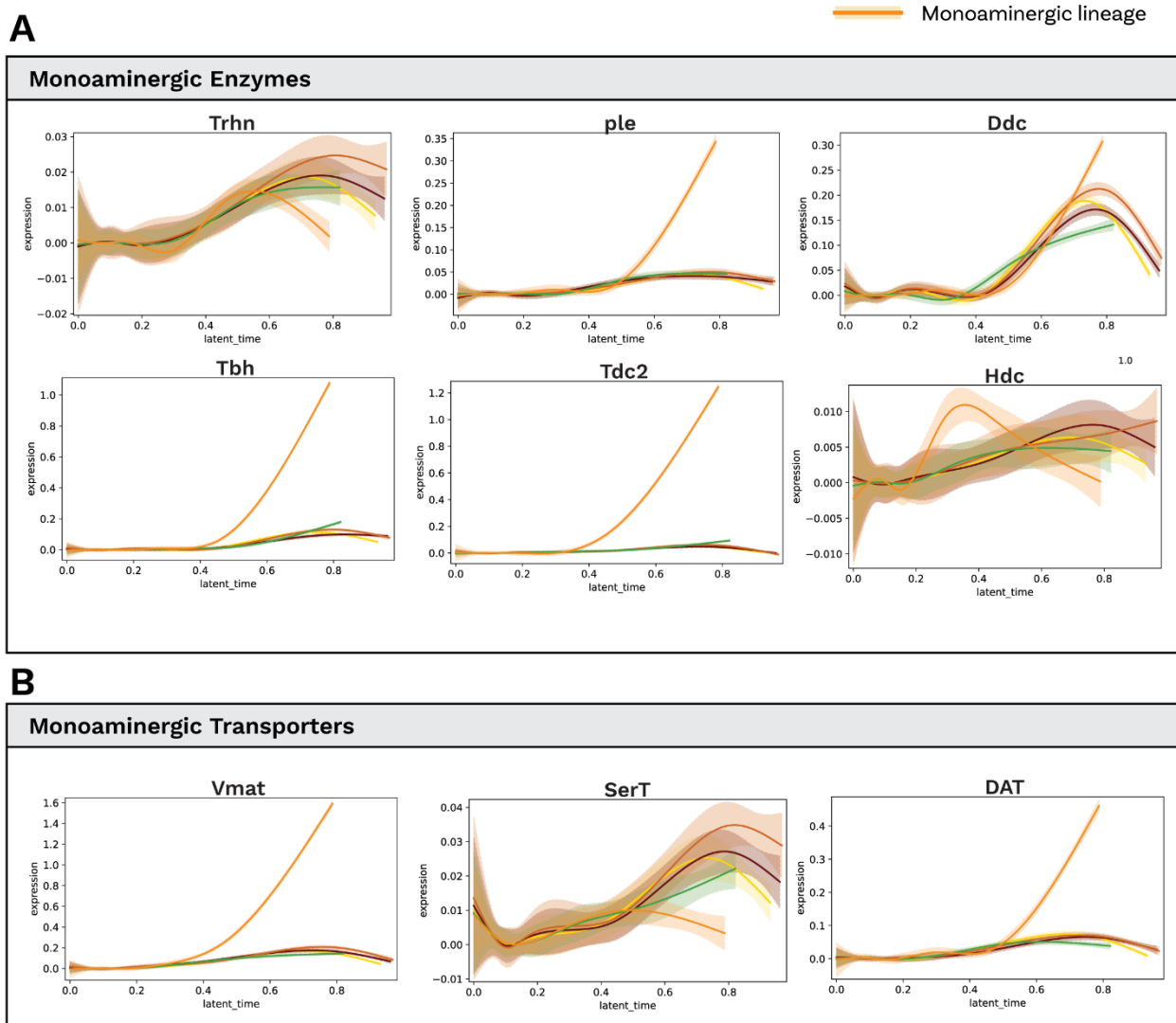
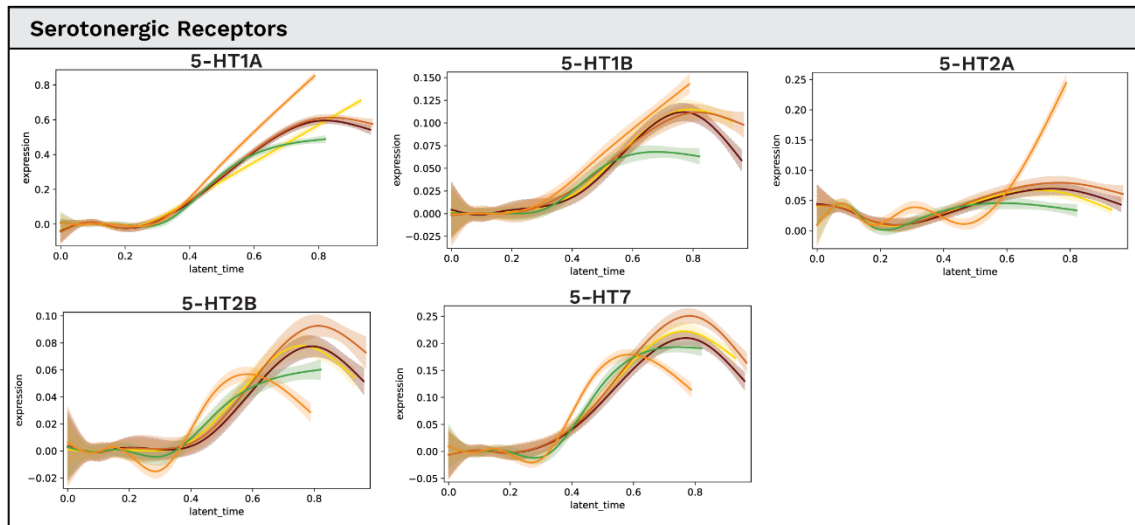


Figure S6. Monoaminergic pathway gene trajectories along latent time in the neurogenic lineage

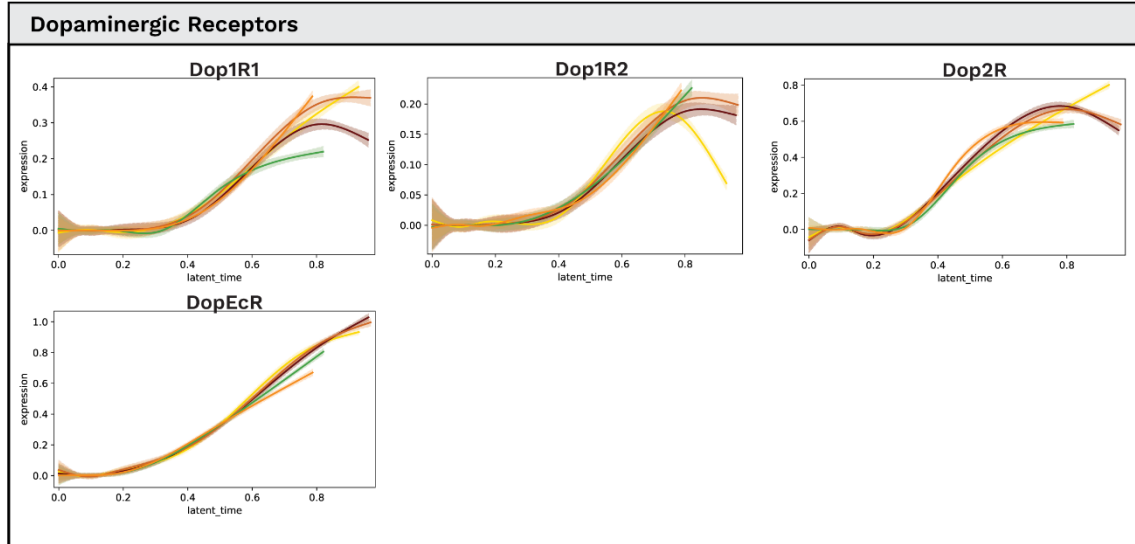
A. Monoaminergic enzymes. Smoothed expression trends across latent time for major neuronal lineages (Acetylcholine, GABA_{1/2}, Glutamate, Monoamine). The monoaminergic lineage is highlighted in orange with a light-orange ribbon. Genes shown: *Trhn*, *ple*, *Ddc*, *Tbh*, *Tdc2*, *Hdc*. In the monoaminergic lineage, *ple* and *Ddc* (dopamine synthesis) and *Tdc2* and *Tbh* (tyramine/octopamine synthesis) rise strongly at late latent time, whereas *Trhn* and *Hdc* show modest or transient changes. Non-monoaminergic lineages remain low or show only brief increases. **B. Monoaminergic transporters.** Trajectories for *Vmat* (vesicular monoamine transporter), *DAT* (dopamine transporter) and *SerT* (serotonin transporter). All three increase toward late latent time in the monoaminergic lineage; *Vmat* and *DAT* are minimal or transient in other lineages, while *SerT* exhibits a more variable profile. Traces show lineage-wise smoothed means; ribbons indicate uncertainty. Axes: scaled expression vs. latent time.

A

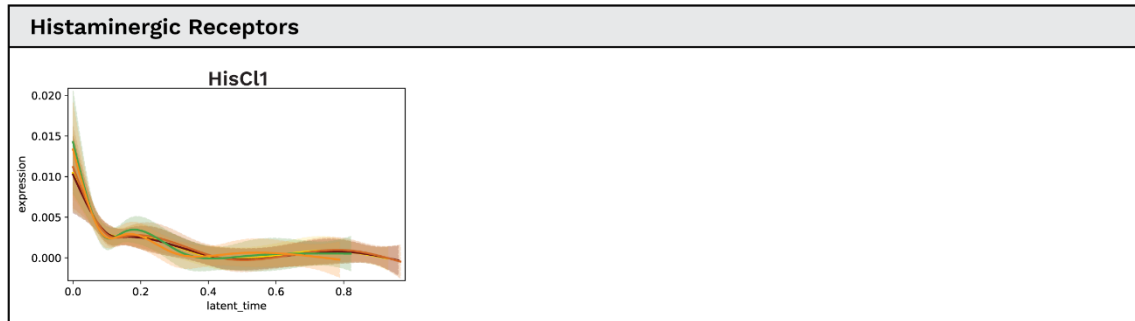
— Monoaminergic lineage



B



C



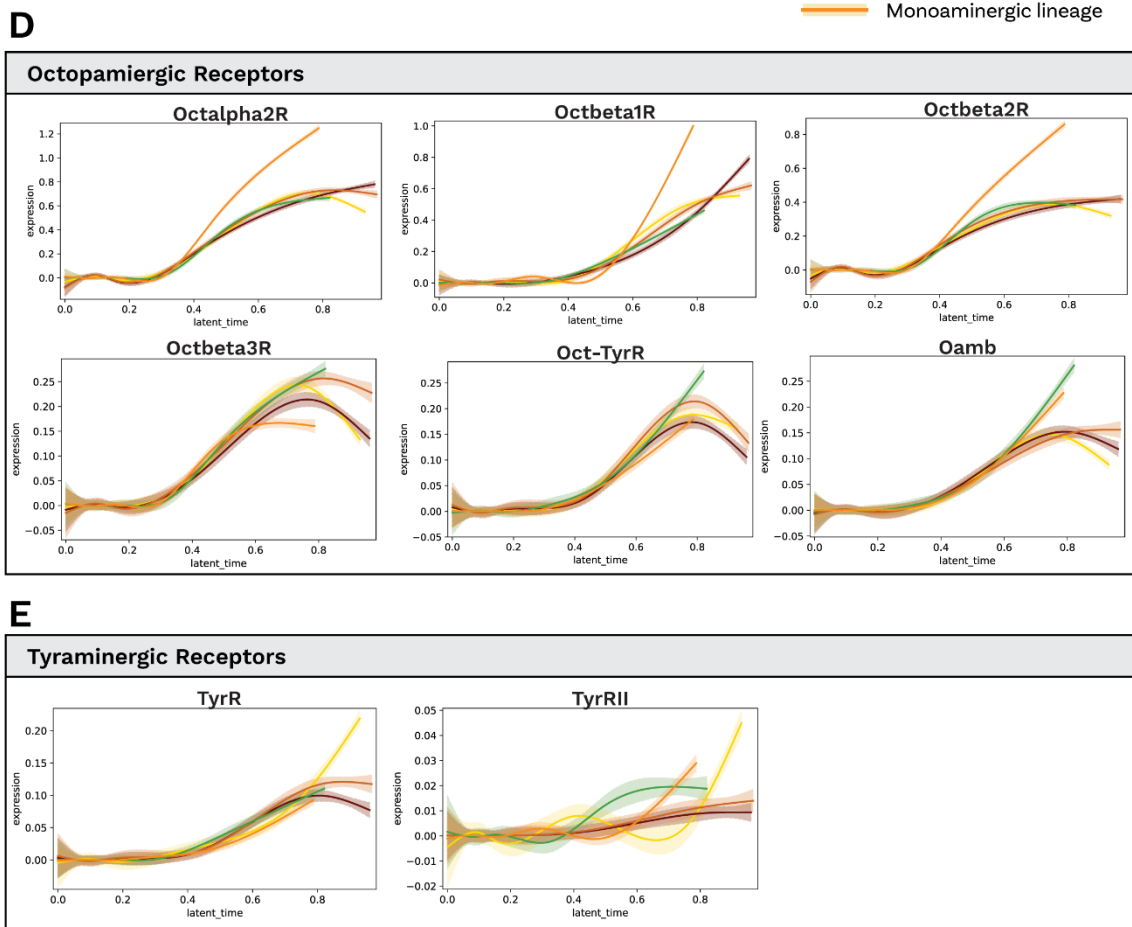
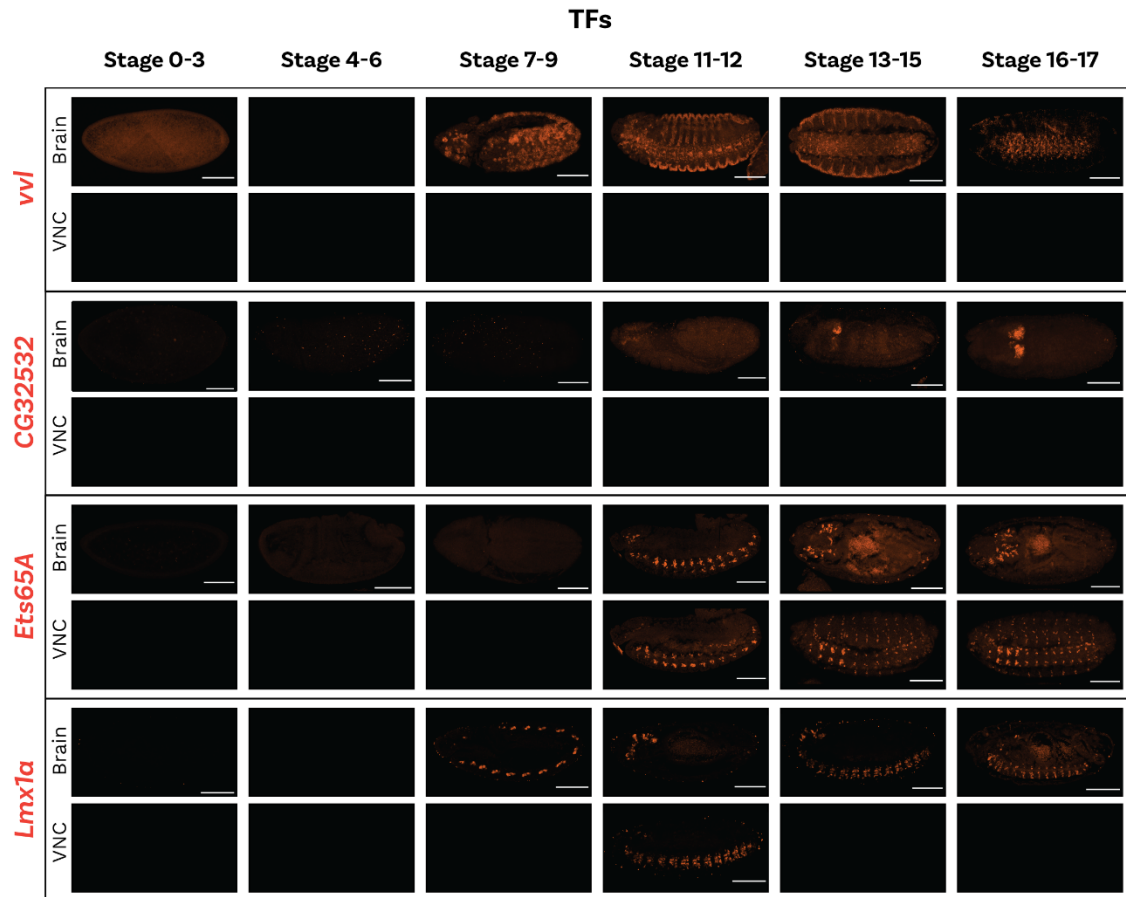


Figure S7. Monoamine receptor trajectories across neuronal lineages and latent time

Curves show lineage-wise smoothed expression across latent time for major neuronal lineages (Acetylcholine, GABA_1/2, Glutamate, Monoamine). The monoaminergic lineage is highlighted in orange with a light-orange ribbon indicating uncertainty. Axes: scaled expression (y) vs latent time (x).

A. Serotonergic receptors. B. Dopaminergic receptors. C. Histaminergic receptors. D. Octopaminergic receptors. E. Tyraminergetic receptors.



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83 **Figure S8. HCR of candidate TFs across the monoaminergic lineage**

84 Whole-mount HCR staining across six embryonic windows (stages 0–3, 4–6, 7–9, 11–
85 12, 13–15, 16–17). For each TF, the top row shows the brain and the bottom row the
86 ventral nerve cord (VNC). Images are lateral views; anterior left. White scale bars
87 shown (100 μ m). *Vvl*, *CG32532*, *Ets65A*, *Lmx1a*. Signal is absent before gastrulation
88 and then becomes detectable in patterned CNS domains from stage ~7–9 onward. By
89 stages 11–12 and 13–15 each TF exhibits characteristic spatial enrichment—e.g.,
90 focal brain expression (*CG32532*), segmental VNC stripes (*Ets65A*), and combined
91 brain/VNC domains (*Lmx1a*)—that persist until stage 16–17.

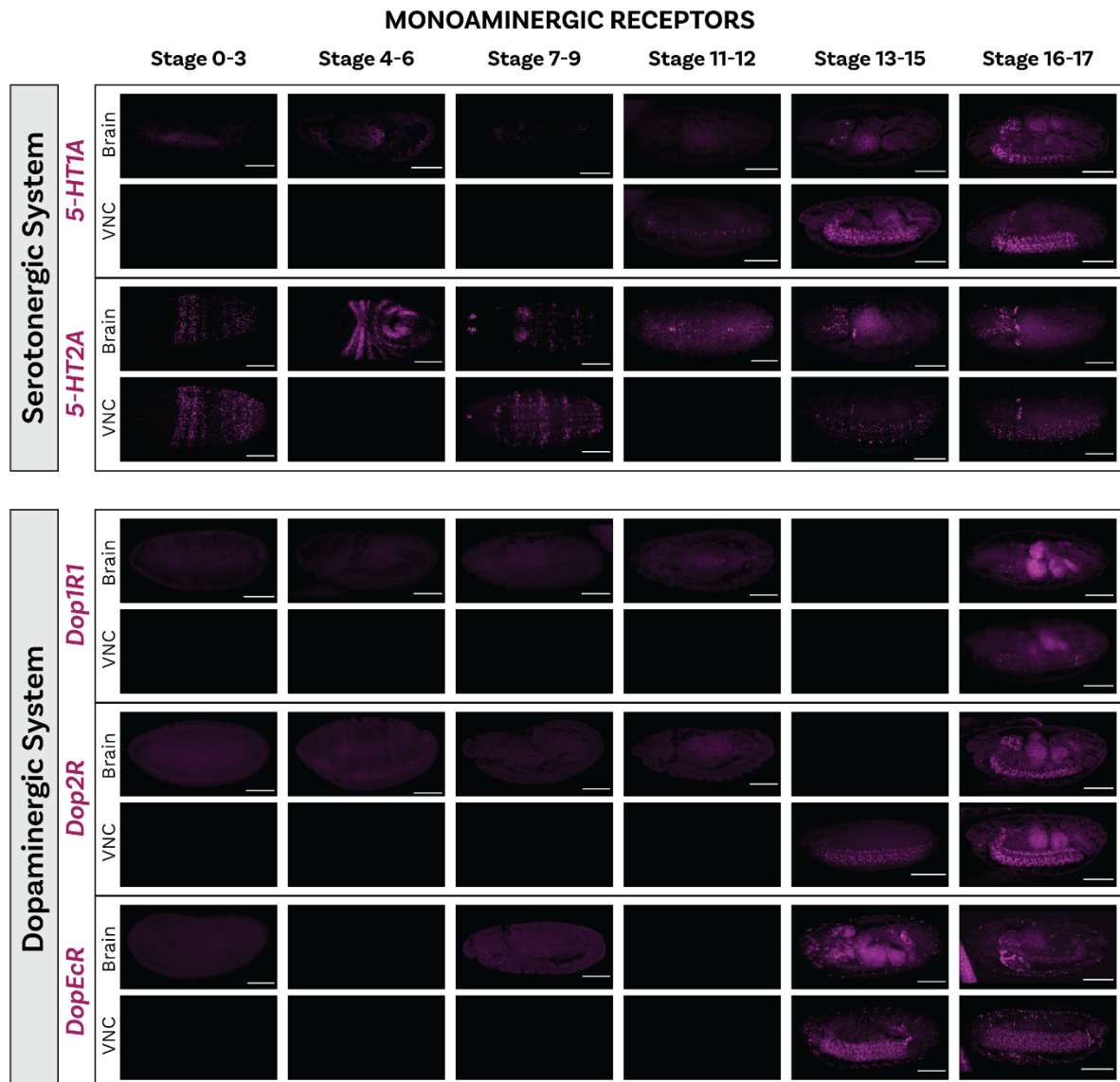
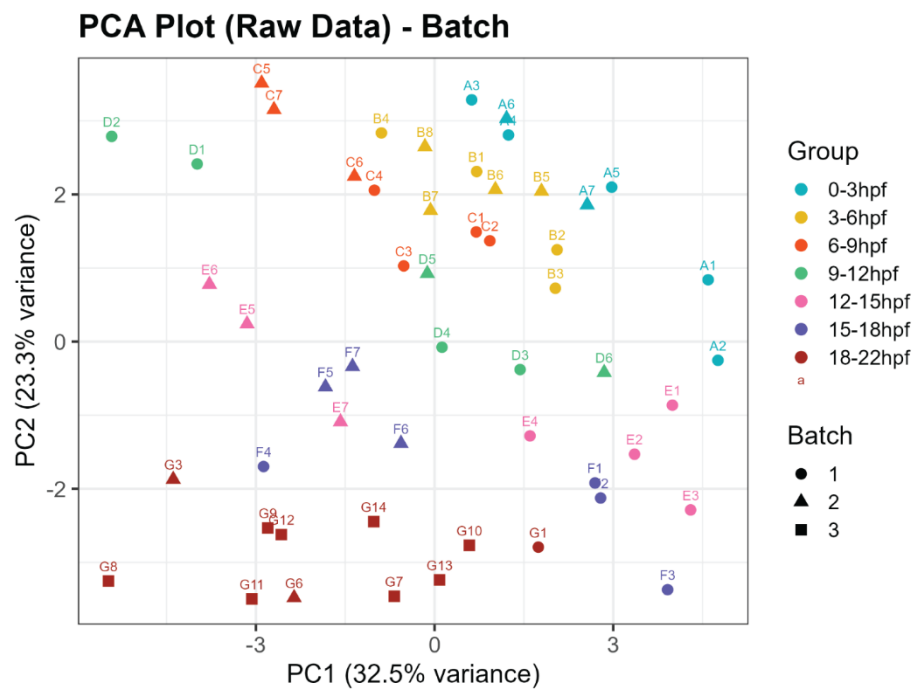


Figure S9. HCR of serotonergic and dopaminergic receptors across embryogenesis

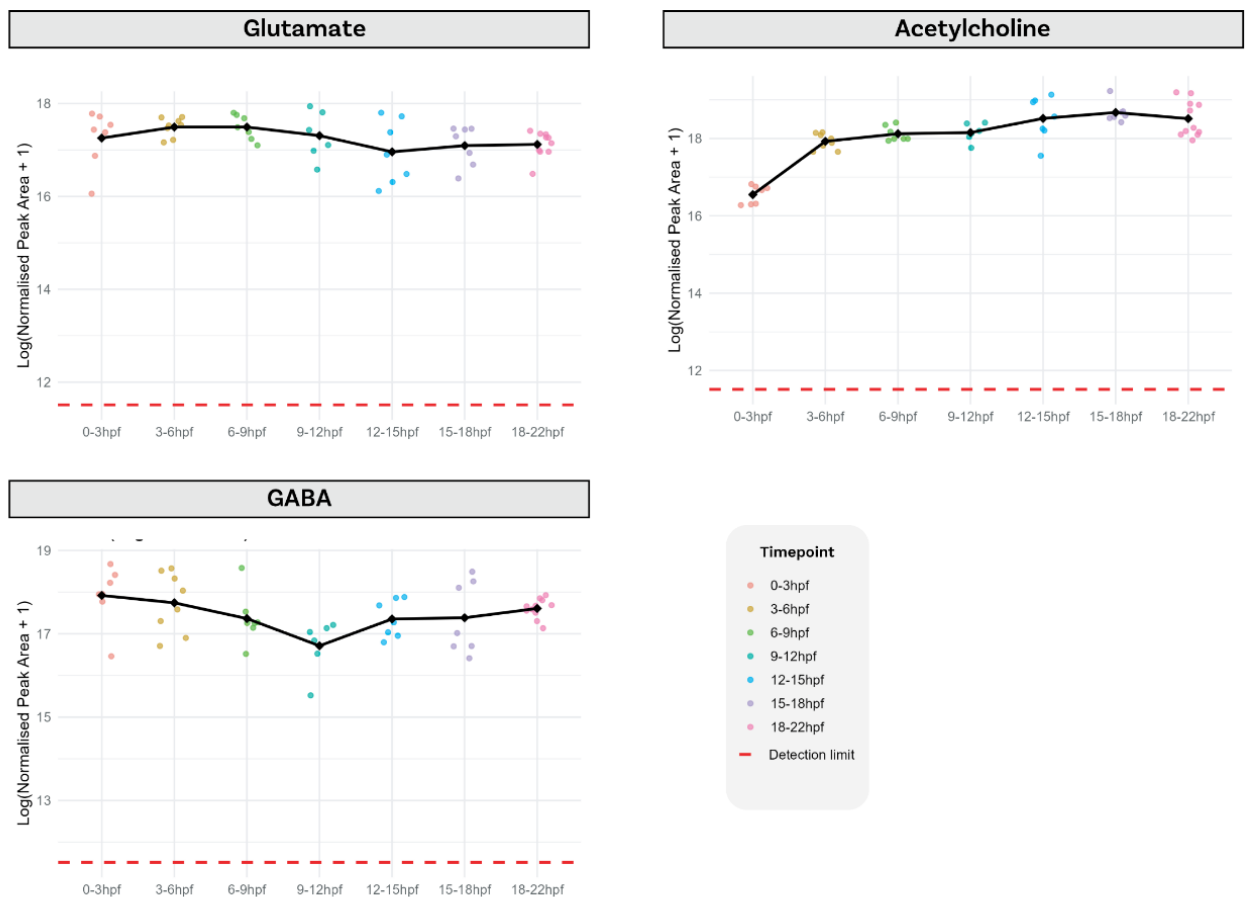
Whole-mount HCR staining for serotonergic and dopaminergic receptors across six windows (stages 0–3, 4–6, 7–9, 11–12, 13–15, 16–17). For each gene, the top row shows the brain, the bottom row the ventral nerve cord (VNC). Images are lateral views; anterior left. Scale bars indicated (100 μ m). **Serotonergic receptors.** *5-HT1A* is undetectable early and emerges in the CNS from stage 13-15, strengthening through stages 16–17 in brain and segmental VNC domains. *5-HT2A* shows early, widespread expression (from stages 0-3), including non-neural tissues, and later consolidates within CNS territories. **Dopaminergic receptors.** *Dop1R1* and *Dop2R* become detectable in the CNS from stages 16–17. *DopEcR* exhibits slightly broader CNS expression from stage 13-15 onwards.



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106 **Figure S10. PCA of targeted mass spectrometry data by developmental window**
 107 **and batch**

108 Principal components analysis of the raw measurements across seven time windows
 109 (0–3 hpf, 3–6 hpf, 6–9 hpf, 9–12 hpf, 12–15 hpf, 15–18 hpf, 18–22 hpf). Each point is
 110 a sample (labels denote sample IDs). Colour encodes developmental Group; shape
 111 encodes Batch (● batch 1, ▲ batch 2, ■ batch 3). Axes show the variance explained
 112 (PC1 = 32.5%, PC2 = 23.3%). Samples separate primarily by developmental window,
 113 with batches intermingled.

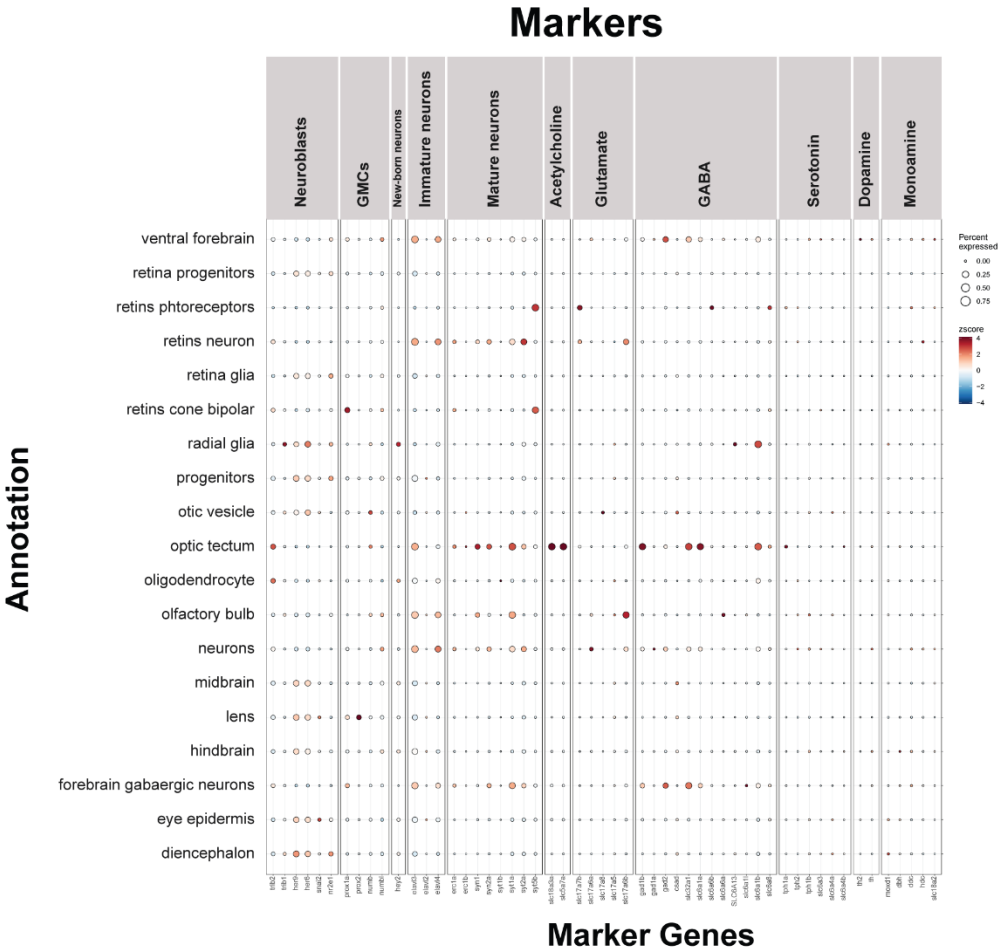


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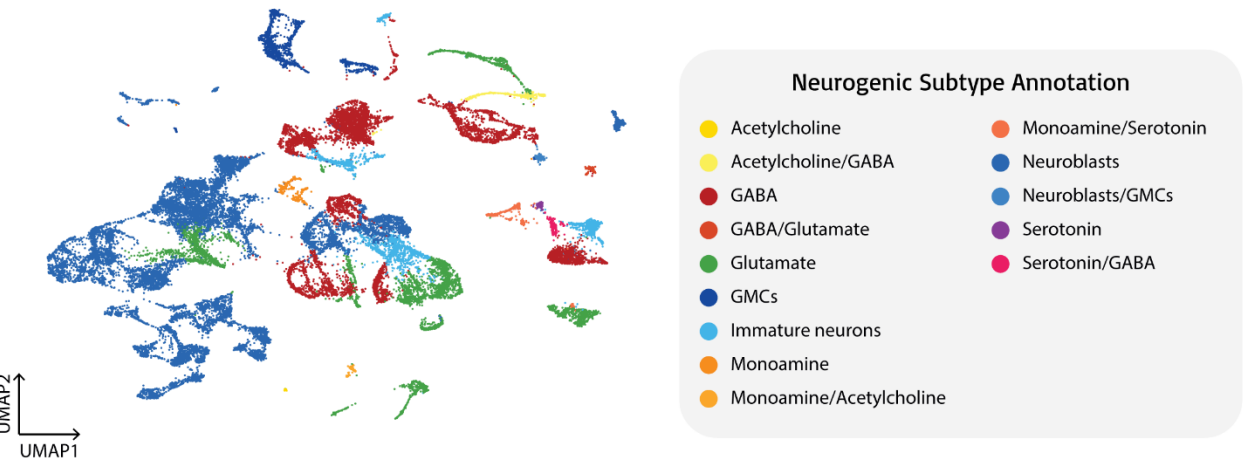
115 **Figure S11. Targeted mass spectrometry of other neurotransmitters**

116 Levels of glutamate, acetylcholine, and GABA were quantified across indicated
 117 timepoints using targeted mass spectrometry. Data are presented as mean from $n \geq 6$
 118 biological replicates.

A



B Neurogenic Annotation - CNS



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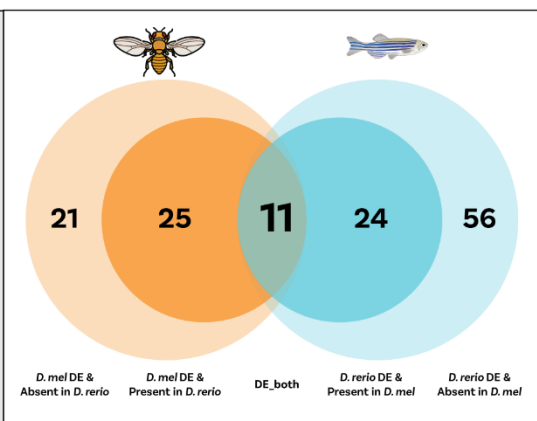
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Figure S12. Zebrafish CNS neurogenic landscape—marker concordance and subtype annotation

A. Marker concordance. Dot plot of orthologous marker genes evaluated across CNS-derived clusters. Rows show anatomical/lineage annotations (e.g., diencephalon, forebrain GABAergic neurons, hindbrain, retina, neuroblasts), and

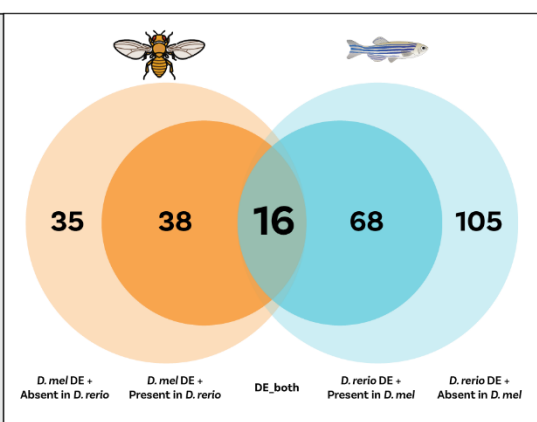
125 columns group markers by neuronal state (Neuroblasts, GMCs, Immature/Mature
126 neurons) and transmitter systems (Acetylcholine, Glutamate, GABA, Serotonin,
127 Dopamine, Monoamine and mixed categories). Dot size indicates the fraction of cells
128 expressing the gene in a cluster; colour is the z-scored average expression. **B.**
129 **Neurogenic subtype annotation (CNS).** UMAP of CNS cells coloured by inferred
130 neurogenic/neuronal subtype. Labels denote transmitter classes and developmental
131 states (Acetylcholine, Acetylcholine/GABA, GABA, GABA/Glutamate, Glutamate,
132 Monoamine, Monoamine/Acetylcholine, Monoamine/Serotonin, Serotonin,
133 Serotonin/GABA, Neuroblasts, Neuroblasts/GMCs, GMCs, Immature neurons).

Monoamine



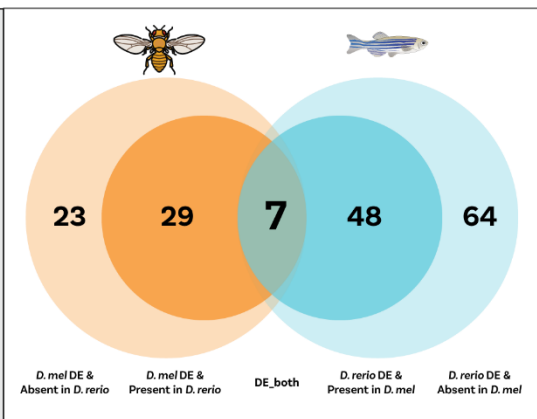
<i>D. melanogaster</i>	<i>D. rerio</i>
cic	cica
Nfl	nfla; nfixb
cbt	klf11b; klf11a
CG4328; Lmx1a	lmx1bb; lmx1al; lmx1ba
FoxP	foxp4
trh	NPAS3
Eip78C	nr1d1
net	toh8
tj	mafa; mafb
Pdp1	hlfa
luna	klf6a

Acetylcholine

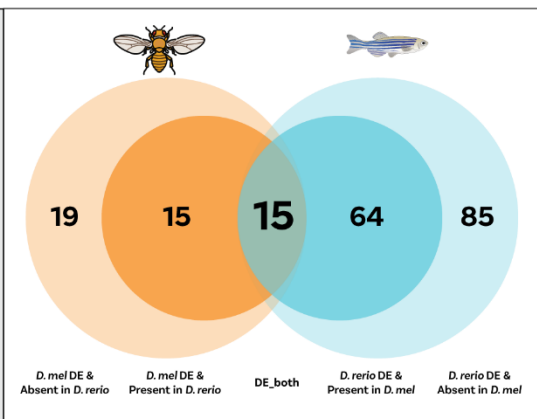


<i>D. melanogaster</i>	<i>D. rerio</i>
<i>Mef2</i>	<i>mef2ca;mef2cb</i>
<i>Kr</i>	<i>bcl6aa;bcl6ab</i>
<i>Hr78</i>	<i>nr2c1</i>
<i>salr</i>	<i>sall3a</i>
<i>oncut</i>	<i>oncut</i>
CG8301;CG12299;cro1;CG18476	znf12a;si.dkey-199m13.4;znf1156;si.ch73-299h12.6;zgc:174315;blf;zgc:16551
<i>ftz-f1</i>	<i>nr5a2</i>
<i>scramb1</i>	<i>plscr3b</i>
<i>FoxP</i>	<i>foxp1a</i>
<i>exex</i>	<i>mnx1</i>
<i>otp</i>	<i>otpb;otpa</i>
<i>Hmx</i>	<i>hmx3a</i>
<i>kn</i>	<i>ebf2;ebf3b</i>
<i>grn</i>	<i>gata3;gata2a</i>
<i>acj6</i>	<i>pou4f1</i>
<i>unc-4</i>	<i>uncx</i>

GABA

[illegible]

Glutamate



<i>D. melanogaster</i>	<i>D. rerio</i>
cic	cica
Mef2	mef2cb; mef2ab; mef2aa; mef2ca
tna	zmiz1a
scrt	scrt2;scrt1b;scrt1a
foxo	foxo3b;foxo3a
onecut	onecut1;onecut3b;onecut2;onecut3a
CG8301	zgc:101130;zgc:113348;BX470259.2
CG43689	myt1a;myt1la;myt1b
zfh1	zeb2b
Nfl	nfixb; nfia
CG13287	prdm8b
tsh	tshz2
Tusp	tulp4a
luna	klf7b;klf7a

Figure S13. Cross-species conservation of transmitter-class TF programs

A–D. EggNOG Orthogroup-level comparison of differentially expressed TFs between *D. melanogaster* (orange) and *D. rerio* (blue) within matched neurotransmitter classes: **A. Monoamine, B. Acetylcholine, C. GABA, D. Glutamate.** Venn diagrams summarise counts of Bilateria eggNOG orthogroups (OGs) containing TFs that are DE in the indicated class (Methods; adjusted $p < 0.05$, $|\log_2FC| \geq 0.25$). Numbers in each sector denote OGs unique to fly (left), unique to zebrafish (right), or shared (middle; DE in both species). The lower captions indicate whether the other species has a detectable member of the OG (“present”) or lacks one in the proteome/annotations (“absent”). Tables to the right list representative conserved TF OGs (center intersection) with the corresponding gene members detected in each species; multiple names in a cell reflect many-to-one/one-to-many mappings within the same OG.