

Supplementary information for Vishwanathan et al.

February 28, 2017

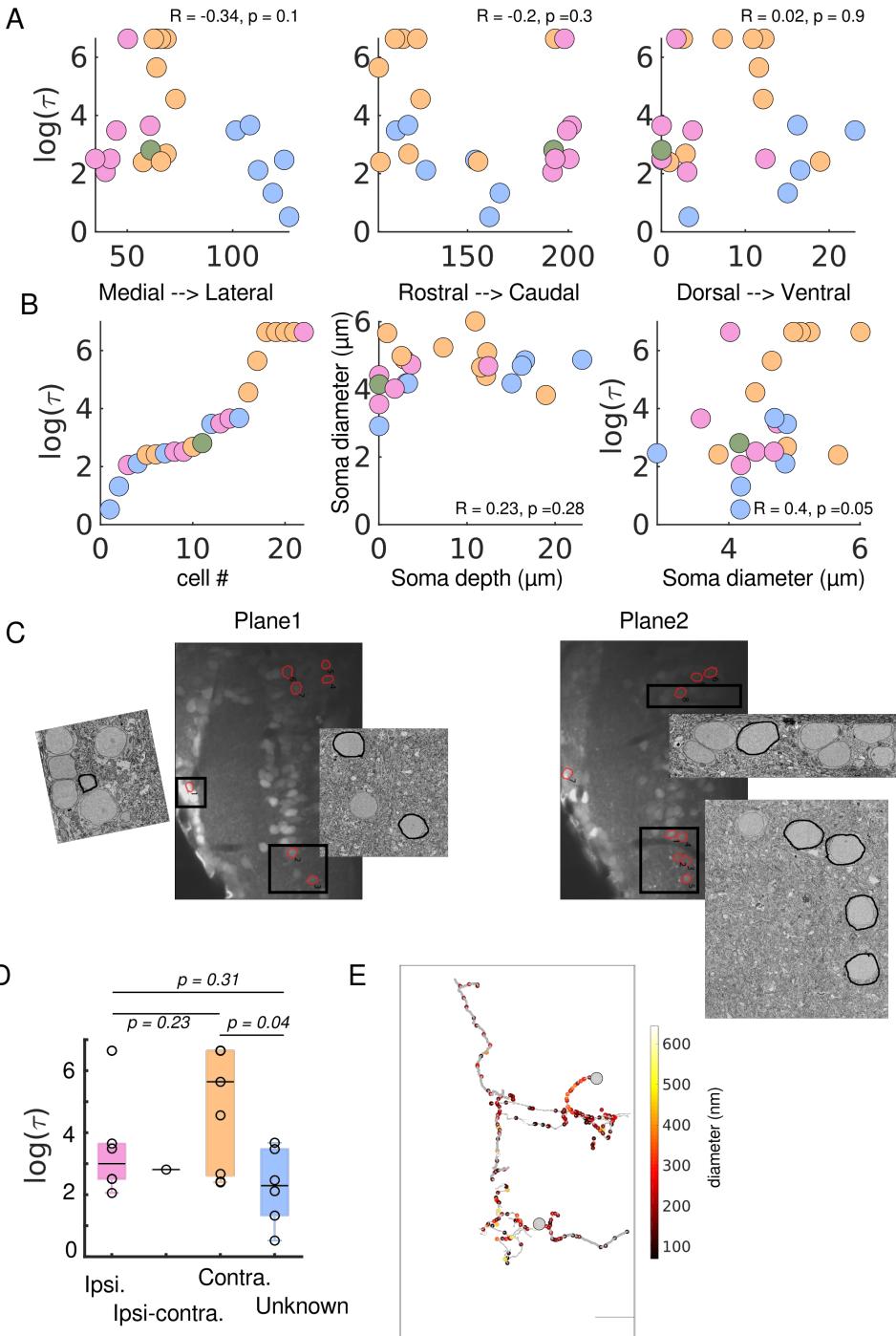


Figure S 1: (A) Spatial distribution of integrator time constants along the rostrocaudal, mediolateral and dorsoventral axis respectively. R values are Pearson coorelation coeffecients. Colors represent the group that the neurons belong to. (Pink - ipsi only, Green - ipsi-contra, Orange - contra only and Blue - unknown).

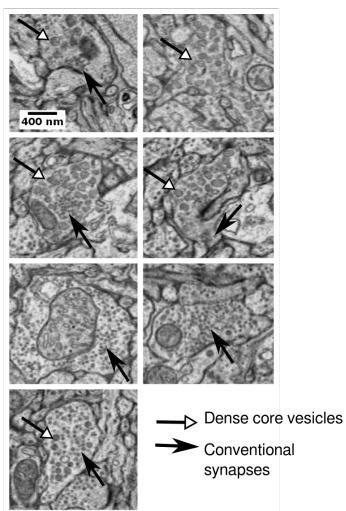
(B) (Left) Graded levels of persistent activity. (Middle) Diameters of integrator somata along depth. (Right) Integrator soma diameter and integrator time constant. R values are Pearson coorelation coeffecients.

(C) Two functionally imaged planes with integrator neurons circled in red. Black boxes are representative areas where corresponding EM images are shown as insets. Insets are representative EM locaiton, after registration, with the functionally identified cells circled in black.

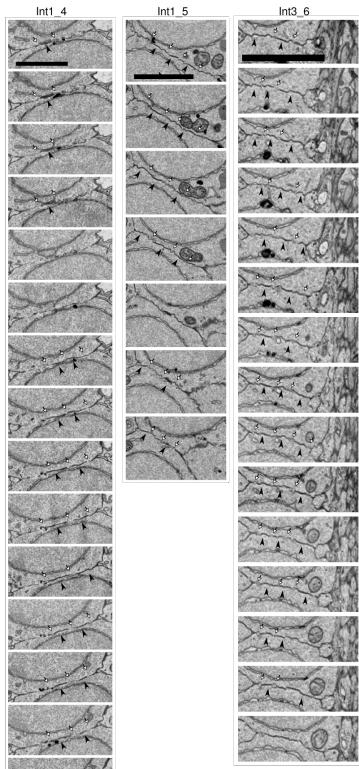
(D) Box plot of integrator time constants for all cells in assigned groups. Black line is the median. P values reported from Kolmogorov-Smirinov test.

(E) Diameter profiles along an example ipsi-only (top) and contra-only (bottom) integrator neuron. Color of each dot represents the thickness of the neurite at that location. Large grey circle represents the soma of the cell. All thin neurites are dendritic and all thick neurites are axonal. Scale bar = $20\mu m$.

A Types of synapses



B Putative tight junctions between somata



C Primary cilia

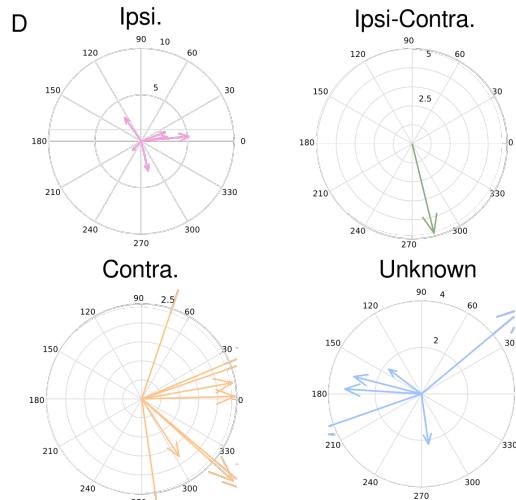
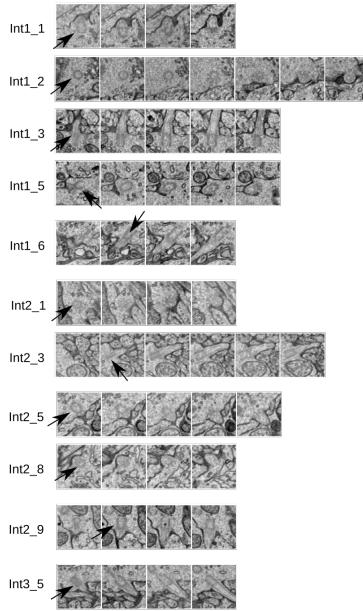


Figure S 2: (A) Examples of types of synapses in the imaged volume. Closed arrows show conventional synapses and open arrows show dense core vesicles within the same bouton.

(B) Successive images of cellular junctions between cell somata. Closed arrow head shows cell junctions indicated by darkening of the membrane at the same location in multiple sections. Open arrows show the separation of the membrane by the lack of darkening. Scale bar = 500nm.

(C) Primary cilium of integrator neuron. The primary cilium is visible over multiple sections as seen by presence of ordered microtubules that emerges very close to the Golgi complex of the cells.

(D) Orientation of primary cilium, centered at the somata. Black arrow show the beginning of the cilium. Distance of concentric rings is in microns. Colors represent the group that the neuron belongs to. (Pink - ipsi only, Green - Ipsi-contra, Orange - contra only and Blue - unknown).

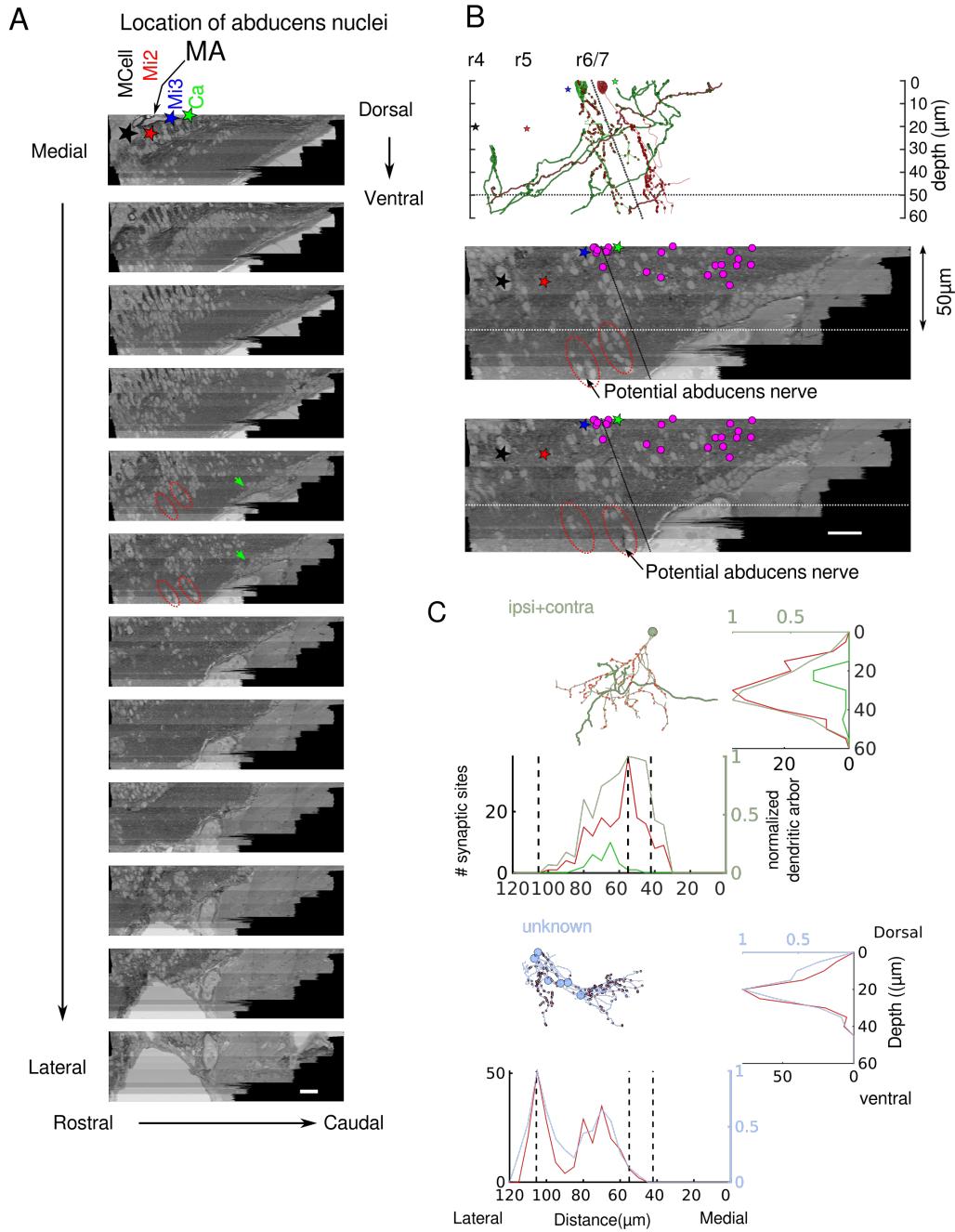


Figure S 3: (A) Location of abducens nuclei from low-res EM images. The frames are ordered from medial to lateral, $10\mu\text{m}$ apart. Red circles (dotted line) show the potential location of both abducens nuclei. Green arrow shows the location of the inferior olive. MA -Mauthner Axon. Rhombomeres r4-7 were identified based on the location of the reticulo-spinal cells (Black star - MCell, red star - Mi2, blue star - Mi3 and green star - Ca). Scale bar $20\mu\text{m}$.

(B) (Top) Lateral view of two ipsilaterally projecting integrator neurons with axonal projections towards the abducens nuclei. The axons project to the same depth as the abducens nuclei (dotted horizontal black line). Black dotted line is the border between r6 and r7, with tilt calculated based on [1, 2]. (Bottom) Dotted white line is $50\mu\text{m}$ ventral to the Mauthner axon plane showing corresponding region from top panel. Potential abducens nerve was identified as dark myelinated axon exiting the volume ventrally. Scale bar = $20\mu\text{m}$.

(C) Depth stratification of ipsi-contra and unknown projecting groups. For each panel, below the trace of all neurons is the stratification of the dendrites (red) and axons (green) along the mediolateral axis. To the right is the stratification along the dorsoventral axis. Black dotted line are the locations of the stripes from figure 1.

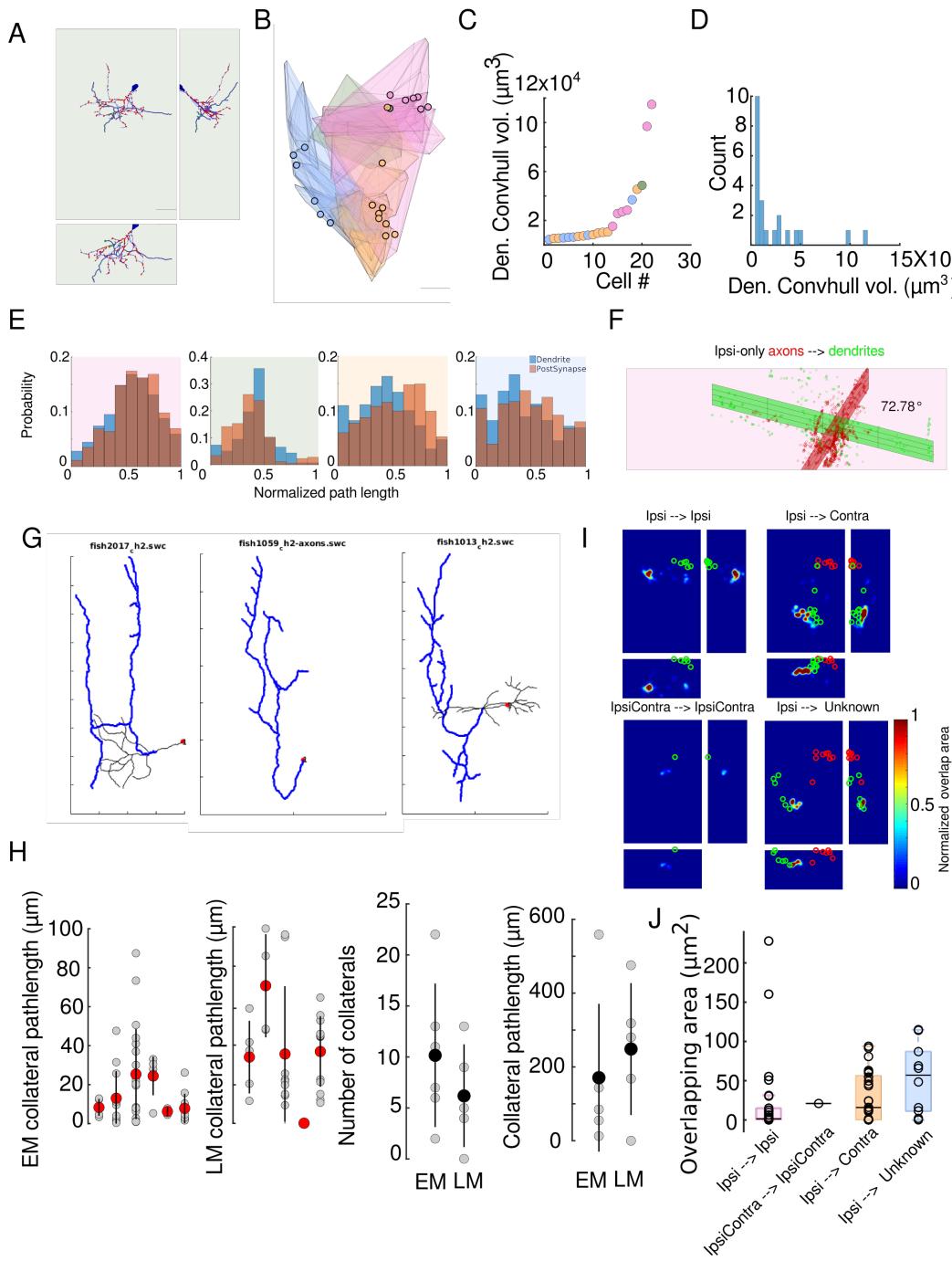


Figure S 4: (A) Three views of one integrator neuron that had both ipsilateral and contralateral projections.
 (B) Convex hulls of all neurons color coded by the group. Colored circle is location of somata. (Pink - ipsi only, Green - ipsi-contra, Orange - contra only and Blue - unknown).
 (C) Convex hull volume of dendrites of integrator neurons.
 (D) Distribution of the convex hull volume of the integrator cells.
 (E) Distribution of the normalized postsynaptic pathlength and the distribution of the normalized dendritic pathlength. Background color (Pink - ipsi only, Green - Ipsi-contra, Orange - contra only and Blue - unknown).
 (F) Angle between best fit planes of axons (green) and dendrites (red) of ipsi-only neurons.
 (G) Example traces of integrator neurons with ipsilateral projecting axons from light microscopic (LM) dye fills from [2], that were used for comparison with EM cells.
 (H) (Left, two panels) Collateral path length of all EM, LM cells respectively. Grey circles are values for each cell, and red circle with line is the mean \pm standard deviation. (Right, two panels) Number of collaterals and total pathlength of collaterals for EM and LM traces.
 (I) Spatial overlap of axons and dendrite from each group. Green circle - location of somata of presynaptic neuron (location of neuron with axon), red circle - location of somata of postsynaptic neuron.
 (J) Box plot with actual overlapping area for neuron pairs from (I).

Cell group	# Cells	Average length (μm)	Average axonal length (μm)	Average dendritic length (μm)	Convex hull volume (μm^3)	Axon diameters (μm)	Dendrite diameter (μm)	Axon initiation site (μm)
Ipsi. only	6	648.8 \pm 160.42	260.15 \pm 146.1	388.65 \pm 59.98	3.08 \times 10 ⁵	0.21 \pm 0.15	0.25 \pm 0.15	99.62 \pm 54.83
Ipsi-contra.	1	838.66	292.89	545.76	0.486 \times 10 ⁵	0.2 \pm 0.11	0.21 \pm 0.12	63.31
Contra. only	9	359.59 \pm 58.86	66.94 \pm 14.71	292.65 \pm 65.25	1.09 \times 10 ⁵	0.16 \pm 0.08	0.22 \pm 0.12	22.35 \pm 21.69
unknown	6	221.47 \pm 48.7	NA	221.47 \pm 48.7	0.67 \times 10 ⁵	NA	0.22 \pm 0.12	

Table S 1: Anatomical features of integrator neurons

Supplementary experimental procedure

Two-photon calcium imaging.

A *nacre* mutant zebrafish larva, 6 days of age, was anesthetized in 100 or 200 mg/L tricaine-methanesulfonate (MS222, VWR TCT0941-025G) for about 1 minute and then quickly mounted dorsal side up with droplets of 1.7% low temperature agarose (Sigma A0701-100G) on the lid of a 35mm petri dish containing a bed of hardened 1% agarose (Invitrogen 15510-027). The larva was then covered in 50 mg/L MS222. The larva was bulk-loaded [3, 4] with calcium sensitive dye Oregon Green 488 BAPTA-1 AM (5 mM, in DMSO with 5% pluronic, Invitrogen, O-6807) by inserting a capillary through the dorsal skin surface over the lateral edge of the right side of the hindbrain just caudal to the cerebellum, at ~30° decline relative to the dorsal surface. Following recovery, the animal was immobilized in low melting agar and was positioned to view a monitor with light gratings. The hindbrain of the animal was imaged at 790nm on predefined planes at 0.98 Hz. The imaging was performed on three pre-defined planes ~ 8 μ m apart, below the level of the Mauthner neuron, unilaterally.

The integrator neurons were identified in a manner similar to a previously described method [5, 1]. Briefly, correlations for every pixel over a 5min acquisition period was determined for eye position and eye velocity. A region-of-Interest (ROI) was then drawn over the pixels where the majority were correlated to one of these variables. The fluorescence was reported as change in fluorescence for each frame to the average fluorescence, divided by the average. Saccadic fluorescence traces were displayed over an 8 sec window starting 1 sec before the end of the saccade. The firing rates were computed over a 6 second fixation period beginning 1 sec after the end of the saccade. The firing rates were computed by deconvolving the average fluorescent traces with a calcium impulse response function (CIRF) as described previously [6]. The integrator time constants were calculated by fitting an exponential function (Matlab *ezfit*) to the firing rates. The time constants for the fits were capped at 100 sec.

Serial section electron microscopy.

The animal was immersed in a fixative mixture of 2% paraformaldehyde and 2.25% glutaraldehyde buffered in 0.07M cacodylate buffer. The skin over the hindbrain was removed for good ultrastructure preservation and homogeneous staining of the tissue. Then it was thoroughly washed in 0.1M cacodylate buffer with 4% sucrose before staining. The tissue was stained using a conventional ROTO procedure [7]. Briefly, it was stained in 1% reduced osmium tetroxide with potassium ferrocyanide for 2 hr on ice. It was then washed 4x 30 min in 0.1M cacodylate buffer, also on ice. This was then followed by amplification in 1% sodium thiocarbohydroazide (TCH) for 15 min followed by 3x5 min washes in water and another round of incubation in 1% osmium tetroxide for 1 hr. The tissue was then washed 3x15 min in water and incubated with 1% aqueous uranyl acetate overnight. The following day the tissue was washed 3x15 min in water and incubated for 30 min in Lead aspartate solution, followed by washing and dehydrated with a series of ethanol, followed by propylene oxide (PO). The tissue was then infiltrated in decreasing gradients of PO and EPON for 24 hrs and baked for 48 hrs at 60 C. Following hardening, the tissue block face was coarsely trimmed and a rectangular mesa was defined for serial sectioning. Care was taken to orient the specimen to permit sectioning along the horizontal axis. Serial sections from the above animal were collected approximately from the level of the Mauthner axon at a thickness of 45 nm. The serial sections were then adhered to a silicon wafer, using double sided carbon tape (TEDpella), the wafers were coated with a thin film of carbon to make them conductive.

Registration of light microscope and electron microscope volumes

The EM images were aligned using the TrakEM2 plugin in Fiji [8]. Briefly, individual images were imported into the TrakEM2 framework and montaged, first, using affine transforms, followed by elastic transforms. The images were then registered in z using a similar approach, where the first pass was performed using affine transforms, followed by elastic transforms. All the alignment was performed on a machine with 32 virtual cores and 120GB RAM.

To locate the cells that were involved in the integrator circuit, we made use of the fact that large, gross morphological features were easily identified in both the LM and EM stacks. Once enough such features were identified in the LM stack, we located these same landmarks in the high-res EM stack. Each pair of landmarks (one from the LM volume and one from the EM volume) was then used to calculate a global affine transform that was applied to transform the LM volume to be overlaid on the EM volume using the TrakEM2 plugin in Fiji [8]. Following this registration, we were able to reliably locate the same cells from the LM and the EM volumes.

Reconstructions

The reconstruction of the identified neurons was performed using the TrakEM2 plugin in Fiji/ImageJ. One expert (AV) traced all neurons and annotated all pre- and postsynaptic sites. AV noted difficult locations while tracing. A second expert (EA) identified locations at which the skeletons looked unusual (sharp turns or closely spaced branch points). EA reviewed the images at all of these locations (typically a few per neuron), and reached consensus with AV. At most locations the original tracing of AV was judged correct. A few locations were judged ambiguous, in the sense that image defects made it impossible to interpret the locations with certainty. At such ambiguous locations, we erred on the side of incomplete tracing. A more detailed second round of tracing was then carried out using a more accurate semi-automated reconstruction method similar to the ‘eyewire’ platform (<http://eyewire.org>). This approach has been used to obtain multiple coverage for single cells. This process revealed that the axon of one cell was previously under-reconstructed. Similarly, another axon from a different cell was over-reconstructed. The remaining, vast majority 20/22 cells remained the same.

The skeletonized tree structure was exported from TrakEM2 as *.swc files. These trees were then imported into Matlab using custom scripts to import .swc files as trees. For the comparison of EM traces with LM traces, the LM traces were traced using the Simple Neurite

Tracer [9] plugin in Fiji, and exported as *.swc file. In total, we reconstructed 9.29 mm of neurite (2.45 mm of axonal length and 6.84 mm of dendritic length).

Ipsi- and contralateral projection (“ipsi-contra”) - One neuron had axons with both ipsilateral and contralateral projections (Sup. Fig. 5A). This integrator neurons was located more lateral to the neurons from *ipsi-only group*. The ipsilaterally projecting axon resembled the axons of the ipsi-only group of neurons (Fig. 3A), with similar tilt in the rostro-caudal axis. This neuron contained a neurite that crossed the midline. We infer that this neurite is a putative contralaterally projecting axon. This cannot be confirmed with certainty, because its presynaptic sites are on the contralateral side of the hindbrain, outside the imaged volume. Our inference is based on a number of cues as mentioned previously.

Analysis

All traces were exported as .swc files from TrakEM2. All tree lengths was reported as pathlengths unless noted otherwise. Similarly, all lengths to a pre- or post synaptic site was reported as pathlengths. We defined the axon initiation site as the parent node of the first presynaptic site. One cell from ipsi-only group was defined as an axon, based on diameter of the axons and the ‘bare’ initial segment. All neurites that were not axonal were defined as dendritic. All nodes of the tree were thus divided as axonal nodes or dendritic nodes. Pathlengths were then generated for a tree over all axonal nodes or all dendritic nodes, and the length of the tree was the sum of all axonal length and dendritic length. A collateral was defined as all those segments of an axon, that emerged from the central axonal tree. All tree nodes that were axonal were then divided as those that were part of a collateral and those that were not. The completeness of cells was decided based on the number of neurites that exited the cells.

The diameter of the trees was generated by drawing a line segment across the cross-section of the tree at a random location along the tree. Many such line segments were drawn across the entire extent of the tree, where the tracer was blinded to the fact whether the neurite was an axon or a dendrite. The measurements were then separated as dendritic or axonal. The values were reported as a mean across all dendritic or axonal measures for a tree.

Arbor densities of the dendrites was computed by projecting all the axonal or dendritic nodes along the desired plane and reported in a normalized scale. Arbor volumes were computed using the Matlab function *convhull*. To infer the neurotransmitter identity from the stripe organization of the cell bodies, we annotated the location of all the cell bodies from a low-resolution stack. The cell density was then computed by projecting all the cells along the desired axis. This process picked out the peaks that were visible in the EM images. The location of a stripe was defined as the local peak that emerged from the cell density projecting analysis. The planar organization of the postsynapses and presynapses were fit to a plane using the *planefit* function, available on the Matlab central repository.

To locate the border of r6/7 we followed a similar approach that was performed previous using light microscopic imaging and the expression of the *hox* genes [10, 1, 2]. Reticulospinal cells were identified based on the ultrastructure, that was very different form the remaining cells in the area. These cells were rich in mitochondria and gave rise to a large myelinated axon that joined the remaining fibers along the midline. We fit a plane to the reticulospinal neurons, and identified a point on this plane that bisected Mi3 and Ca. This point was move by $0.37\mu m$ caudally for every micron ventrally based on [1, 2].

References

- [1] Kayvon Daie, Mark S Goldman, and Emre R F Aksay. Spatial patterns of persistent neural activity vary with the behavioral context of short-term memory. *Neuron*, 85(4):847–860, February 2015.
- [2] Melanie M Lee, Aristides B Arrenberg, and Emre R F Aksay. A Structural and Genotypic Scaffold Underlying Temporal Integration. *The journal of neuroscience*, 35(20):7903–7920, May 2015.
- [3] E Brustein, N Marandi, Y Kovalchuk, P Drapeau, and A Konnerth. "In vivo" monitoring of neuronal network activity in zebrafish by two-photon Ca²⁺ imaging. *Pflügers Archiv*, 446(6):766–773, 2003.
- [4] D Smetters, A Majewska, and R Yuste. Detecting action potentials in neuronal populations with calcium imaging. *Methods (San Diego, Calif.)*, 18(2):215–221, June 1999.
- [5] Andrew Miri, Kayvon Daie, Aristides B Arrenberg, Herwig Baier, Emre Aksay, and David W Tank. Spatial gradients and multidimensional dynamics in a neural integrator circuit. *Nature Neuroscience*, 14(9):1150–1159, September 2011.
- [6] Andrew Miri, Kayvon Daie, Rebecca D Burdine, Emre Aksay, and David W Tank. Regression-Based Identification of Behavior-Encoding Neurons During Large-Scale Optical Imaging of Neural Activity at Cellular Resolution. *Journal of Neurophysiology*, 105(2):964–980, February 2011.
- [7] Juan Carlos Tapia, Narayanan Kasthuri, Kenneth J Hayworth, Richard Schalek, Jeff W Lichtman, Stephen J Smith, and JoAnn Buchanan. High-contrast en bloc staining of neuronal tissue for field emission scanning electron microscopy. *Nature Protocols*, 7(2):193–206, February 2012.
- [8] Albert Cardona, Stephan Saalfeld, Johannes Schindelin, Ignacio Arganda-Carreras, Stephan Preibisch, Mark Longair, Pavel Tomancak, Volker Hartenstein, and Rodney J Douglas. TrakEM2 software for neural circuit reconstruction. *PLoS ONE*, 7(6):e38011, 2012.
- [9] Mark H Longair, Dean A Baker, and J Douglas Armstrong. Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics*, 27(17):2453–2454, September 2011.
- [10] Leung-Hang Ma, Charlotte L Grove, and Robert Baker. Development of oculomotor circuitry independent of hox3 genes. *Nature Communications*, 5:4221, 2014.