

Supporting Information Figure Legends

Figure S1. Visualization of developing spinal *pet1:EGFP*+ neurons and descending raphe projections.

An anesthetized 60 hpf *Tg(-3.2pet1:EGFP)^{ne0214}* larva was embedded laterally in agarose. Images centered on the midbody (body segment 15) were collected using a confocal microscope every two hours between 60 and 72 hpf. The larva was maintained at 28.5°C when not being imaged. Arrows indicate the most caudal descending raphe projection at each age and arrowheads indicate *pet1:EGFP*+ cells as they first became visible. **A:** At 60 hpf, raphe projections were present in the most rostral region of the field of view (arrow). Two faintly *pet1:EGFP*+ cells were visible in the ventral spinal cord (A; dashed lines represent the dorsal and ventral boundaries of the spinal cord, approximately same position in all panels). **B and C:** Raphe projections extended further caudally (arrows) and additional cell bodies were detected (arrowheads) at 62 (B) and 64 hpf (C). **D and E:** Raphe projections continued to grow in a caudal direction at 64 and 68 hpf (D and E, arrows) and one additional cell was observed (arrowhead) at 68 hpf (E). **F and G:** Raphe fibers extended across the entire field of view and no new cells were detected at 70 (F) and 72 (G) hpf. Scale bar = 50 μ m.

Figure S2. Spinal 5-HT immunoreactivity precedes both *pet1:EGFP* and *tph2:mCherry* transgene expression, and later becomes restricted to cells that coexpress *pet1:EGFP* and *tph2:mCherry*. Whole-mount *Tg(-3.2pet1:EGFP)^{ne0214}* and *Tg(tph2:nfsB-mCherry)^{y226}* double transgenic larvae were labeled with antibodies to 5-HT (blue) and confocal images were collected from the midbody region of the spinal cord (dashed lines in A represent the dorsal and ventral boundaries of the spinal cord, approximately same position in all panels). Black arrowheads indicate cells that are 5-HT+ but did not express either transgene, white arrows indicate cells that were positive for only *pet1:EGFP* expression (green), black

arrows indicate a cell that coexpressed *pet1:EGFP* and *tph2:nfsB-mCherry* (*tph2:mcherry*; red) but did not contain 5-HT, and white arrowheads indicate cells that were positive for all three markers. **A-D:** Antibodies to 5-HT revealed cells in the ventral 48 hpf spinal cord (A, black arrowheads) before *pet1:EGFP* (B) or *tph2:mCherry* (C) expression was detected (merge in D). **E-H:** At 60 hpf, 5-HT antibody labeling (E) that did not overlap with *pet1:EGFP* (F) or *tph2:mCherry* (G) expression persisted (black arrowheads). 5-HT- cells that expressed only *pet1:EGFP* (white arrows) or coexpressed *pet1:EGFP* and *tph2:mCherry* (black arrows) were observed in the ventral spinal cord (merge in H). **I-L:** At 72 hpf, *pet1:EGFP*+ cells (J) colabeled with *tph2:mCherry* expression (K) and 5-HT (I, white arrowheads), although a portion did not colabel (white arrow). 5-HT antibody labeling continued, but was reduced in cells that did not express either transgene (black arrowheads). **M-P:** By 96 hpf, 5-HT antibody labeling (M) was restricted to cells that coexpressed *pet1:EGFP* (N) and *tph2:mCherry* (O, merge in P, white arrowheads). Because *pet1:EGFP* was expressed in cells that did not coexpress *tph2:mCherry* (H and L, white arrows), but *tph2:mCherry* was only detected in *pet1:EGFP*+ cells (H, L, and P, black arrows and white arrowheads), expression from the *pet1* promoter likely preceded expression from the *tph2* promoter. Further, 5-HT was detected in cells that coexpressed both transgenes (L and P, white arrowheads) and not in cells that expressed only *pet1:EGFP* (H and L, white arrows). This suggested that 5-HT synthesis occurred after *tph2:mCherry* expression. Scale bar = 20 μm .

Figure S3. 5-HT antibody labeling of ISNs progresses in a rostral to caudal direction. *Tg(-*

3.2pet1:EGFP)^{ne0214} larvae between the ages of 48 and 72 hpf were labeled with antibodies to 5-HT (red) and imaged in regions of the spinal cord centered on body segments 7 (rostral), 15 (midbody), and 23 (caudal). **A-F:** 5-HT was detected in ventral spinal cells in all regions at 48 (A-C) and 52 hpf (D-F). Expression of *pet1:EGFP* (green) was only observed in descending raphe processes in the rostral spinal cord (A and D). **G-I:** *Pet1:EGFP*+ cells that did not colocalize with 5-HT were observed in the rostral

region (G), but not the midbody (H) or caudal (I) region at 56 hpf. **J-L:** At 60 hpf *pet1:EGFP+* cells were detected in the midbody (K) and caudal (L) regions and began to colocalize with 5-HT in the rostral region (J, yellow cells). **M-O:** A subset of *pet1:EGFP+* cells were labeled with 5-HT antibodies in the rostral (M) and midbody (N) regions, but not in the caudal region (O), at 64 hpf. **P-U:** 5-HT antibody labeling continued to colocalize with *pet1:EGFP* expression (yellow), but was reduced in *pet1:EGFP-* cells (red cells) in all spinal cord regions at 68 (P-R) and 72 hpf (S-U). Projections from the raphe extended to the midbody region (Q and T) but had not reached the caudal spinal cord (R and U) at 68 and 72 hpf. Scale bar = 50 μ m.





