SUPPLEMENTARY INFORMATION

for:

Comprehensive catecholaminergic projectome analysis reveals single neuron integration of zebrafish ascending and descending dopaminergic systems

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Characterization of modified th:rasGFP BAC constructs.

(a) BLAST of the end sequences against zebrafish Zv7 genome assembly revealed that the insert contains 154 kb upstream of the transcription start site and terminates within intron 7 of the th gene (Chromosome 25, Ensembl 2006).

(b) To determine if the BAC chosen for modification carried th or the paralogous th2 gene, PCR primers were designed for th (orange arrows) and th2 (blue and green arrows). B1 and B2 were independent clones of the same BAC. Positive and negative control templates were genomic DNA and water respectively. The BAC used in our analysis gave a PCR product with th primers only.

(c) To generate th:rasGFPsv (shuttle vector), an approximately 1 kb fragment of th promoter (th A-Box) and the Ras membrane anchor tagged mGFP were cloned into the shuttle vector pLD53.SCA-E-B, which was subsequently used in the recombination process. Resultant clones were screened using the combinations of primers shown as blue and red arrows (left panel). A second recombination step was not carried out to remove the shuttle vector backbone. Correct clones were further identified by long-range PCR based on combinations of primers 1-3 which revealed six correct clones, of which one was used for data generation (right panel). (d) To reduce possible influences of vector sequences, we engineered th:rasGFPvf (vector-free) using the BAC recombineering strategy by Warming et al. Following the isolation of modified BAC clones from Gal counter selection plates, PCR amplification and sequencing across the respective homology arms were performed to verify the orientation of the transgene (upper panel). Pulsed-field electrophoresis of NotI digested unmodified th BAC and its derivative th:rasGFPvf in 1.2% TBE agarose indicated no aberrant BAC insert rearrangement after the recombination event (lower panel).
**SUPPLEMENTARY FIGURE S2**

Overview of projection behavior of single neurons for each catecholaminergic group.

The projection behaviors (columns - see top) of each catecholaminergic group (rows - see left) are indicated quantitatively by the color code representing the percentage (color code at bottom) of total neurons per analyzed group that display each projection characteristic. The total number of neurons analyzed is shown in the rightmost column. In the event that individual neurons possess multiple, distinct projections, the percentages of one horizontal line may add up to more than 100. The prominence of mainly red/pink and orange bars indicates that specific neuronal groups (retinal amacrine cells, olfactory, subpallial, preoptic, pretectal, DC1, DC3 and DC7 dopaminergic groups, and noradrenergic sympathetic ganglia) each have their specific projection characteristics. Dopaminergic groups DC2, DC4, DC5, and DC6, and other noradrenergic groups display more varied projection behaviors within each cluster. Dor, dorsal projections; single in endohypothalamic tract indicates contribution of single axon to tract; (#) ipsilateral distal branch that crosses the midline to target the contralateral side; (*) major axon proximal to the soma that targets the contralateral side.
SUPPLEMENTARY FIGURE S3
Locally-projecting noradrenergic neurons.
Visualization of individual GFP-tagged catecholaminergic neurons in genetic mosaic 4 dpf zebrafish larvae by anti-GFP (green) and anti-TH (red) immunohistochemistry. (a-c) Z projections of confocal stacks, except DAB panels. Dorsal (dor), lateral (lat) and transverse (trans) views.
(a) Noradrenergic (NA) neurons in the interfascicular zone (IZ) of the MO typically innervate local targets via short processes and/or a short ventral projection (arrow) (magnified in bottom panels). AP, area postrema; VA, vagal area. (b) Sympathetic ganglia possess bi- or multipolar processes and target locally (magnified in bottom panels). (c) A locally-projecting potential NA neuron of the trigeminal ganglia. The brain was fluorescence-immunolabeled against GFP and TH (leftmost panel; boxed region magnified in inset), followed by diaminobenzidine (DAB) staining for GFP (middle). Rightmost panel shows transverse vibratome section (30 μm thick) of the same brain. Short, local processes radiating away from the trigeminal ganglia were observed. Anterior at left, dorsal at top. Arrowhead indicates soma. Scale bars = 20 μm.
Dopaminergic neurons that form characteristic local projections.

Visualization of individual GFP-tagged catecholaminergic neurons in genetic mosaic 4 dpf zebrafish larvae by anti-GFP (green) and anti-TH (red) immunohistochemistry. (a-c) Z projections of confocal stacks in dorsal (dor) and lateral (lat) views. (a) Olfactory bulb dopaminergic (DA) neurons establish dense local arbors (magnified in bottom panel). (b) A DA retinal amacrine cell arborizes locally (magnified in bottom panel). (c) An example of DA neurons in the anterior preoptic region, which are liquor-contacting and typically contribute to the anterior catecholaminergic tracts (act). They have short local projections, but also contra- and ipsilateral projections (magnified in middle-GFP against black contrast, and right panels). Anterior at left, dorsal at top. Arrowhead indicates soma. Scale bars = 20 μm.
**SUPPLEMENTARY FIGURE S5**

**High individual target variability displayed by DC5 neurons.**

Visualization of individual GFP-tagged catecholaminergic neurons in genetic mosaic 4 dpf zebrafish larvae by anti-GFP (green) and anti-TH (red) immunohistochemistry. (a-d) Z projections of confocal stacks in dorsal (dor) and lateral (lat) views. GFP signals shown in white against black contrast for corresponding red/green images. Small bottom panels in rightmost column show magnification of boxed region.

(a) Both the ipsilateral axonal projection and its contralateral branch target the spinal cord (SC; direction indicated by long arrow) and the fine processes (short arrow) arising from the soma terminate in the endohypothalamic tract (eht). (b) Local processes (short arrows) contribute to the eht and long projections innervate the rhombencephalon (r) and SC (direction indicated by long arrow). (c) Short projections (arrow) terminate within the eht and hypothalamus (H) while the main axon innervates several areas in the rhombencephalon via its branches. (d) The main axon targets the rhombencephalon with a branch extending towards the pretectum (Pr) (see lateral view) and local processes contribute to the eht (arrow).

Dotted lines indicate the outline of corresponding brain. Anterior at left, dorsal at top. Arrowheads indicate DC5 somata. Scale bars = 20 μm.
**SUPPLEMENTARY FIGURE S6**

**Representation of projection behavior classes of single neurons for DC 5.**

Individual DC5 somata were classified based on their major projection behaviors (green). Minor deviations in projection characteristics were taken into consideration (light green and yellow). The rightmost column indicates the number of cells observed for each specific pattern (rows). Single DC5 neurons typically connect the di- and rhombencephalon via the contribution of short processes to the eht and simultaneous innervations of the hindbrain and/or spinal cord. However, different combinations of target choice were observed based on the analysis of 110 somata (rightmost column). Dor, dorsal projections; single in endohypothalamic tract indicates contribution of single axon to tract; (#) ipsilateral distal branch that crosses the midline to target the contralateral side; (*) major axon proximal to the soma that targets the contralateral side.

| Local | Ascending | Descending | Dor | Ventral | Lateral |
|-------|-----------|------------|-----|---------|---------|
| Arborizing | Bi/multi-polar | Single in postoptic commissures | Single in hypothalamus | To subpallium | To posterior tuberculum | To hypothalamus in hypothalamus in hindbrain | To hypothalamus in hypothalamus in hindbrain | To locus coeruleus | To spinal cord | Midline crossing (#) | Dorsal | To preoptic region | To caudal hypothalamus
|       |          |            |     |         |         |                                |                                |                |               |                     |       |                |                  |
|       |          |            |     |         |         |                                |                                |                |               |                     |       |                |                  |
|       |          |            |     |         |         |                                |                                |                |               |                     |       |                |                  |
|       |          |            |     |         |         |                                |                                |                |               |                     |       |                |                  |

**Behavior applicable to all members of one class**
**Behavior observed in a large subset of one class**
**Behavior observed for single or few members of one class**

No. of cells: 110

39, 14, 6, 4, 19, 16, 6, 1
Statistical analysis using Fisher’s Exact Test revealed the major modes of neuron projection pattern of each TH immunoreactive cluster.

To analyze if each CA group possesses typical projection characteristics, and to highlight similarities and differences between neuronal groups, Fisher’s Exact Test was performed using the Genedata Expressionist Analyst module (Genedata AG, Basel, Switzerland). The universe group was taken to be all the projections. Each individual projection characteristic listed in the second row formed the property group. We then tested the selection group, made up of an individual neuronal cluster, against the property group for over- or under-representation of the neuronal projection behavior. The projection traits that are significantly enriched (p < 0.05) within each CA group are marked in blue. Other minor characteristics (p > 0.05) are shown in yellow. The rightmost column shows the total number of neurons analyzed.

**SUPPLEMENTARY FIGURE S7**

Statistical analysis using Fisher’s Exact Test revealed the major modes of neuron projection pattern of each TH immunoreactive cluster.

To analyze if each CA group possesses typical projection characteristics, and to highlight similarities and differences between neuronal groups, Fisher’s Exact Test was performed using the Genedata Expressionist Analyst module (Genedata AG, Basel, Switzerland). The universe group was taken to be all the projections. Each individual projection characteristic listed in the second row formed the property group. We then tested the selection group, made up of an individual neuronal cluster, against the property group for over- or under-representation of the neuronal projection behavior. The projection traits that are significantly enriched (p < 0.05) within each CA group are marked in blue. Other minor characteristics (p > 0.05) are shown in yellow. The rightmost column shows the total number of neurons analyzed.
**SUPPLEMENTARY FIGURE S8**

**Representation of projection behavior classes of single subpallial dopaminergic neurons.**

Individual subpallial dopaminergic somata were classified based on their major projection behaviors (green). Tel-diencephalic descending projections that crossed the midline in two cases within a class comprising 13 somata are indicated (yellow). The rightmost column indicates the number of cells observed for each specific pattern (rows). Subpallial dopaminergic neurons predominantly arborize within the subpallium, but also form contralateral projections across the anterior commissure and descending connections to the hypothalamus. (#) ipsilateral distal branch that crosses the midline to target the contralateral side.

| Local | Descending | Lateral | No. of cells |
|-------|------------|---------|--------------|
| Arborizing | Bi/multi-polar | Single, short | To thalamus/hypothalamus | To locus coeruleus | To hindbrain | Branching | Midline crossing (#) | Ipsilateral | Contralat. in ant. commissure | |
| ![Behavior applicable to all members of one class](green) | ![Behavior observed for single or few members of one class](yellow) | | | | | | | | | 20 |
| | | | | | | | | | 13 |
| | | | | | | | | | 12 |
| | | | | | | | | | 10 |
| | | | | | | | | | 3 |
| | | | | | | | | | 2 |
| | | | | | | | | | **60** |
SUPPLEMENTARY FIGURE S9
Histological analysis of GFP-labeled TH neurons.
Following fluorescence-immunohistochemistry using rabbit anti-TH and chicken anti-GFP, the 4 dpf embryo was stained again for GFP with HRP anti-chicken and diaminobenzidine (DAB; brown).
Top row shows dorsal views (left to right: planes from dorsal to ventral) of olfactory bulb (OB), pretectum (Pr) and tectum (T). A TH neuron in the OB (white arrowhead, middle panel) forms a dense local arbor that innervates locally (black arrowhead, middle panel). The pretectal TH neuron (asterisk/white arrowhead in middle panel; corresponds to Fig. 4a) emanates contra- and ipsilateral projections that terminate within the T (black arrowheads in left and middle panels), as well as locally within the Pr (asterisk/black arrowhead in right panel). The contralateral process traverses the posterior commissure (pc) (black arrowhead, middle panel). A locally projecting TH neuron with weak GFP signal was detected in the subpallium (SP) (white arrowhead, right panel). Bottom row shows transverse views (left to right: from rostral to caudal plane) of the same brain from a 30 μm thick vibratome section containing the pretectal TH neuron and some of its projections. Anatomical regions are annotated in black. Neuron projection path/targets are indicated by black arrowheads. Specific cell somata are labeled in white and indicated by white arrowheads. Asterisks highlight the corresponding landmarks in dorsal and transverse views. Anterior at left (dorsal views), dorsal at top. Scale bars = 20 μm.
SUPPLEMENTARY FIGURE S10
Comparison of our manual separation with automatic tracking software.
The dataset presented in Figure 1 was analyzed by two methods using only the GFP signals.
(a) Isolation of three separate neurons from the raw data using our technique (left panels): Segmentation by combining drawn masks in 3D space and applying the 3D mask to the raw data. Individual neurons and their extensions are distinguishable in the primary data (right panel). AP, area postrema; H, hypothalamus; MO, medulla oblongata. Lateral views of maximum intensity projection. Anterior at left, dorsal at top; 4 dpf larvae; anti-TH (red), anti-GFP (green). Scale bars = 20 μm. (b) Software analysis using FilamentTracer Autopath algorithms from Imaris (Bitplane AG). Top panels show that the soma in the area postrema (purple arrows) was automatically identified by FilamentTracer and manually confirmed, followed by the execution of the algorithm that traced the processes from the soma. White double arrows indicate false representation of projections (due to linking up of background signals) arising from the area postrema. Bottom panels illustrate that the somata (purple arrows) in the dataset were user-defined before the same algorithm was run. Less false projections (white double arrow) were detected, however the program did not recognize a true connection from the area postrema (white broken arrow, bottom right panel). Lateral views in 3D space. Anti-GFP (white), Imaris trace (green).
SUPPLEMENTARY FIGURE S11
Identification of GFP labeled somata as catecholaminergic neurons by double immunolabeling of cell bodies with anti-GFP and anti-TH antibodies.

All somata labeled by th:rasGFP and shown in the main and supplementary figures were also immunoreactive for anti-TH antibody. This is not always obvious from the stacks shown in the figures, because the gray levels of green and red channels were adjusted to highlight the spatial information of fine GFP-labeled processes, which were usually not as well-stained by anti-TH antibody. For this supplementary figure, the red channel representing anti-TH immunoreactivity was adjusted to positively identify TH expression in GFP (green) positive somata. Dorsal view; anterior at left, except 8a (anterior at top); arrowheads indicate somata. Scale bars = 5 μm.
SUPPLEMENTARY TABLE S1

Number of GFP- and TH immunoreactive cells and of embryos analyzed for each catecholaminergic group.

| Catecholaminergic | No. of THir cells labeled | No. of embryos analyzed |
|-------------------|---------------------------|-------------------------|
| Retinal amacrine cells | 17                        | 13                      |
| Olfactory bulb     | 48                        | 39                      |
| Subpallium         | 60                        | 46                      |
| Anterior preoptic  | 17                        | 16                      |
| Preoptic           | 17                        | 14                      |
| Pretectum          | 10                        | 10                      |
| DC1                | 41                        | 36                      |
| DC2 - post. tuberculum | 69                   | 65                      |
| DC4 - post. tuberculum | 42                     | 42                      |
| DC5 - hypothalamus | 110                       | 104                     |
| DC6 - hypothalamus | 51                        | 47                      |
| DC3 - hypothalamus | 48                        | 36                      |
| DC7 - hypothalamus | 26                        | 21                      |
| Locus coeruleus    | 35                        | 31                      |
| Medulla oblongata - interfascicular zone | 9                | 8                       |
| Medulla oblongata - vagal area       | 13                        | 9                       |
| Medulla oblongata - area postrema    | 33                        | 24                      |
| Sympathetic ganglia | 15                        | 11                      |

| Trigeminal ganglia | 9 | 8 |

In some embryos, GFP-labeled cell bodies with clearly distinguishable projections could be analyzed on both left and right halves of the brain. Therefore the number of TH immunoreactive cells documented is higher compared to the number of embryos studied for most catecholaminergic groups.
SUPPLEMENTARY TABLE S2

Comparison of diencephalic cluster 2 and 4 dopaminergic neurons in left and right brain territories.

|       | DC 2 |       | DC 4 |       | PVOa |       |
|-------|------|-------|------|-------|------|-------|
|       | left | right | left | right | left | right | total |
|       | 3    | 2     | 5    | 4     | 8    | 6     | 14    |
|       | 4    | 3     | 3    | 4     | 7    | 7     | 14    |
|       | 5    | 3     | 5    | 3     | 10   | 6     | 16    |
|       | 2    | 4     | 3    | 3     | 5    | 7     | 12    |
|       | 2    | 2     | 4    | 4     | 6    | 6     | 12    |
|       | 3    | 4     | 5    | 4     | 8    | 8     | 16    |
|       | 4    | 3     | 6    | 5     | 10   | 8     | 18    |
|       | 3    | 1     | 3    | 6     | 6    | 7     | 13    |
|       | 3    | 2     | 4    | 3     | 7    | 5     | 12    |
|       | 2    | 2     | 4    | 5     | 6    | 7     | 13    |
|       | 3    | 3     | 5    | 5     | 8    | 8     | 16    |
|       | 3    | 2     | 6    | 5     | 9    | 7     | 16    |
| Average | 3.08 | 2.58 | 4.42 | 4.25 | 7.50 | 6.83 | 14.33 |
| Std. Dev. | 0.90 | 0.90 | 1.08 | 0.97 | 1.62 | 0.94 | 2.02 |
| Two-tailed paired T-test | 0.166 | 0.674 | 0.207 |

DC2 and DC4 neurons were counted in 12 anti-TH immunolabeled brains (rows) from 4 dpf larva using the Spots function in Imaris x64 6.3.1 (Bitplane AG, Zurich, Switzerland). A bounding box to delineate the region of interest was defined as: x = 110, y = 138, z = 48. The diameter for DC2/4 DA somata was estimated to be < 6.5 μm. Manual thresholding was performed only in several cases to deselect background or non-DC2/4 cells, which could be determined based on morphology. The T-tests revealed no significant left-right asymmetry in the number of these neurons.
SUPPLEMENTARY TABLE S3

Comparison of ascending dopaminergic projections in left and right brain territories.

|               | Number of long ascending projections from PVOa (DC2/4) neurons (n = 10/111) |
|---------------|--------------------------------------------------------------------------------|
|               | left | right |
| DC2           | 2    | 3     |
| DC4           | 2    | 3     |
| Total         | 4    | 6     |
| One-tailed paired T-test | 0.278 |

The total number of long ascending projections derived from diencephalic cluster (DC) 2 and DC4 neurons recorded in our analyses. The T-test suggested that DC2 and DC4 neurons from both left and right posterior tuberculum target the subpallium with similar frequency.
### SUPPLEMENTARY TABLE S4

Comparison of subpallial dopaminergic neurons in left and right brain territories.

|                  | Number of subpallial DA neurons per brain (n = 20) |
|------------------|---------------------------------------------------|
|                  | left  | right | total |
| 10               | 9     | 10    | 19    |
| 13               | 10    | 13    | 23    |
| 10               | 8     | 12    | 18    |
| 13               | 13    | 11    | 26    |
| 11               | 12    | 8     | 23    |
| 11               | 8     | 10    | 19    |
| 11               | 10    | 6     | 12    |
| 12               | 12    | 10    | 24    |
| 11               | 10    | 12    | 21    |
| 9                | 8     | 12    | 17    |
| 14               | 10    | 9     | 19    |
| 12               | 15    | 9     | 29    |
| 9                | 9     | 10    | 18    |
| 11               | 10    | 10    | 21    |
| 11               | 16    | 11    | 27    |
| **Average**      | 10.70 | 10.30 | 21    |
| **Std. Dev.**    | 1.89  | 2.47  | 3.93  |
| **Two-tailed paired T-test** | 0.379 |

Neurons were counted in 20 anti-TH immunolabeled brains (rows) from 4 dpf larva using the Spots function in Imaris x64 6.3.1 (Bitplane AG, Zurich, Switzerland). A bounding box to delineate the region of interest was defined as: x = 110, y = 138, z = 48. The diameter for subpallial DA somata was estimated to be < 5 μm. Due to the continuity of subpallial and olfactory DA neurons which appear morphologically similar in the telencephalon, the boundary of these two clusters was not always clear. Therefore the bounding box dimensions were kept constant and began at the most caudal edge of the subpallial DA group in all measurements. The T-test showed similar number of DA neurons on both sides of the subpallium.
SUPPLEMENTARY TABLE S5
Comparison of descending dopaminergic projections in left and right brain territories.

|                     | Number of descending projections from subpallial DA neurons (n = 24/60) |
|---------------------|--------------------------------------------------------------------------|
|                     | left          | right         |
| Total               | 14            | 16            |
| One-tailed paired T-test |               | 0.352         |

The number of subpallial descending projections tabulated in our analyses. The T-test revealed that the basal forebrain is targeted bilaterally at similar frequency.
**SUPPLEMENTARY TABLE S6**

Frequency distribution of neuron projection targets across all analyzed datasets.

| Neuronal groups                        | Frequency | No. of cells |
|----------------------------------------|-----------|--------------|
| Retinal amacrine cells                 | 1         | 17           |
| Olfactory bulb                         | 1         | 48           |
| Subpallium                             | 1         | 60           |
| Anterior preoptic                      | 1         | 17           |
| Preoptic                               | 1         | 17           |
| Precordum                              | 1         | 10           |
| DC1                                    | 1         | 41           |
| DC2                                    | 1         | 69           |
| DC4                                    | 1         | 42           |
| DC5                                    | 1         | 110          |
| DC6                                    | 1         | 51           |
| DC3                                    | 1         | 48           |
| DC7                                    | 1         | 26           |
| Locus coeruleus                       | 2         | 35           |
| Medulla oblongata – interstitial zone  | 1         | 9            |
| Medulla oblongata – vagal area         | 1         | 13           |
| Medulla oblongata – area postrema     | 1         | 33           |
| Sympathetic ganglia                    | 1         | 15           |

Tabulation of the number (i.e., sample size; yellow columns indicate sample sizes greater than 30 where data was present) of neurons analyzed (top row) for each target territory of a catecholaminergic cluster (left column), and the frequency at which the sample sizes are recorded. The sum of frequencies for the occurrence of sample sizes ranging from one to 92 cells was graphically analyzed (data not shown). Saturation was achieved for most dopaminergic neuronal groups (such as amacrine cells, olfactory bulb, subpallial and diencephalic clusters), but some targets may have been missed where modest numbers of neurons were observed.
SUPPLEMENTARY TABLE S7
Frequency distribution of classes of neuron projection patterns across all analyzed datasets.

| Neuronal groups                      | Sample Size | No. of cells |
|--------------------------------------|-------------|--------------|
|                                      | 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 |              |
| Retinal amacrine cells               |             | 1            | 17          |
| Olfactory bulb                       |             | 1            | 48          |
| Subpallium                           | 1 1 1 1     | 1            | 60          |
| Anterior preoptic                    | 3 1 4       | 1            | 17          |
| Preoptic                             | 4           | 1            | 17          |
| Pretectum                            | 1 1         |              | 10          |
| DC1                                  | 2           | 1            | 41          |
| DC2                                  | 1 2 4 1 1 2 1 |          | 69          |
| DC4                                  | 1 1 1 1     | 1            | 42          |
| DC5                                  | 1 1 2       |              | 110         |
| DC6                                  | 1 2         | 1            | 51          |
| DC3                                  | 1           | 1            | 48          |
| DC7                                  | 1           | 1            | 26          |
| Locus coeruleus                      | 2 2 3 1 2 1 |              | 35          |
| Medulla oblongata - interfasicular zone | 2 1 1 |          | 9           |
| Medulla oblongata - vagal area       | 1 2         |              | 13          |
| Medulla oblongata - area postrema    | 1 2         | 1 1         | 33          |
| Sympathetic ganglia                  | 1           | 1            | 15          |
| Trigeminal ganglia                   | 1           |              | 9           |

Tabulation of the number (i.e., sample size; (i.e., sample size; yellow columns indicate sample sizes greater than 30 where data was present) (top row) for each class of projection pattern observed within a catecholaminergic cluster (left column), and the frequency at which the sample sizes are recorded. The sum of frequencies for the occurrence of sample sizes ranging from one to 48 cells was graphically analyzed (not shown). The left-shift of the curve suggests that the saturation of projection patterns was not achieved for all the neuronal groups analyzed. However for most projection behaviors observed in dopaminergic groups and TH immunoreactive ganglia, saturation was likely achieved due to the sampling of a high number of datasets.
SUPPLEMENTARY NOTES

Supplementary Note 1 Comparison to mammalian catecholaminergic systems projection behavior

For easier comparison of the projection behavior of catecholaminergic (CA) neurons identified for zebrafish in our study, we very briefly summarize the previously published information for mammalian systems and provide a summary on the correlations between zebrafish and mammalian CA groups.

The mammalian CA cell groups in the CNS are numbered in a caudal (A1) to rostral (A17) order. The caudal hindbrain noradrenergic (NA) groups, A1 and A2, of the medulla oblongata (MO) have ascending projections to the forebrain and primarily to the hypothalamus. The prominent more rostral NA neurons of the locus coeruleus (LC, A6) have both ascending and descending projections. Noradrenergic projections innervate most parts of the CNS, including the spinal cord, cerebellum, hypothalamus, thalamus, hippocampus, striatum and neocortex. In contrast, the fibers of the A5 and A7 cell groups mainly project within the brain stem and to spinal cord regions. The dopaminergic (DA) neurons of the retrorubral area (A8), substantia nigra pars compacta (A9), and ventral tegmental area (A10) of the di- and mesencephalon send out ascending projections to forebrain targets via the mesocortical, mesolimbic, and nigrostriatal pathways. DA neurons are also located in the diencephalon (A11 to A15), olfactory bulb (A16) and retina (A17). A12 cell group DA neurons projecting to the median eminence and to the hypophysis constitute the tuberoinfundibular system. The A11 DA cell group is a source of diencephalospinal projections.

The incerto-hypothalamic CA system mainly consists of projections from ventral thalamic A13 DA neurons, which are joined by axons of DA groups A11 and A14 to target regions in the hypothalamus. Finally, projections of the A14 DA cell group form the periventricular DA system and innervate, apart from the periventricular and preoptic area, the hypophysis. The olfactory bulb and retina DA neurons project locally.

Based on anatomical and gene expression data, the following correlations of zebrafish and mammalian CA groups may be postulated: A1-A3 may correlate with zebrafish medulla oblongata NA neurons; A6 correlates with zebrafish LC NA group; A8-A10 mesencephalic DA groups are likely absent in zebrafish; A11 correlates with the posterior tubercular and hypothalamic DC2, DC4, DC5 and DC6 groups in zebrafish; A12 and A14 correlate with the hypothalamic groups DC3 and DC7, and more DA neurons develop at later stages in these brain areas in zebrafish; A13 correlates with ventral thalamic DC1 DA neurons in zebrafish (in adults also termed DC0 by Rink and Wullimann); A15 correlates with anterior preoptic and preoptic groups in zebrafish; dispersed striatal DA neurons exist in mammals, but have not been identified in zebrafish.
assigned a number in the mammalian nomenclature. They may correlate with zebrafish subpallial DA neurons. A16 correlates with the olfactory bulb group in zebrafish; A17 correlates with the retinal amacrine DA group in zebrafish.

**Supplementary Note 2 Projection patterns of locally-projecting noradrenergic neurons**

Locally-projecting NA neurons such as those in the interfascicular zone of the medulla oblongata (n = 9) (Supplementary Fig. S3a) and the sympathetic ganglia (n = 15) (Supplementary Fig. S3b) were also identified. Furthermore the trigeminal ganglia, for which there is no published evidence that they are catecholaminergic, were found to be th-positive in 4 dpf fish. Short, local processes radiating away from the brain were observed to arise from GFP-labeled trigeminal ganglia (n = 9) (Supplementary Fig. S3c). TH and Dopamine-beta-hydroxylase expression have been previously reported for neurons of the rat trigeminal ganglion.

**Supplementary Note 3 Dopaminergic groups with local and intermediate length projections**

The majority of the examined pretectal DA neurons arborize locally (n = 10/10) and also innervate the tectum (n = 9/10). Some contribute to the posterior commissure (n = 7/10) while connecting the left and right tectum (Figs. 2, 4a).

The second population with local and intermediate length projections is DC1, which in the 4 dpf larvae consists of a dorso-rostral portion of small DA neurons which locate to the ventral thalamus and a ventro-caudal portion of similar small, non-liquor contacting DA neurons of the periventricular posterior tuberculum. Due to lack of a clear anatomical boundary, both populations have been grouped together and termed DC1 in larvae, while in adult zebrafish they can be clearly distinguished as ventral thalamic DA group (termed DC0 in the adult brain) and periventricular posterior tubercular DA group (termed DC1 in the adult brain). The DC1 neurons typically send short proximal processes into the eht (n = 41/41; Fig. 2). 15% of DC1 somata in addition send descending projections to the rhombencephalon (Fig. 4b). Less than 10% of documented neurons have more complex branching activities and contralateral projections via the poc (Fig. 4b; Supplementary Fig. S2). In these examples, the single neurons target more than one brain territory apart from the eht, such as the hypothalamus, pretectum, tectum and hindbrain (Fig. 2). Essentially all of the dorso-rostral portion of DC1, which likely correlate with ventral thalamic DA neurons, contribute to the eht, while a minority also projects to pretectum/tectum. The projection behavior of this ventral thalamic population is thus similar to A13 in mammals, which predominantly contributes to the incerto-hypothalamic system. Most of the ventro-caudal portion of DC1, which
may correlate with the periventricular posterior tubercular DA neurons, contribute to these "minor" projection patterns labeled in blue and grey in Figure 2. These periventricular posterior tubercular DA neurons are located in the proximity of DC2 neurons, but can clearly be distinguished by their small size from DC2 neurons, which are large and pear-shaped. At 4 dpf we did not detect ascending projections to the telencephalon which originate from periventricular posterior tubercular DA neurons.

Supplementary Note 4 Dopaminergic groups with predominant local projections

Several THir groups located along the rostral to caudal extent of the hypothalamus contribute to local projection networks and terminate their processes within the vicinity (Figs. 2, 4). Anterior preoptic DA neurons are liquor-contacting (n = 17/17) and mostly contribute to the anterior CA tracts (n = 13/17) and postoptic commissure (poc) (n = 5/17) (Supplementary Fig. S4c). Preoptic DA neurons contribute by and large to the poc (n = 11/17) and eht (n = 6/17) (Fig. 4c). DC 3 neurons of the paraventricular organ are liquor-contacting (n = 48) and contribute chiefly to the eht (n = 28/48) and caudal hypothalamus (n = 14/48) (Fig. 4d). The liquor-contacting DC 7 somata that line the posterior recess possess sparse processes that target locally within the caudal hypothalamus (n = 26) (Fig. 4e). Dopaminergic projections in the ventral diencephalon are located close to the ventral surface of the hypothalamus, and may thus target the pituitary. However, our data could not resolve direct contributions to the pituitary without analysis of further markers. For adult as well as larval zebrafish, dopaminergic projections have been detected close to the pituitary stalk, and were reported to enter the pituitary by 5 dpf.

SUPPLEMENTARY METHODS

Image processing and analysis: Procedure

To document the complete brain, two overlapping confocal stacks were recorded. The fore- and hindbrain confocal stacks were stitched using XuvTools. All raw datasets of whole brain stacks were stored in HDF5 files (http://www.hdfgroup.org). As a hierarchical file format, HDF5 allows the storage of multiple n-dimensional datasets and metadata within a single file, thus providing a powerful basis for batch processing tasks. The image processing pipeline was created with multiple small C++ programs that engaged the appropriate keys to read input datasets and write their output into the same file with a different key. Following the data analysis pipeline
described in the Methods, the noise was reduced by filtering in z-direction using a Gaussian kernel with standard deviation of 3.7 μm in a small number of datasets for better visualization.

**Image processing and analysis: Quality control**

To ensure that an appropriate region around the neuron soma and projections was segmented, two quality control procedures were used:

1. Oversized masks (i.e., masks that contain too much background signals) can easily be identified in the created final dorsal and lateral maximum intensity projections (MIP) (Figs. 1c-e). Furthermore rotating MIP-movies of the masked datasets were created, therefore allowing a more detailed quality control of highly branching regions (Supplementary Movie 1).

2. Undersized masks (i.e., masks that accidentally crop important information such as parts of a soma or projections) were identified by special visualization (QC in Figs. 1c-e). For example, in Figure 1c (see dorsal MIP of QC panel) green represents the raw channel segmented only by the lateral mask $L$ (Fig. 1b), and shows the background signal only from those layers, that are near the desired structure; cyan (Fig. 1c) represents the raw channel segmented with the final mask $M$ (Fig. 1b). In this visualization one can identify missing regions simply by searching for overlaps of cyan/blue masks and continuous pixel-signals in the green channel.

In addition we have performed a comparison of our technique against the FilamentTracer module from the standard software Imaris (Bitplane AG, Zurich) and concluded that our approach was less likely to link non-specific signals into the CA axonal projections (Supplementary Fig. S10). We found that algorithm-generated traces may vary drastically depending on user-defined thresholds. These attempts led us to further conclude that our large number of non-uniform datasets and the large file sizes of individual recordings inhibited the regular application of commercially available software. In contrast our image processing method requires no user intervention, apart from the application of the segmentation masks on MIPs of unprocessed images. The strength of our approach lies in its ability to present the original immunolabeled neuron and its fine processes, instead of a computational representation of how its axonal projections may appear.
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