Materials and Methods

Ethics Statement

All animal experiments were approved by the government of Lower Saxonia and were conducted in accordance with legal regulations (EU Directive 2010_63).

Fish maintenance

Fish were raised and maintained under a 14 hours light / 10 hours dark cycle at 28°C according to standard protocols (1,2). To microinject expression constructs and to establish transgenic lines, fish of the *brass* line *brs*$^{b2/b2}$ were used. For microscopy, embryos were treated with 200 μM 1-phenyl 2-thiourea (PTU) (Sigma-Aldrich) starting from 1 dpf to inhibit melanin synthesis.

The stable fish lines that we established by using a PC specific enhancer element are listed in Figure S1. *olig2*:EGFP and *olig2*:DsRed fish lines were kind gifts from Dr. Bruce Appel and were used for the labeling of EC cells, the UAS:Venus line expresses the membrane targeted FynVenus-fluorescent protein under the control of 5xUAS Gal4-binding sites. PC specific expression driven by the enhancer element was confirmed by antibody stainings using the PC specific anti-ZebrinII antibody (kind gift of R. Hawkes) (Fig. 1A3).

Microinjection

Glass capillaries (GC100F-10, Harvard Apparatus) were pulled into microinjection needles by using a vertical needle puller (model 700C, David Kopf Instruments), which were used in a Femtojet Express Microinjector (Eppendorf) equipped with a micromanipulator (Narishige). Plasmid DNA was microinjected into the soma of one-cell stage wild-type embryos (= P0 generation, mosaic) at a concentration of 25 ng/ml (injection volume about 1.5 nl). For the generation of stable transgenic fish, transgene cassettes flanked by recognition sites of the Tol2 transposase were used and the DNA was supplemented with 25 ng/ml mRNA encoding the Tol2 transposase to increase genomic integration of the transgene cassette (3).

To establish stable transgenic lines, the injected P0 generation was raised to adulthood and crossed with wild type fish to screen embryos of the F1 generation for inherited fluorescent protein expression under a fluorescence stereomicroscope (MZ16FA or

M205FA, Leica Microsystems).

Isolation of regulatory elements from the zebrafish carbonic anhydrase-related protein VIII (*Car8*) gene

Using deletion analysis, we found that 258 bp (base pairs) sequence located about 2kb upstream of the *car8* start ATG is sufficient to induce specific gene expression in zebrafish Purkinje neurons (Fig. S1A). To achieve the strong co-expression of two target cDNAs, we used two tandem sequences of this car8 enhancer for WGA experiments and four tandem sequences for GECI and optogenetic experiments. A detailed characterization of the enhancer will be provided elsewhere.

Isolation of regulatory elements from the zebrafish *olig2* gene

Genomic DNA 2 kb upstream and 10 kb downstream of the *olig2* open reading frame (ORF) were retrieved from a previously established *olig2:EGFP* BAC (4) by homologous recombination (vector #1370). Subsequently, the open reading frame of EGFP was exchanged to KalTA4 (24) by conventional cloning (vector #1736) and the entire cassette containing the putative enhancer element of the zebrafish *olig2* gene was cloned into a pBluescript backbone (Stratagene) between recognition sites for the Tol2 transposase (vector #1737).

Immunohistochemistry in the adult zebrafish brain

Immunohistochemistry was performed according to previous studies (5). Adult zebrafish were sacrificed by an overdose of Tricaine and brains were explanted and fixed in Bouin fixative (saturated picric acid (Sigma-Aldrich) : formalin (Sigma-Aldrich) : acetic acid (VWR) = 15 : 5 : 1) over night. After a series of ethanol and Xylene washes, the brains were embedded in paraffin and sectioned (10 μm) using a microtome (Jung AG). Paraffin sections were dewaxed in Xylene and gradually hydrated in ethanol/PBS series. After washing in PBS, the sections were incubated for 30 min with 0.1% Pronase (Roche) at RT, immersed in 0.3% H_2O_2 in methanol for 30min, washed in PBS and blocked by 5% skim milk in PBS for 30 min. These pretreated sections were incubated for 16 hrs at 4°C either with a goat anti-WGA antibody (Vector Labs, antibody was preabsorbed with acetone powder of zebrafish

larvae) diluted by 1:200 with 5% skim milk in PBS or with a 1:200 dilution of a rabbit anti-tagRFP antibody (Evrogen). After washing in PBS, the sections were incubated with a biotinylated anti-goat or anti-rabbit IgG (1:500 dilution, Vector Labs) respectively, followed by staining using a Vectastain Elite ABC kit (Vector Labs). Signals were visualized with diaminobenzidine peroxidase (Sigma-Aldrich) reaction. After washing in distilled water, sections were immersed in ethanol and then Xylene and mounted with Entellan new (Merck Millipore) or aqueous mounting solution (Vectashield mounting medium, Vector Labs). For immunofluorescence of adult brain sections, 1:200 dilutions of chicken anti-GFP antibody (Aves Labs) and anti-chicken IgG FITC antibody (Jackson ImmunoResearch) were used and similar procedures were performed. With respect to anatomical nomenclature and abbreviations, we referred to the zebrafish brain atlas (46).

Whole mount immunohistochemistry of zebrafish larvae

Whole mount immunohistochemistry was performed according to previous studies (6). Zebrafish larvae were fixed in 4% PFA (Applichem) for 4 hours at RT. After washing in PBS with 1% TritonX-100, the samples were incubated for 30 min with 10 µg/ml proteinase K (Fermentas) at RT followed by washing in PBS/1% TritonX-100 and fixation in 4% PFA for 30 min. The larvae were washed in PBS/1% TritonX-100, blocked in 5% skim milk/PBS/1% TritonX-100 for 6 hours, and subsequently incubated for 16 hrs at 4°C in 5% skim milk/PBS/1% TritonX-100 with one of the following antibodies: 1:100 dilution of goat anti-WGA antibody (preabsorbed in acetone powder of zebrafish larvae); 1:100 dilution of rabbit anti-tagRFP antibody; 1:200 dilution of chicken anti-GFP; 1:200 dilution of mouse anti-ZebrinII antibody (obtained from R. Hawkes). In case of the anti-ZebrinII antibody, larvae were sometimes pretreated in hybridization buffer at 65°C for 2 hours for antigen retrieval. After washing in PBS/1% TritonX-100, the samples were incubated for 16 hrs at 4°C with one of the corresponding secondary antibodies at a dilution of 1:100: anti-goat Alexa488; anti-rabbit Alexa546; anti-chicken FITC; anti-mouse Alexa546; (all from Invitrogen). BODIPY 630/650 (Invitrogen) was used to counterstain cellular membranes for clarifying anatomical structures. After intense washing in PBS/1% TritonX-100, the larvae were mounted in 1.5% low-melting agarose (Biomol) in PBS and subjected to

image recording. For the anatomical nomenclature and abbreviations, we referred to the zebrafish brain atlases (7,8).

mRNA *in situ* hybridization

mRNA *in situ* hybridization was performed according to previous studies (9). Digoxigenin (DIG) labeled riboprobes were generated using the DIG RNA labeling kit according to the manufacturer's instructions (Roche). Zebrafish larvae were fixed in freshly prepared 4% PFA in PBST (PBS/0.1% Tween20), washed in PBST, dehydrated in methanol and stored at -20°C. After rehydration (75, 50, 25% MeOH/PBST), the specimens were washed twice in PBST followed by incubation in 10 µg/ml proteinase K and fixed again in 4% PFA for 25 min. After washing in PBST (5x 5 min each) the specimens were pre-hybridized in hybridization buffer (50% formamide, 5xSSC, 5 mg/ml torula yeast RNA, 100 µg/ml heparin, 0.1% Tween-20) for 2 hours and then hybridized over night with the riboprobe in hybridization buffer both at 65°C. Subsequently, samples were washed in 50% formamide/2xSSCT, 2xSSCT and 0.2xSSCT. For probe detection, the embryos were incubated in 0.2% blocking solution (Roche) for 2 hours followed by over night incubation in anti-DIG antibody conjugated with alkaline phosphatase (1:7000 dilution, Roche) in 0.2% blocking solution at 4°C on a vertical rotator. After washing with PBST, the hybridized probe was detected via color reaction with 450 µg/ml NBT and 175 µg/ml BCIP (Roche) in AP buffer (100 mM pH 9.5 Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$). Washing in PBST stopped the color reaction. Samples were fixed in 4% PFA in PBST for 20 min, then immersed in 50% Glycerol or subjected to additional staining procedures.

In case of adult sections, freshly prepared paraffin sections (10 µm) were used. After dewaxing and rehydration, the specimens were treated with 10 µg/ml proteinase K, followed by fixation in 4% PFA for 25 min. The samples were treated with 0.1M pH8.0 triethanolamine / 0.25% acetic anhydride, washed in 4xSSC and pre-hybridized in hybridization buffer at 65°C for 1 hour followed by hybridization over night at 65°C in hybridization buffer containing the riboprobe. Washing, antibody incubation and probe detection were performed as described above.

Microscopy

Confocal sections were recorded with a TCS SP8 microscope (Leica Microsystems, Wetzlar) and processed using the LAS AF software. 3D-images and projections from z-stack recordings were constructed by the same software. For documentation of expression data from paraffin sections, vibratome sections and whole mount *in situ* hybridization samples, stereomicroscopes (MZ16FA, M205FA Leica Microsystems, Wetzlar) or a transmitted light epifluorescence microscope (Zeiss Axiosxop) equipped with an Axiocam HRc camera (Carl Zeiss AG, Jena) were used.

Retrograde tracing of neuronal projections

For post mortem DiI-labeling, adult zebrafish brains were fixed with 4% PFA at RT over night. After washing with PBS, DiI crystals (Invitrogen) were applied to the target region with a microneedle pulled from a glass capillary tube (GC100F-10) and a micromanipulator. If necessary, parts of the brain were removed to correctly target the DiI cristals. Samples were incubated in 0.4% PFA for 2 ~ 4 weeks at 37°C. Subsequently, specimens were embedded in low-melting agarose and sectioned (200 µm) using a vibratome (Leica VT1000S). For staining retrogradely labeled tissue for WGA expression, floating slices were washed in PBS/1% TritonX-100 and then incubated for 30 min with 10 µg/ml proteinase K at RT. After washing in PBS with 1% TritonX-100, the sections were fixed again in 4% PFA for 30 min followed by washing in PBS/1% TritonX-100 and blocking in 5% skim milk in PBS/1% TritonX-100 for 6 hours. Afterwards, these samples were incubated for 16 hour at 4°C with a 1:100 dilution of a goat anti-WGA antibody (preabsorbed with acetone powder from zebrafish larvae) in 5% skim milk in PBS/1% TritonX-100. After washing in PBS with 1% TritonX-100, the sections were incubated in a 1:100 dilution of an anti-goat IgG antibody conjugated to alkaline phosphatase (Vector Labs) for 16 hrs at 4°C. After several washes in PBS/1% TritonX-100, signals were developed via color reaction with 450 µg/ml NBT, 175 µg/ml BCIP and 5 mM levamisole (Sigma) in AP buffer. The color reaction was stopped by washing with PBS/1% TritonX-100 and fixation in 4% PFA/PBS for 20 min and then subjected to image recording.

For fluorogold application, zebrafish were anesthetized with 0.02% MS222 (Thomson and Joseph) in 30% Danieau's solution (0.12 mM MgSO4, 0.18 mM $Ca(NO_3)_2$, 0.21 mM KCl, 1.5 mM HEPES pH 7.2 and 17.4 mM NaCl). Then 0.2 µl, 4% fluorogold

(Biotium) in PBS was injected into one hemisphere of the telencephalon with a microneedle pulled from a glass capillary tube (GC100F-10) attached to a Hamilton syringe (701N). After recovery from anaesthesia, zebrafish were maintained in 0.0002% methylene blue (Merck) for 3 days to avoid bacterial infections. Subsequently, the specimens were sacrificed by an overdose of Tricaine and the brains were explanted and fixed in 4% PFA over night at RT. The fixed brains were embedded in paraffin after a series of ethanol and Xylene equilibration washes, and sectioned using a microtome. Paraffin sections (10 μm) were dewaxed in Xylene and mounted with Entellan New for detection of fluorogold by image recording.

Calcium imaging

For *in vivo* calcium imaging larvae were kept in 30% Danieau's solution containing 75 μM PTU from 1 dpf onwards to prevent pigmentation but yet to allow for performing OKR and OMR in a reproducible manner. tagRFP-T and fluctuating GCaMP5G fluorescence were monitored under a confocal microscope (TCS SP8, Leica Microsystems) simultaneously to observing animal behavior. To monitor the entire Purkinje cell layer, the pinhole was opened to an optical thickness of 31.5 μm for the analysis of behavior-related calcium imaging and to a thickness of 13.6 μm for the developmental analysis of Purkinje cell activities respectively. For high-speed recording images were acquired every 69 milliseconds, while conventional recording was performed every 275 milliseconds respectively. Subsequent ratiometric image analysis was performed using the ImageJ software (http://rsbweb.nih.gov/ij/).

Optogenetics

Fish for optogenetics combined with behavioral monitoring were kept in 30% Danieau's solution. When optogenetics were combined with calcium imaging, 75 μM PTU was added to the medium from 1 dpf onwards. A confocal microscope equipped with a resonance scanner (TCS SP8, Leica) was used to allow for image recording at high temporal resolution (75 milliseconds) of larval behaviors. Illumination of Arch was performed using a 561 nm laser, while ChR2 was illuminated using the 488 nm laser. To simultaneously record larval behaviors, illumination with a 458 nm laser (0.1%) was used for Arch experiments and a 633 nm laser (0.3%) was used for ChR2 experiments.

The PC layer could be visualized by monitoring the fluorescent protein fusion of the respective opsin (561 nm for Arch-tagRFP-T, and 488 nm for ChR2-Venus), which was used to set ROIs for targeting optogenetic illumination to specific cerebellar regions.

Stimulation of stereotypic behavior

A small projector (PK102 Pro, Optoma) was connected to a computer for playing various Matlab (Mathworks) /Psychtoolbox-3 (http://psychtoolbox.org/HomePage) -custom-programmed movies (Movie S1, for example). For example, to induce OKR the width of each displayed moving column was programmed in Matlab such that it appeared with equal width on a bended screen. The angle of the moving eyes or the coordinates of the tail movements were calculated and plotted using the Image J plug-in "Moment Calculator" (http://rsbweb.nih.gov/ij/plugins/moments.htm).

Statistical Analysis

Data were expressed as means ± standard deviation (SD). An ANOVA was used to test results for statistical significance. Post-hoc analysis using Bonferroni correction for multiple tests was used unless otherwise mentioned. Differences were considered significant with $p < 0.05$.

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SI Figure Legends

Figure S1. Information about PC specific enhancer and list of fish lines

(A) Schematic representation of the genomic localization of the 258 bp *car8* enhancer about 2kb upstream of the translational start (ATG) of zebrafish carbonic anhydrase VIII. (B) Fish lines are listed, in which transgenes are expressed under the control of bidirectional PC specific tandem enhancer elements. See also method section, fish lines. Abbrebviations: bp: base pairs, kbp: kilo base pairs, GI: globin intron, pA: polyA.

Figure S2. WGA expression in eurydendroid cells and absence of WGA expression in granule cells

(A) (A1) WGA expression detected by immunohistochemistry colocalizing with GFP fluorescence in eurydendroid cells in the cerebellum of 9 dpf double transgenic larvae Tg(*olig2*:EGFP) x Tg(FyntagRFP-T:PC:WGA). A yellow box in the upper row image (*olig2*:EGFP + WGA) indicates the area from which higher magnification recordings were performed that are shown in the lower row. While GFP localizes to both the nucleus and the cytosol, WGA signals are often only found in the cytosol. Thus some eurydendroid cells are outlined by WGA signals (see image of higher magnification, white square). This finding shows that WGA was transported in an anterograde manner. Thickness of optical section: 2.361 μm (upper images) and 1.068 μm (lower images). n = 4. (A2) WGA expression was absent from cerebellar granule cells as no co-localization of immunohistochemical detection of WGA expression and GFP fluorescence in the cerebellum of 9 dpf double transgenic larvae Tg(*gata1*:EGFP) x Tg(FyntagRFP-T:PC:WGA) could be observed. This shows that WGA was not transported in a retrograde manner. Thickness of optical section: 1.271 μm. n = 4. (B) WGA expression detected by immunohistochemistry colocalizing with GFP fluorescence in eurydendroid cells of 1 month old double transgenic Tg(*olig2*:EGFP) x Tg(FyntagRFP-T:PC:WGA) zebrafish (upper figures, arrows). WGA expression was excluded from GFP-expressing granule cells in the cerebellum of 1 month old double transgenic Tg(*gata1*:EGFP) x Tg(FyntagRFP-T:PC:WGA) zebrafish. These findings indicate an anterograde but not retrograde trans-neuronal transport of WGA in zebrafish. Red color in the merged figures represents the signal of WGA and green color represents that of the *olig2*:EGFP or *gata1*:EGFP expression; n = 3.

Figure S3. PC efferent mapping in the adult zebrafish brain (additional cross sections)

Cross sections of adult zebrafish brains from the transgenic strain Tg(FyntagRFP-T:PC:WGA) processed for detection of WGA expression by immunohistochemistry. Low and high magnification figures are aligned from the top-left (rostral) to the bottom-right (caudal) respectively. n = 10. See list of abbreviations for names of anatomical structures. A: anterior thalamic nucleus, AON: anterior octaval nucleus, CCe: corpus cerebelli, Ce: cerebellum, CM: mammillary body, CP: central posterior thalamic nucleus, Dc: central zone of dorsal telencephalic area, DIL: diffuse nucleus of the inferior lobe, DON: descending octaval nucleus, DP: dorsal posterior thalamic nucleus, DTN: dorsal tegmental nucleus, EW: Edinger-Westphal nucleus, GC: central gray, Hc: caudal zone of periventricular hypothalamus, Hd: dorsal zone of periventricular hypothalamus, IGL: internal granule cell layer, IO: inferior olive, LLF: lateral longitudinal fascicle = lateral lemniscus, LRN: lateral reticular nucleus, MLF: medial longitudinal fascicle, MON: medial octavolateralis nucleus, NLV: lateral valvular nucleus, NR: red nucleus, NIII: oculomotor nucleus, ON: octaval nucleus, PPa: parvocellular preoptic nucleus, anterior part, PPv: periventricular pretectal nucleus, ventral part, SO: secondary octaval population, SRF: superior reticular formation, TS: semicircular torus, TeO: optic tectum, TL: longitudinal torus, TPp: periventricular nucleus of posterior tuberculum, Val: valvular cerebellum, VL: ventrolateral thalamic nucleus, VM: ventromedial thalamic nucleus, VT: ventral thalamus, Vv: ventral nucleus of ventral telencephalic area, X: vagal nerve.

Figure S4. Controls for WGA immunostaining

As control, WGA immunohistochemistry was performed on cross sections of brains from adult transgenic Tg(FyntagRFP-T:PC:empty) zebrafish demonstrating the absence of unspecific staining throughout the brain. Anti-tagRFP-T staining is shown in the inset to control for staining procedures. n = 6.

Figure S5 PCs project via short axons to ECs nearby

Transgenic Tg(*olig2*:EGFP) embryos were injected with the expression construct tagRFP-T:PC:WGA to mark individual PCs in a mosaic manner. At 9 dpf larvae were processed by immunohistochemistry for WGA expression and analyzed for

neighborhood relations of PCs and GFP-expressing ECs. CLSM analysis showed that WGA-containing axons from individual PCs were very short usually contacting the nearest ECs. One of the longest PC axon observed is shown leaving the PC soma (white arrowhead) and contacting an EC at 8.04 μm distance (white arrow). Thickness of each optical section: 1.068 μm. n = 20 axons from 5 larvae. Average length: 1.91 +/- 2.79 (standard deviation) μm. The figure shows a representative view of the 3D reconstructed images.

Figure S6. Axon tracing of eurydendroid cells

Axons of PCs and ECs were traced by using cell type specific enhancers driving fluorescent protein expression. (A) The regulatory element of zebrafish *olig2* drives expression in cerebellar eurydendroid cells. Double transgenic Tg(*olig2*:DsRed) x Tg(5xUAS:FynVenus) zebrafish embryos were injected with an *olig2*:KalTA4 expression plasmid at the one-cell stage and analyzed for co-expression of FynVenus (yellow fluorescent protein Venus fused with membrane targeting signal of Fyn kinase) and DsRed at 5 dpf by CLSM. The white arrow indicates a FynVenus and DsRed double positive cell. Thickness of optical section: 1.271 μm. n = 5. (B) In order to trace individual axons of eurydendroid cells double transgenic Tg(FyntagRFP-T:PC:empty) x Tg(5xUAS:FynVenus) transgenic embryos were injected with the *olig2*:KalTA4 expression plasmid. At 5 dpf projections of individual ECs were traced by CLSM. ECs of the lateral cerebellum send long axons to the torus semicircularis (left images) or the hypothalamus (right images). Lower images show the respective confocal transverse sections counterstained by BODIPY630/650. Yellow signals of these axons are encircled by a red square and enlarged images are shown on the right side. Red: PCs, Yellow: FynVenus positive ECs. Arrowhead: eurydendroid somata, Arrow: axon terminals. n = 3 for each projection. See also Figure 2. (C) ECs in the rostro-medial cerebellum send long axons to the thalamus, the nucleus of the medial longitudinal fasciculus, the nucleus ruber or the reticular formation. Fluorescence emitted by overlaying dorsal PCs was eliminated by the analysis software for clarity of EC-derived fluorescent signal (LAS AF software, Leica). Red: PCs, Yellow: FynVenus positive ECs, See also Figure 2. Square: ROIs (see Figure 3).

Figure S7. Late development of the zebrafish cerebellum

(A) Comparison of fluorescent protein expression patterns in transgenic Tg(FyntagRFP-T:PC:empty) and Tg(*olig2*:GFP) zebrafish at different juvenile and adult stages by stereomicroscopy. The upper row of images shows PCs while the lower row displays ECs at different developmental stages indicated above. The red square image is a horizontal confocal section (thickness of optical section: 7.232 μm) of the upper image, which shows the caudal part of the adult cerebellum ("S"-like shape in this figure: encircled by white line). Note that the morphology of the PC layer changed and appeared to rearrange by rotation of the lateral domains to more posterior positions. Asterisks having the same color indicate the larval and adult position of the corresponding tissue. (B) This was further supported by injection of a FyntagRFP-T:PC:empty expression construct into wild type embryos followed by analyzing the position of red fluorescent PC domains in the lateral cerebellum which appeared in the caudal cerebellum (white arrow head) several weeks later (n = 3).

Figure S8. WGA immunostaining of zebrafish brains with mosaic expression of WGA only in the caudal cerebellar lobe or valvula cerebelli

Transient transgenic Tg(tagRFP-T:PC:WGA) zebrafish with red fluorescence only in the caudal cerebellar lobe (A) or valvula cerebelli (B) were raised to adulthood and processed for WGA expression by immunohistochemistry. (A) Fish expressing WGA only in the caudal cerebellum (white arrow) showed immunohistochemical WGA signals in the octaval nuclei (black arrow), but not in the thalamus or the medial longitudinal fasciculus. The cerebellar corpus is encircled by the red oval. n = 4. (B) Fish expressing WGA only in the valvula cerebelli (white arrow) also showed immunohistochemical WGA signals in the octaval nuclei (black arrow) but not in the thalamus or the medial longitudinal fasciculus. The cerebellar corpus is encircled by the red oval. n = 4.

Our WGA-mapping and axon tracing of ECs showed that the valvular cerebellum connects to the octaval nucleus on the ipsilateral side only, while the caudal part of the cerebellum projects axons to both the octaval nucleus on the ipsilateral (predominantly) and the contralateral side. This efferent organization is very close to that of mammals, where the anterior lobe of the cerebellar vermis projects to the octaval nucleus only ipsilaterally (10,11,12). The mammalian caudal vermis instead connects to both the ipsilateral and contralateral octaval nucleus via the fastigial nucleus (10,12). Thus the

valvular cerebellum of the zebrafish may correspond to the vermis of the anterior lobe in mammals, while the caudal zebrafish cerebellum may be equivalent to the mammalian caudal vermis. In addition, the lateral aspects of this caudal PC region in zebrafish connect directly and not via ECs to the respective ipsilateral octaval nuclei as does the mammalian flocculus connecting directly to each ipsilateral octaval nucleus without involving deep cerebellar nuclei (10,11). Thus the caudolateral PC region may well correspond to the mammalian flocculus. Furthermore, in zebrafish the restricted rostro-medial region of the PC layer projects to motor-related neuronal structures including the medial longitudinal fascicle, the red nucleus and the reticular formation. Thus, based on connectivity this part should be equivalent to the mammalian paravermis, which also connects to motor-related structures including the thalamus, the red nucleus and the reticular formation (10,12).

Figure S9. Stimulation of cerebellum-dependent behaviors on the stage of a CLSM
To stimulate cerebellum-dependent behaviors such as OKR or OMR a small projector was connected to a computer for displaying various MatLab/Psychtoolbox-3 programmed movies (Movie S1, for example). These movies were projected onto a circular white screen and displayed to the visual field of larvae mounted in low melting agarose and facing towards the screen. Note that the width of black and white stripes on the PC monitor was adjusted such that they appeared with the same width of stripes on the circular screen. During OKR, the trunk of the zebrafish was restrained by low-melting agarose (pink square) while the eyes were free to move. In contrast during OMR the head was restrained while the trunk was free to exert swimming movements.

Figure S10. Analysis of OKR-induced Ca^{2+}-activity and OMR-induced Ca^{2+}-activity in PCs at high temporal resolution
(A) (A1) Transgenic Tg(tagRFP-T:PC:GCaMP5G) larvae were stimulated to perform OKR, while changes of fluorescence intensity of the GECI GCaMP5G were monitored at high temporal resolution (69ms / frame, ca. 14.5 fps, left graphs). Elevation of neuronal activity was observed to occur in PCs of the caudal cerebellum simultaneously with the onset of saccade movements. (A2) The graphs on the left show an example of neuronal activities during leftward saccade. Note that the temporal resolution is different between A1 and A2. The ROIs and corresponding colors are the same as in

Figure 3. n = 5 larvae. The graph on the right shows quantitative data of the laterality. n = 14 events. (B) (B1) Transgenic Tg(tagRFP-T:PC:GCaMP5G) larvae were stimulated to perform OMR, while changes of fluorescence intensity of the GECI GCaMP5G were monitored at high temporal resolution (69 ms / frame, ca. 14.5 fps, graphs on the left). Elevation of neuronal activity was observed to occur in PCs of the rostro-medial cerebellum simultaneously with the initiation of swimming movements. (B2) The graphs on the left show an example of Ca^{2+}-activity in PCs during induction of right-oriented swimming movement (note in Figure 3C induction of left-oriented swimming is shown). Note that the temporal resolution is different between B1 and B2. The ROIs and corresponding colors are the same as in Figure 3. n = 5 larvae. The graph on the right shows quantitative data of the laterality. n = 16 events.

Figure S11. *In vivo* validation of PC optogenetics by GECIs

Signals of GECIs were recorded during irradiation of the entire PC layer or of focal ROIs by CLSM. The temporal resolution of recordings were 75, 275 and 1,500 milliseconds for each validation, with all settings showing successful manipulation of PC physiology by optogenetic illumination. In this figure we show the results of 275 milliseconds recording for Arch and 1,500 milliseconds recording for ChR2. This is because 1,500 milliseconds intervals allow for sequential illumination of 488 nm and 561 nm laser and thus we could avoid the effect of excitation laser (488 nm) on R-GECO1 signal during optogenetic activation. Therefore both graphs in 5A as well as graphs in 5B and 5C are displayed at different temporal resolution along the y-axis. (A) Illumination of the entire PC layer by CLSM reversibly suppressed (Tg(Arch-tagRFP-T:PC:GCaMPG5G) larva irradiated with 561 nm, upper row), or triggered (Tg(ChR2-Venus:PC:R-GECO1) larva irradiated with 488nm, lower row) neuronal activity of PCs. n = 5 larvae each. (B) ROI irradiation of the PC layer of Tg(Arch-tagRFP-T:PC:GCaMPG5G) larva by 561 nm laser allowed for restricted suppression within this selected ROI (see Fig. 3 for color-coding). These graphs indicate a representative example of left caudal PC layer irradiations. The orange line marks the actual illumination period and the bidirectional arrow indicates the suppression of neuronal activity in the selected ROI. n = 5 larvae. (C) ROI irradiation of the PC layer of Tg(ChR2-Venus:PC:R-GECO1) larva by 488 nm laser allowed for restricted activation of PC activity within a selected ROI. These graphs indicate a

representative example of left caudal PC layer irradiations. The blue line marks the actual illumination period and the bidirectional arrow indicates the activation of neuronal activity in the selected ROI. n = 5 larvae.

Figure S12. Optogenetic *in situ* manipulation of PC activity during OKR

(A) OKR-monitoring during optogenetic suppression of PCs in a Tg(Arch-tagRFP-T:PC:GCaMPG5G) larva by 561 nm laser. Dunnett's test was used for post-hoc analysis. (A1) Suppression of PCs in the entire cerebellum or bilateral caudal regions during OKR resulted in the reduction of saccade events, while illumination outside this caudal ROI did not affect saccade number. control1: illumination outside o fthe cerebellum (hindbrain) of Tg(Arch-tagRFP-T:PC:GCaMPG5G) larva, control2: illumination of the entire PC layer of Tg(tagRFP-T:PC:GCaMP5G) larva. n = 3 larvae, 6 trials each, one representative example is shown. (A2) The optogenetic impairment of saccades is fast and reversible. One example during right-directed OKR-movie presentation, saccades to the left and right caudal irradiation is shown. n = 3 larvae. The image center of each eye is calculated and the relative angle of the furthest point of the eye ridge from this center is plotted. (A3) Suppression of PCs in the left or right caudal PC layer induced a significant decrease of saccade events towards the right or the left respectively (an example is indicated by the red arrow). n = 3 larvae, 6 trials each, one representative example is shown. (B) OKR monitoring during optogenetic activation (488 nm) of PCs of a Tg(ChR2-Venus:PC:R-GECO1) larva. Activation of PCs in the left or right caudal PC layer resulted in a significant decrease of saccade events toward to the left or the right respectively (red arrows). control1: illumination outside of the cerebellum (hindbrain) of Tg(ChR2-Venus:PC:RGECO-1) larva, control2: illumination of the entire PC layer of Tg(tagRFP-T:PC:GCaMP5G) larva. n = 3 larvae, 6 trials each, one representative example is shown. Dunnett's test was used for post-hoc analysis.

Abbrebviations

A: anterior thalamic nucleus

AON: anterior octaval nucleus

Arch: Archaerhodopsin 3

BAC: bacterial artificial chromosome

CCe: corpus cerebelli

Ce: cerebellum

ChR2: Channelrhodopsin 2

CLSM: confocal laser scanning microscopy

CM: mammillary body

CP: central posterior thalamic nucleus

Dc: central zone of dorsal telencephalic area

DIL: diffuse nucleus of the inferior lobe

DON: descending octaval nucleus

DP: dorsal posterior thalamic nucleus

DTN: dorsal tegmental nucleus

EC: eurydendroid cells

EW: Edinger-Westphal nucleus

GC: central gray

GECI: genetically encoded calcium indicator

GI: globin intron

Hc: caudal zone of periventricular hypothalamus

Hd: dorsal zone of periventricular hypothalamus

IGL: internal granule cell layer

IO: inferior olive

LLF: lateral longitudinal fascicle = lateral lemniscus

LRN: lateral reticular nucleus

MLF: medial longitudinal fascicle

MON: medial octavolateralis nucleus

NLV: lateral valvular nucleus

NR: red nucleus

NIII: oculomotor nucleus

OKR: optokinetic response

ON: octaval nucleus

OMR: optomotor response

ORK: open reading frame

pA: polyA

PC: Purkinje cells/neurons

PFA: paraformaldehyde

PPa: parvocellular preoptic nucleus, anterior part

PPv: periventricular pretectal nucleus, ventral part

PTU: 1-phenyl 2-thiourea

ROI: region of interest

RM: rostro-medial cerebellum

SO: secondary octaval population

SRF: superior reticular formation

TS: semicircular torus

TeO: optic tectum

TL: longitudinal torus

TPp: periventricular nucleus of posterior tuberculum

Val: valvular cerebellum

VL: ventrolateral thalamic nucleus

VM: ventromedial thalamic nucleus

VT: ventral thalamus

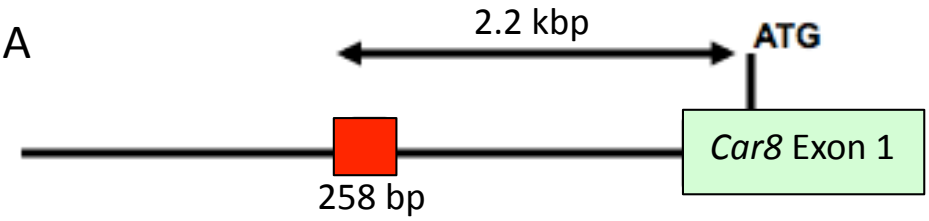
Vv: ventral nucleus of ventral telencephalic area

WGA: wheat germ agglutinin

X: vagal nerve

Figure S1

A


2.2 kbp
ATG
Car8 Exon 1
258 bp

B

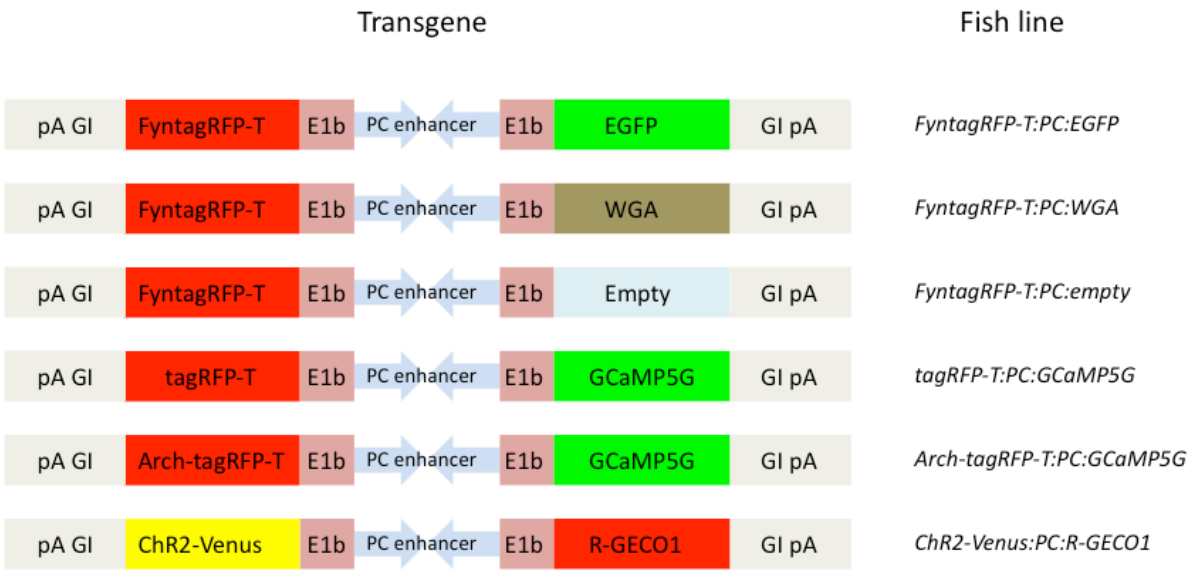

Transgene
Fish line
pA GI
FyntagRFP-T
E1b
PC enhancer
E1b
EGFP
GI pA
FyntagRFP-T:PC:EGFP
pA GI
FyntagRFP-T
E1b
PC enhancer
E1b
WGA
GI pA
FyntagRFP-T:PC:WGA
pA GI
FyntagRFP-T
E1b
PC enhancer
E1b
Empty
GI pA
FyntagRFP-T:PC:empty
pA GI
tagRFP-T
E1b
PC enhancer
E1b
GCaMP5G
GI pA
tagRFP-T:PC:GCaMP5G
pA GI
Arch-tagRFP-T
E1b
PC enhancer
E1b
GCaMP5G
GI pA
Arch-tagRFP-T:PC:GCaMP5G
pA GI
ChR2-Venus
E1b
PC enhancer
E1b
R-GECO1
GI pA
ChR2-Venus:PC:R-GECO1

Figure S2

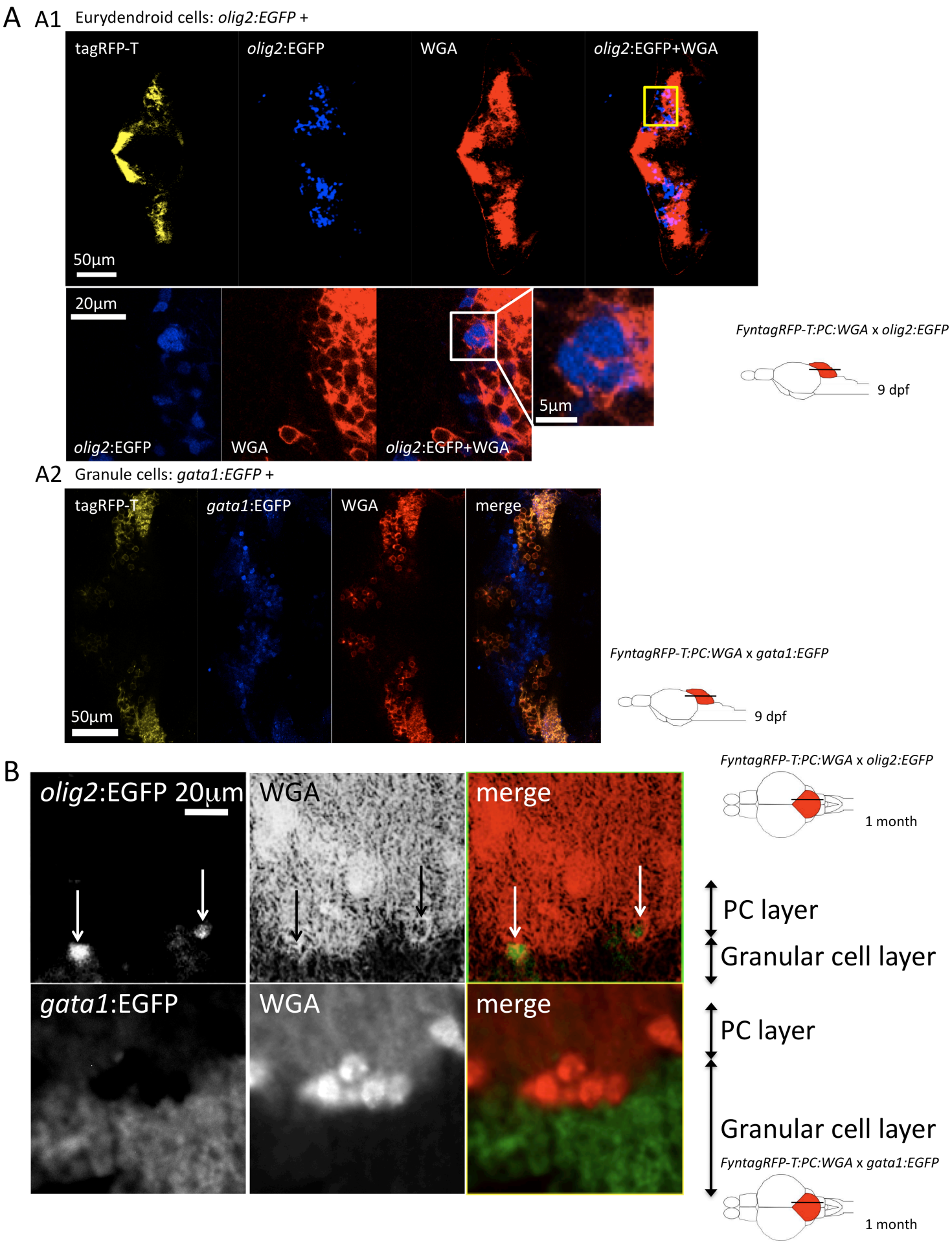

A
A1
Eurydendroid cells: olig2:EGFP +
tagRFP-T
olig2:EGFP
WGA
olig2:EGFP+WGA
50µm
20µm
olig2:EGFP
WGA
olig2:EGFP+WGA
5µm
FyntagRFP-T:PC:WGA x olig2:EGFP
9 dpf
A2
Granule cells: gata1:EGFP +
tagRFP-T
gata1:EGFP
WGA
merge
50µm
FyntagRFP-T:PC:WGA x gata1:EGFP
9 dpf
B
olig2:EGFP 20µm
WGA
merge
FyntagRFP-T:PC:WGA x olig2:EGFP
1 month
PC layer
Granular cell layer
gata1:EGFP
WGA
merge
PC layer
Granular cell layer
FyntagRFP-T:PC:WGA x gata1:EGFP
1 month

Figure S3

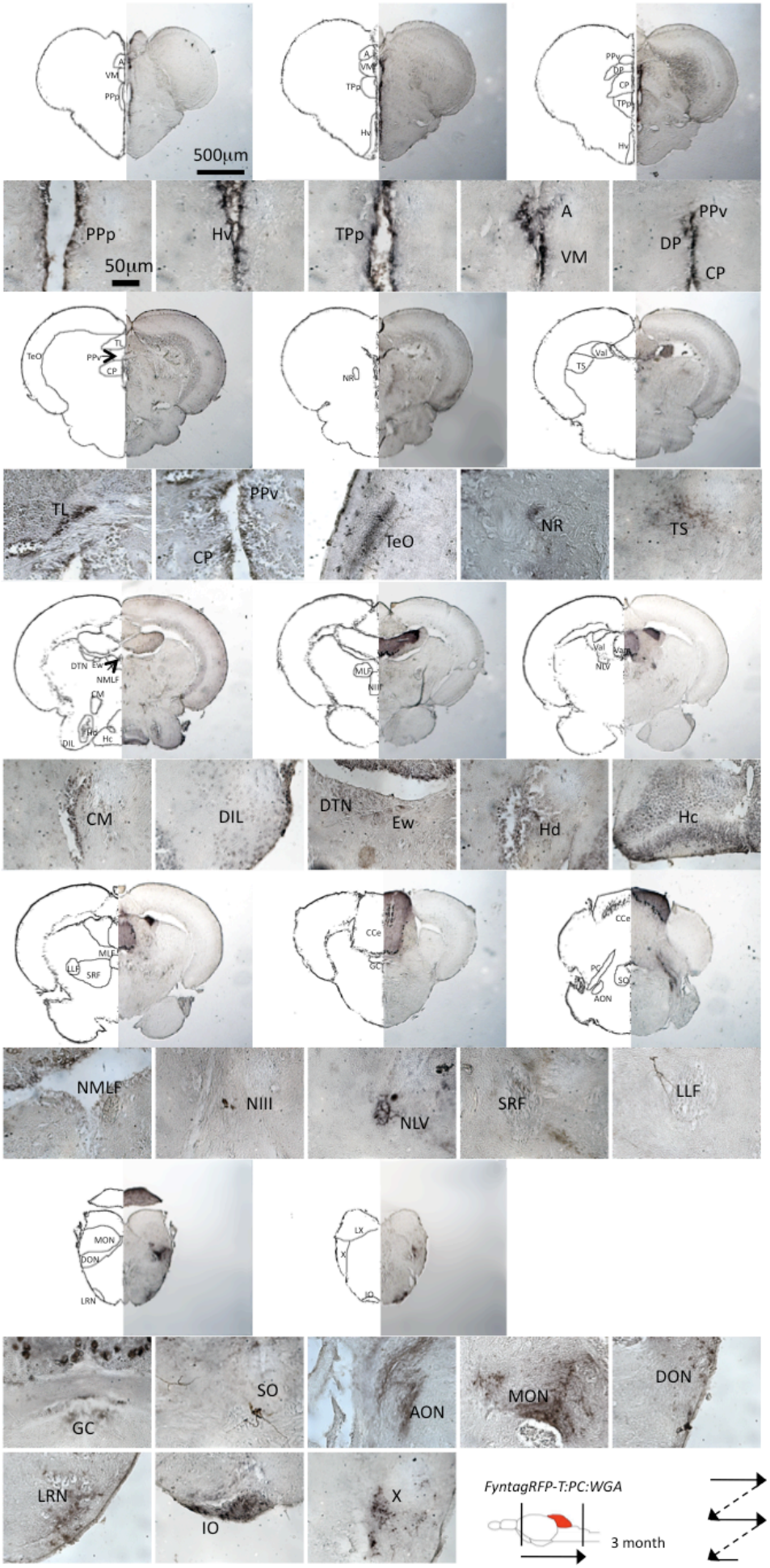
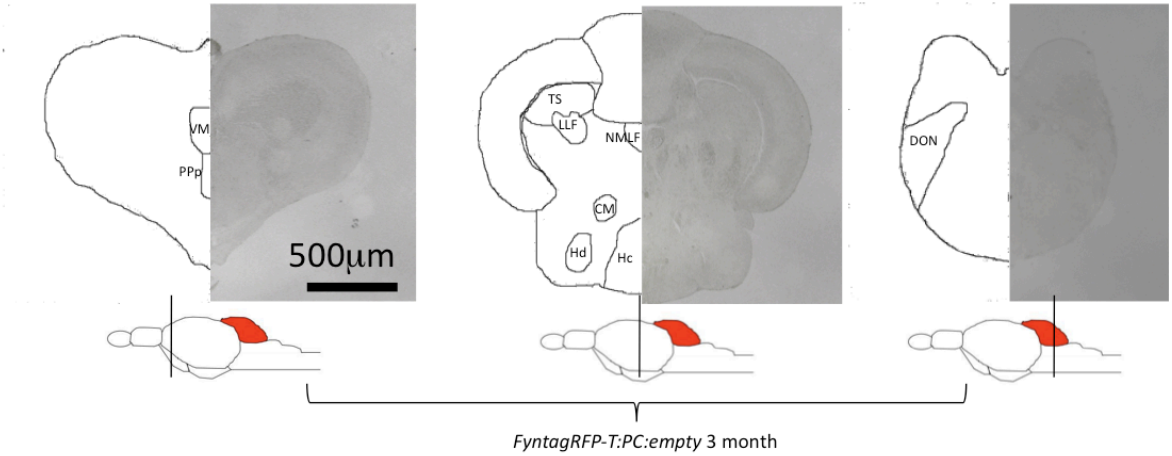

500µm
50µm
PPp
Hv
TPp
A
VM
PPv
DP
CP
TeO
TL
NR
Val
TS
DTN
Ew
NMLF
MLF
NIII
NLV
CM
DIL
Hd
Hc
CCe
GC
LLF
SRF
AON
SO
MON
DON
LRN
IO
LX
X
FyntagRFP-T:PC:WGA
3 month

Figure S4

Negative control



FyntagRFP-T:PC:empty 3 month

tagRFP-T

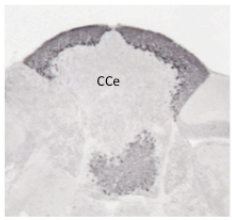


Figure S5

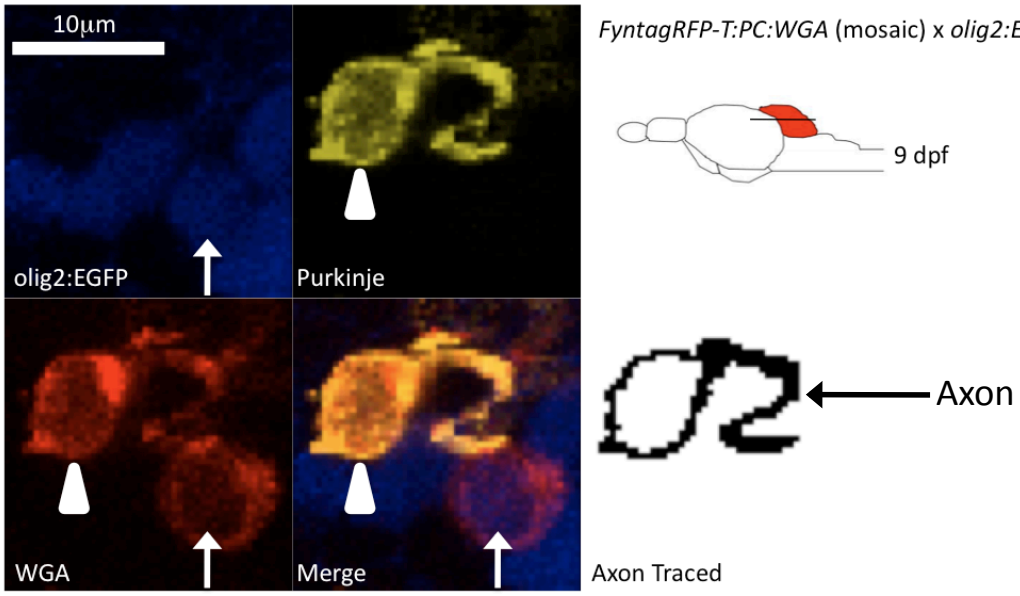
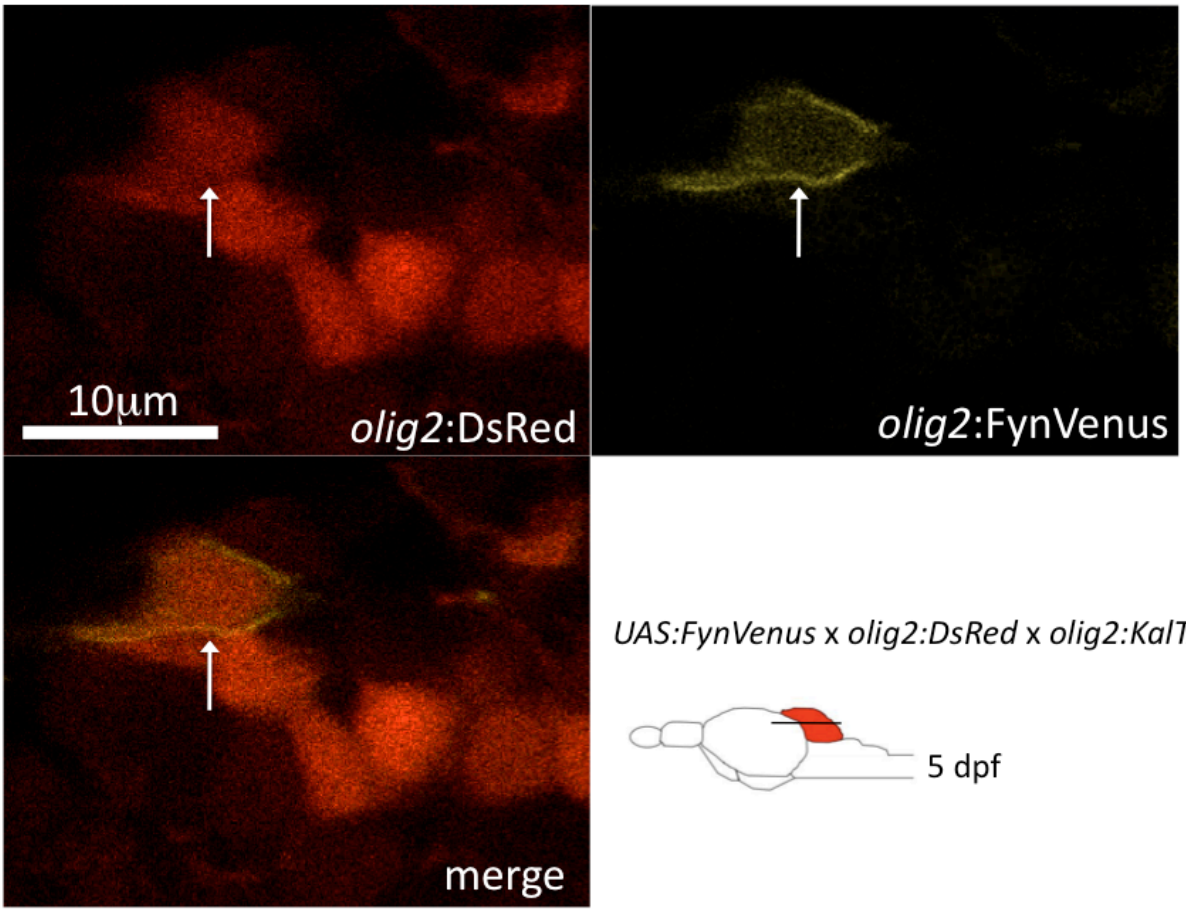
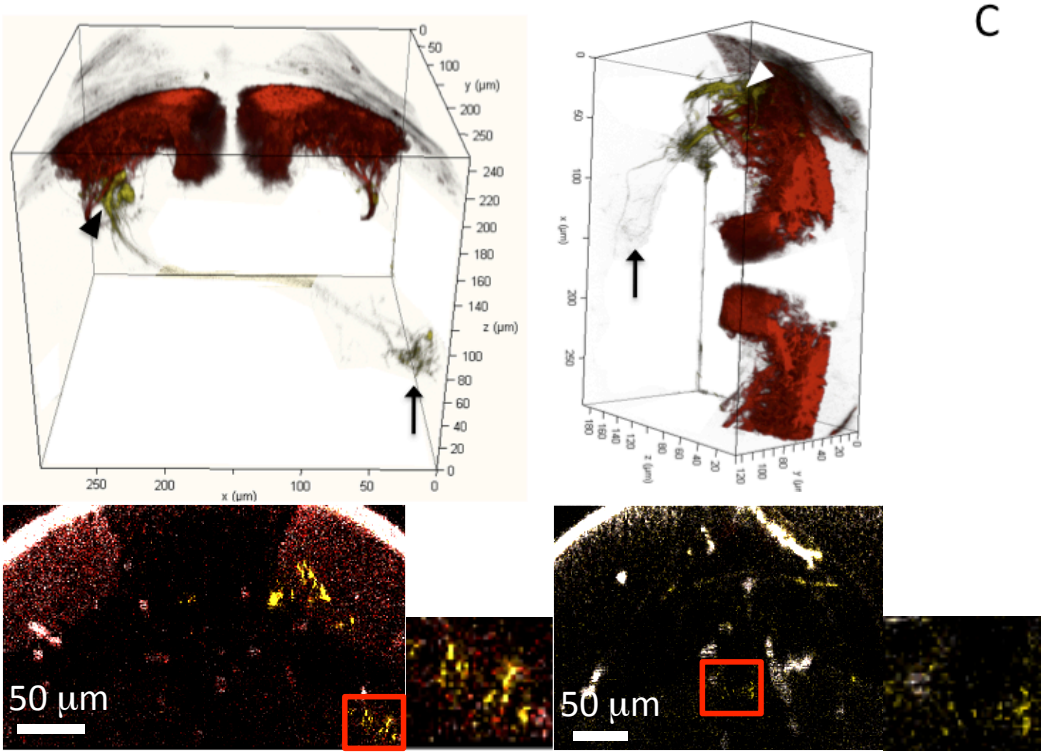

10μm
olig2:EGFP
Purkinje
WGA
Merge
FyntagRFP-T:PC:WGA (mosaic) x olig2:EGFP
9 dpf
Axon
Axon Traced

Figure S6

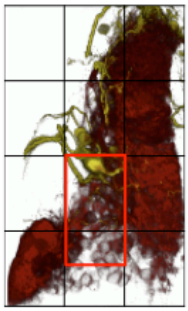
A



B



C



UAS:FynVenus x *FyntagRFP-T:PC:empty* x *olig2:KalTA4* (mosaic) 5 dpf

Figure S7

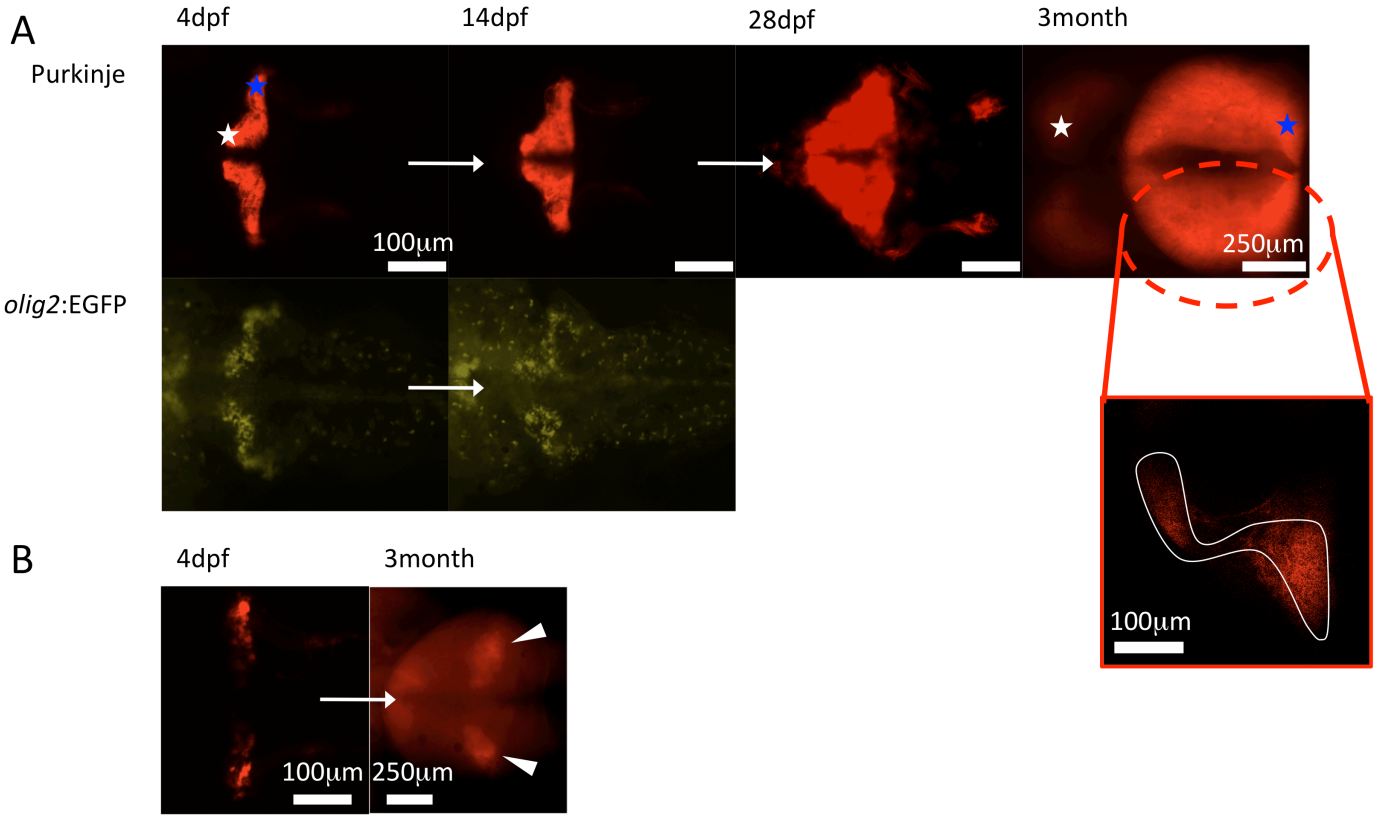
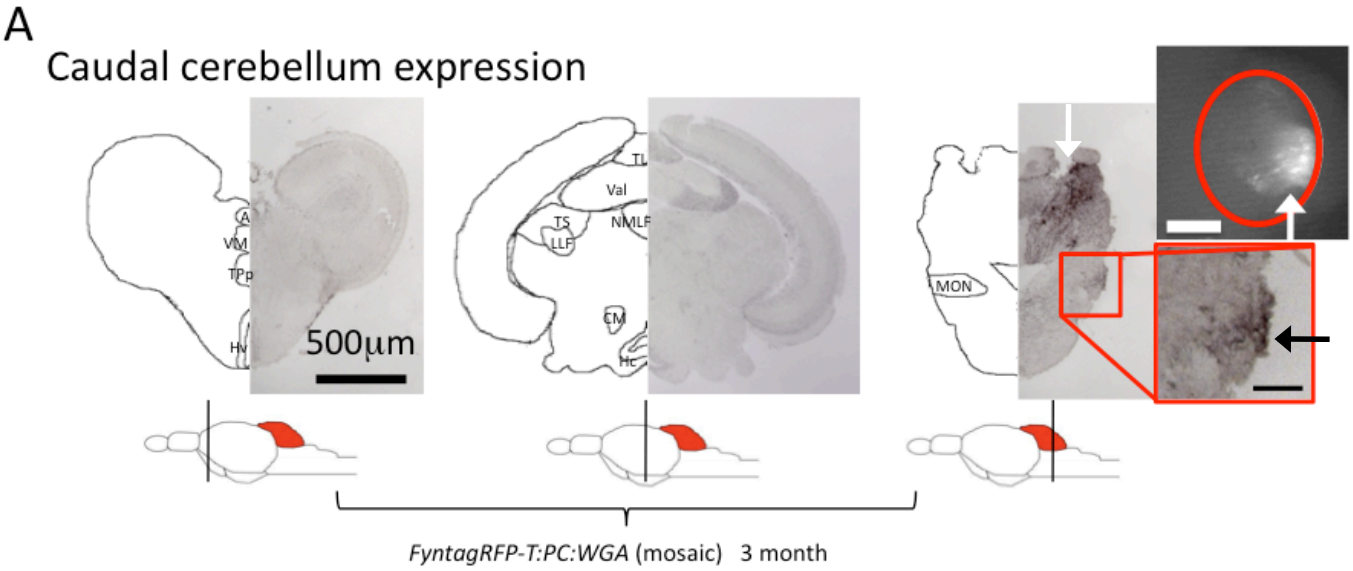

A
4dpf
14dpf
28dpf
3month
Purkinje
100μm
250μm
olig2:EGFP
B
4dpf
3month
100μm
250μm
100μm

Figure S8


A
Caudal cerebellum expression
500µm
TL
Val
TS
NMLF
LLF
CM
Hc
MON
FyntagRFP-T:PC:WGA (mosaic) 3 month

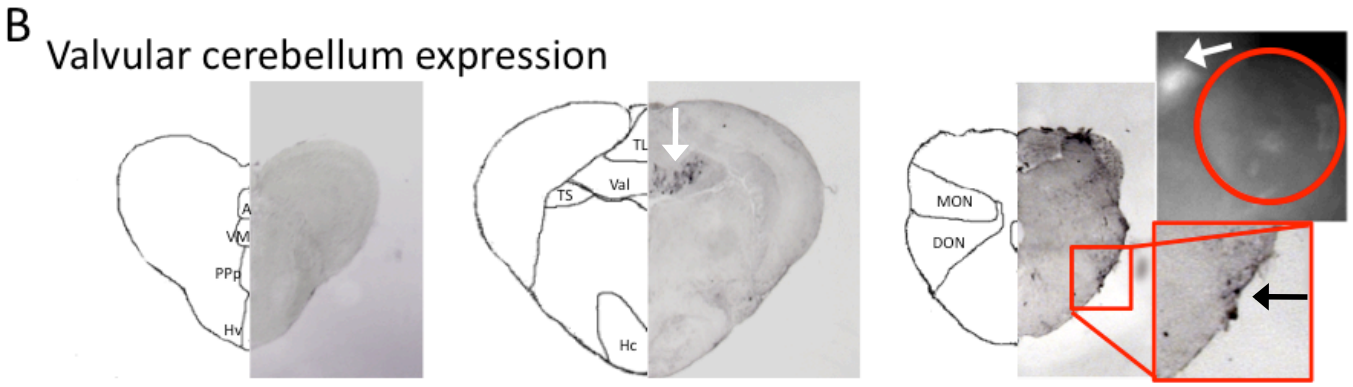

B
Valvular cerebellum expression
TL
Val
TS
Hc
MON
DON

Figure S9

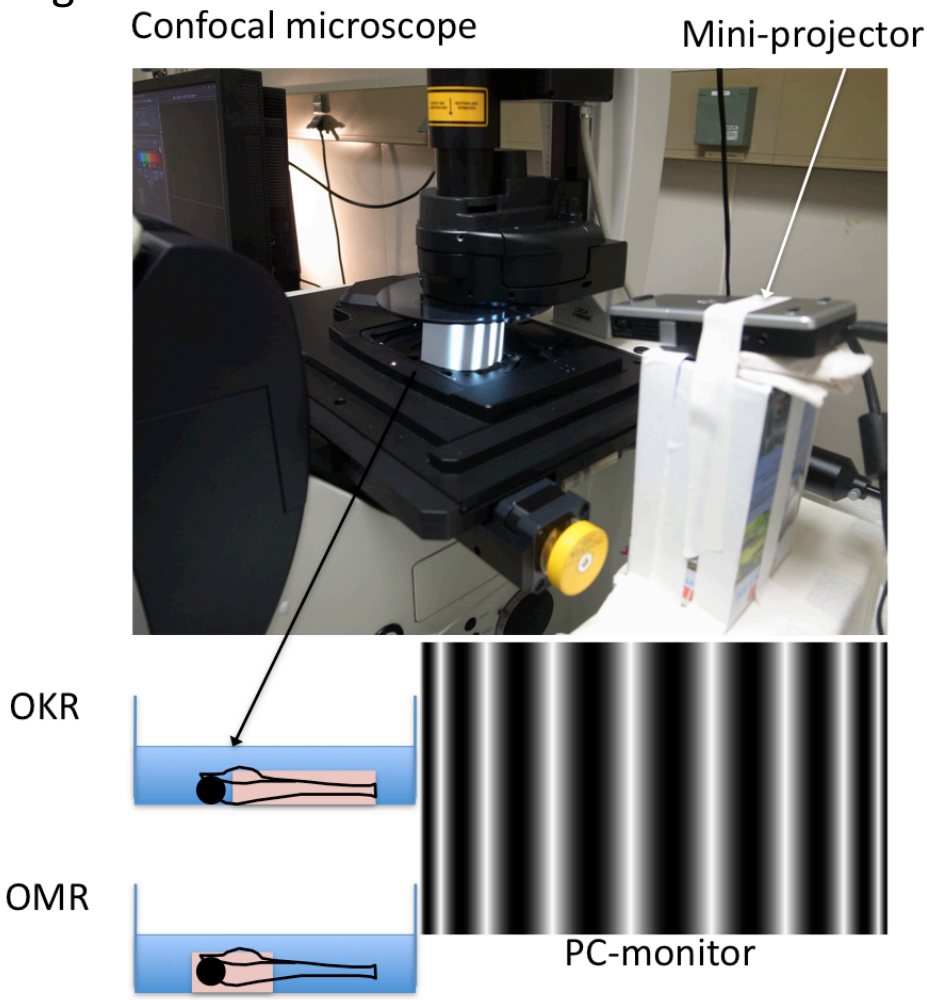


Figure S10

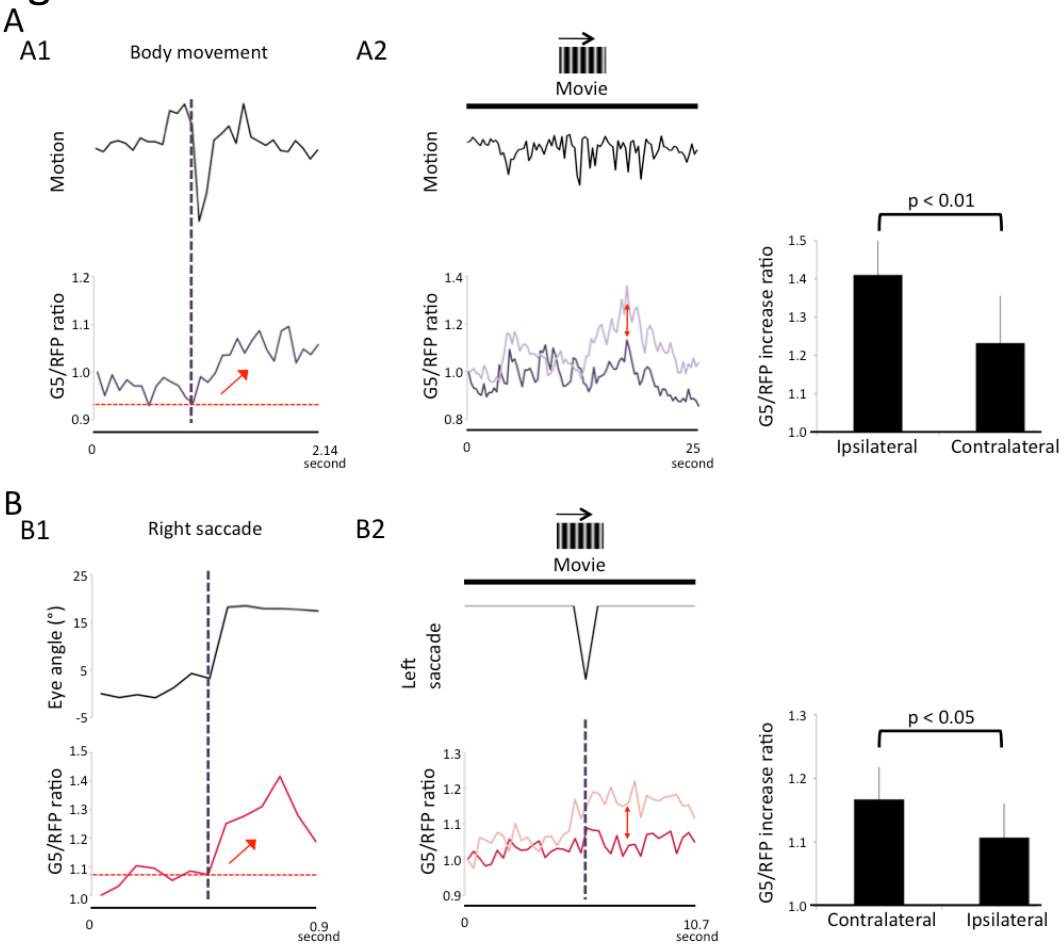

A
A1
Body movement
Motion
G5/RFP ratio
1.2
1.1
1.0
0.9
0
2.14
second
A2
Movie
Motion
G5/RFP ratio
1.4
1.2
1.0
0.8
0
25
second
p < 0.01
G5/RFP increase ratio
1.5
1.4
1.3
1.2
1.1
1.0
Ipsilateral
Contralateral
B
B1
Right saccade
Eye angle (°)
25
15
5
-5
G5/RFP ratio
1.5
1.4
1.3
1.2
1.1
1.0
0
0.9
second
B2
Movie
Left saccade
G5/RFP ratio
1.3
1.2
1.1
1.0
0.9
0
10.7
second
p < 0.05
G5/RFP increase ratio
1.3
1.2
1.1
1.0
Contralateral
Ipsilateral

Figure S11

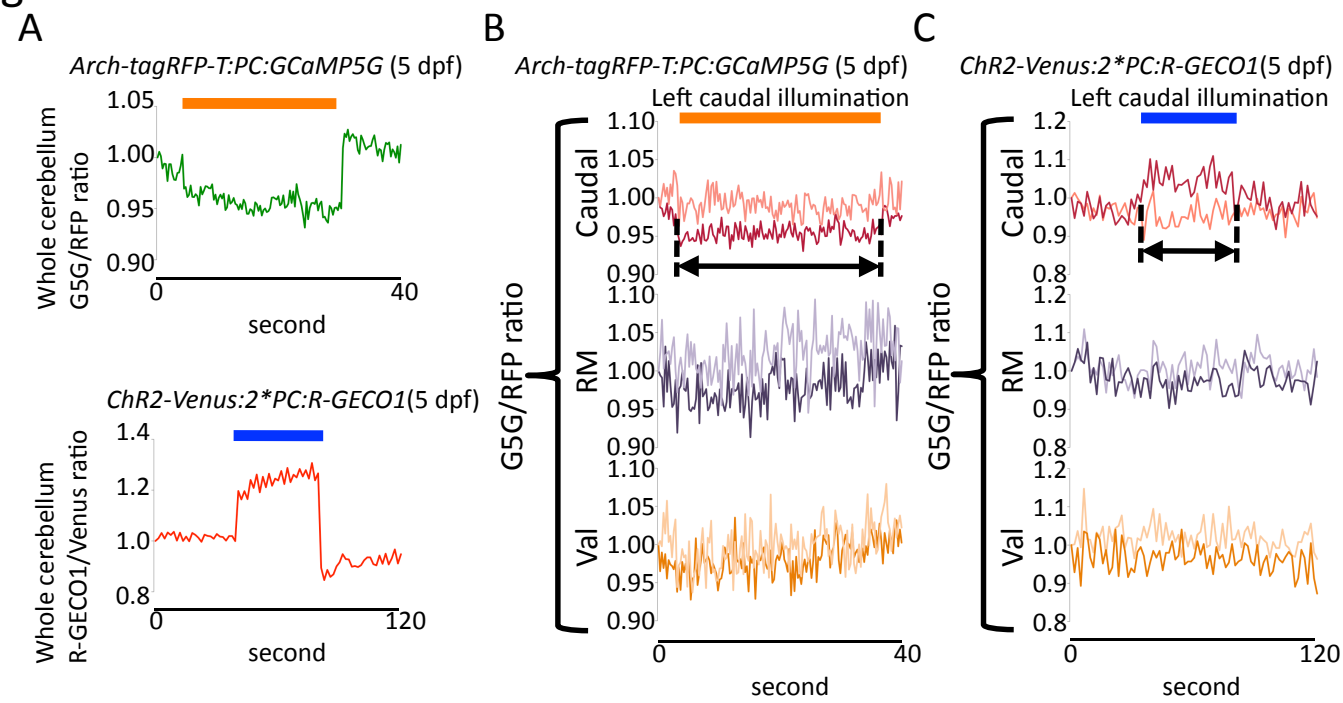
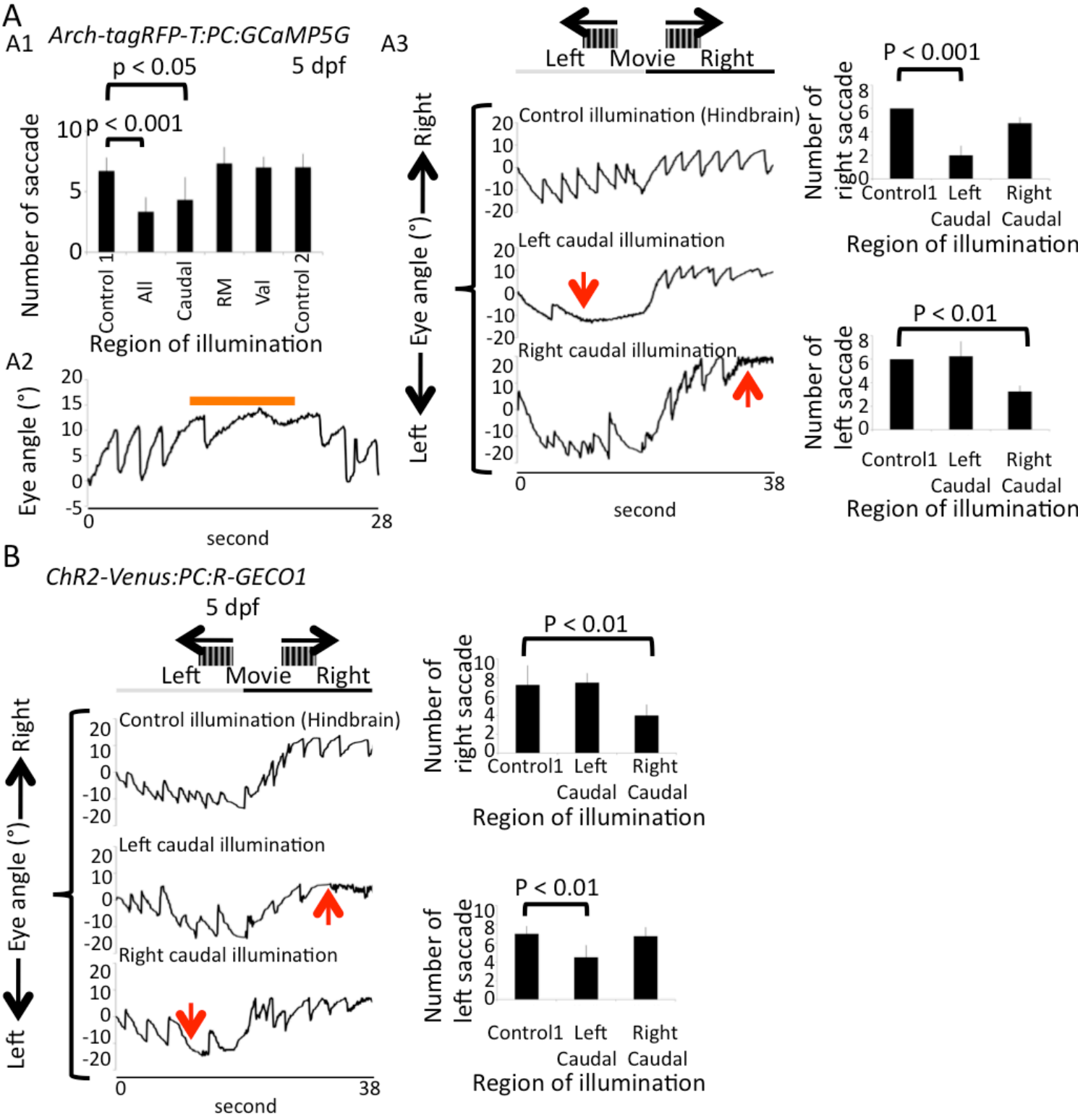

A
Arch-tagRFP-T:PC:GCaMP5G (5 dpf)
Whole cerebellum G5G/RFP ratio
1.05
1.00
0.95
0.90
0
40
second
ChR2-Venus:2*PC:R-GECO1(5 dpf)
Whole cerebellum R-GECO1/Venus ratio
1.4
1.2
1.0
0.8
0
120
second
B
Arch-tagRFP-T:PC:GCaMP5G (5 dpf)
Left caudal illumination
G5G/RFP ratio
Caudal
RM
Val
1.10
1.05
1.00
0.95
0.90
0
40
second
C
ChR2-Venus:2*PC:R-GECO1(5 dpf)
Left caudal illumination
G5G/RFP ratio
Caudal
RM
Val
1.2
1.1
1.0
0.9
0.8
0
120
second

Figure S12


A
A1
Arch-tagRFP-T:PC:GCaMP5G
5 dpf
p < 0.05
p < 0.001
Number of saccade
Control 1
All
Caudal
RM
Val
Control 2
Region of illumination
A2
Eye angle (°)
second
A3
Left Movie Right
Control illumination (Hindbrain)
Left caudal illumination
Right caudal illumination
Right
Left
Eye angle (°)
second
P < 0.001
Number of right saccade
Control1 Left Caudal Right Caudal
Region of illumination
P < 0.01
Number of left saccade
Control1 Left Caudal Right Caudal
Region of illumination
B
ChR2-Venus:PC:R-GECO1
5 dpf
Left Movie Right
Control illumination (Hindbrain)
Left caudal illumination
Right caudal illumination
Right
Left
Eye angle (°)
second
P < 0.01
Number of right saccade
Control1 Left Caudal Right Caudal
Region of illumination
P < 0.01
Number of left saccade
Control1 Left Caudal Right Caudal
Region of illumination