Supporting Online Material for

Filtering of Visual Information in the Tectum by an Identified Neural Circuit

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Supporting Online Material

Materials and Methods

Transgenic lines used

The following transgenic lines were used (designations according to official zebrafish nomenclature; previously published synonyms or abbreviated names are in parentheses): *Tg*(*UAS:GCaMP1.6*)*s1993t* (a.k.a. *UAS:GCaMP1.6*); *Tg*(*UAS:KillerRed*)*s1996t* (a. k. a. *UAS:KillerRed*)*; Tg*(*Atoh7:Gal4-VP16*)*s1992t* (a. k. a. *Atoh7:Gal4* or *Ath5:Gal4*)*; Et*(*-1.5hsp70l:Gal4-VP16)s1013t* (a. k. a. *Gal4s1013t*)*; Et*(*fos:Gal4-VP16*)*s1038t* (a. k. a. *Gal4s1038t*); *Et*(*-1.5hsp70l:Gal4-VP16*)*s1156t* (a. k. a. *Gal4s1156t*); *Tg*(*UAS-E1b:Kaede*)*s1999t* (a.k.a. *UAS:Kaede*), *Tg*(*5xUAS:TeTxLC-CFP*)*zf85* (a.k.a. *UAS:TeTxLC-CFP*).

Generation of transgenic lines

Zebrafish were maintained at 28.5 °C in the *Tüpfel long fin nacre* (*TLN*) genetic background. *Nacre* unpigmented larvae (*mitfa*$^{-/-}$) where used for all imaging experiments.

To generate the *UAS:GCaMP1.6* line, a fragment encoding GCaMP1.6 (*S1*) was cloned into the Tol2 vector *pT2KXIG* (a gift from K. Kawakami) containing a *14xUAS* site and a minimal promoter derived from heat shock cognate 70-kDa protein (*hsp*) promoter (600bp) (*S2*). To generate stable a line, wild-type *TLN* embryos were injected at the one-cell stage with a solution of 25 ng/ml DNA, 50 ng/μl transposase mRNA (prepared using the Ambion mMESSAGE mMACHINE T7 kit) and 0.04% Phenol Red. F1 embryos were pooled and screened for the transgene by crossing them with Gal4 carriers. F0 founder animals were then mated to wild-type TLN fish to create stable lines.

UAS:KillerRed fish were generated similarly, using KillerRed coding sequence (Evrogen JSC), but using *UAS-E1b* minimal promoter sequences (*S3*).

To generate the *Atoh7:Gal4* construct, *Gal4-VP16-SV40polyA* (*S3*) was amplified and subcloned between a *BamHI* fragment containing 7kb upstream of the *atoh7* gene and a *NotI/ApaI* fragment containing 5kb downstream of the *atoh7* gene (*S4*). To generate the *Tg*(*Atoh7:Gal4*)^{s1992t} line, plasmid DNA (25ng/μl) was injected in 1X I-SceI buffer containing 0.5 units/μl of I-SceI.

Mosaic labeling of tectal neurons

To label single SINs, *TLN* larvae where injected at 2-4 cell stages with DNA plasmids *UAS:PSD-95-GFP*; *UAS:DsRed-Express* (*S5*) and *Dlx5/6:Gal4 (S6)* at 25 ng/ml each. The *Dlx5/6:Gal4* construct drives the expression in a subset of tectal neurons and it represents a truncated version of the isolated *dlx5/6* enhancer and promoter sequence (E. R. and H. B., manuscript in preparation).

To generate embryos transiently expressing GCaMP3 in single or few PVNs in the tectum, TLN larvae where injected at the 2-4 cell stages with DNA plasmids *UAS:GCaMP3 (S7)* and a full length version of *Dlx5/6:Gal4 (S6)* at 15 ng/μl each together with 50 ng/μl Tol2 transposase mRNA. After injection, embryos were raised until 5 days post fertilization and then mounted in low-melting agarose for imaging.

Local application of Bicuculline

For the pharmacological experiments, 6-7 dpf larvae were mounted upright in 2% low-melting agarose, and a window was cut in the agar on top of the larval midbrain. Using a sharp tungsten needle we cut the skin over the tectum midline. The skin was then gently pulled sideways to expose the tectum. Bicuculline was added at 40 μM final

concentration. To avoid a systemic distribution of the drug, larvae were imaged only very briefly after start of drug exposure (<10 min). Under these conditions, we did not observe effect of the drug on retinal ganglion cells. In contrast, direct injection of the drug into the eye cup disinhibited retinal ganglion cell discharges. Control Ca^{++} recording experiments were performed after bicuculline washout.

Surgical removal of the eye

Eye enucleation was performed on 48 hours post fertilization embryos. The embryos where mounted on their sides in low-melting agarose (1.5%) and anesthetized with 0.04% Tricaine solution. A small window was cut in the agar, on top of the eye to be removed, using a fine scalpel. The eye tissue was removed with a sharp tungsten needle until no retinal pigmented epithelium was left. Embryos were then released from the agar and let develop until 6-7 dpf before Ca^{++} imaging recordings.

Ca^{++} imaging

We performed imaging experiments on live zebrafish larvae (6-8 dpf) mounted in low-melting agarose (2%). The larvae were mounted upright in a custom-build cuvette with one eye up against a glass coverslip. GCaMP1.6 imaging was performed on the contralateral tectum in a medial position in the dorso-ventral access with a Zeiss LIVE Laser Scanning Microscope equipped with a 63X (NA 1.0) water immersion objective. An Argon 488nm laser was used at minimal laser power (0.5-3%) to avoid GCaMP photo-bleaching and stimulating the visual system of the animal. Under these conditions, we sometimes observed a visual response at the onset of the scanning laser illumination that quickly disappeared, probably due to habituation of the fish to the illumination. We routinely discarded the first 4 sec of our recordings and considered responses to stimuli

only thereafter. Most images were acquired at 10 Hz and contained 256x256 pixels. In some cases, images were acquired at 4 Hz and contained 512x512 pixels.

A wide LCD screen synchronized with the acquisition was used for visual stimulation and positioned in front of the glass edge of the cuvette. Black moving bars or a full-screen flash were used as stimuli. We synchronized visual stimulation and data acquisition in the LIVE system. The visual artifact was apparent in the recording but easily filtered out temporally.

Image analysis

Data analysis was performed using custom-made routines written in Matlab. The measured fluorescence intensities were converted to relative changes in fluorescence intensity (ΔF/F) in each pixel and frame. The baseline fluorescence intensity, F, was estimated by averaging of five frames before stimulus onset. Single exponential correction for decrease in the GCaMP fluorescence (entering the dark state) was applied. Trials with motion artifacts were discarded. ROIs in the superficial and deep tectum were selected automatically based on previously published anatomical data (*S8*). A minimum of twelve trials per condition was average to produce the final response for a given stimulus.

For definition of superficial and deep ROIs, the shape of the tectum was automatically split in three portions using a custom-built script based on Bezier functions. Based on previous anatomical observations (*S9*), the “superficial layer” was defined as the most superficial third of the neuropil, while the “deep layer” was the deepest third. Values of the peak ΔF/F responses were compared using *t*-test with a cutoff of $p < 0.05$. The ratios were calculated by dividing the peak-values for the superficial layer to the deep

layer, and averaged across trials. The values of the ratios were compared to a normal distribution around one, in different conditions.

Measurements of optomotor responses

Behavioral assays were performed as previously described (*S10*). Ten larvae were placed in custom-built acrylic tanks, or "racetracks," which allowed the larvae to swim in only two directions. Images were captured before and after each stimulus. These images were analyzed using custom-written scripts in Matlab. The position of each larva was determined and measured in respect to the target position toward which the larvae were driven by the moving bars. The experiment was repeated over two consecutive set of stimuli moving in opposite directions.

Measurements of prey capture

Prey capture assay where essentially performed and scored as previously described (*S11*). Ten larvae where assayed for each experimental point. Between 30 and 60 *Paramecium multimicronucleatum* where added per dish, along with a single larva**.** The number of remaining paramecia was determined at hourly intervals for 3 hours.

Photo-ablation of SINs with KillerRed

Light-induced cell ablation was performed by using a green laser (563nm) at maximum power on a small region where cells were expressing the protein KillerRed (*S12*). Monitoring the photobleaching of the KillerRed fluorescence, followed by TUNEL and Annexin V staining, enabled us to define optimal conditions for photo-induced killing without toxicity in the controls (non-expressor larvae). The duration of the illumination (5-10min) corresponded typically to twice the duration for the complete photobleaching of the KillerRed.

Immunohistochemistry, *in situ* hybridization, and apoptosis detection

Embryos at appropriate stages were fixed in 4% PFA in PBS and processed for immunohistochemistry according to published protocols (*S9*). The following primary antibodies and concentrations were used for whole-mount immunohistochemistry: antibody to GFP (chick anti-GFP) 1:500; antibody to Reelin (mouse anti-Reelin 40-189) 1:500; antibody to GABA (rabbit anti-GABA) 1:2,000. GABA immunostaining required fixation in 0.1% glutaraldehyde/4%PFA in PBS. Secondary antibodies conjugated to Alexa-488 or Alexa-555 were selected accordingly and used at 1:500 dilutions. Nuclei were counterstained with DAPI. Whole-mount in situ hybridization was performed according to standard protocols using *gad67* riboprobe as described (*S13*) and larvae were then sectioned using a vibratome (20 µm thickness). Apoptosis was detected by whole-mount TUNEL assay using the ApoTag kit and NBT/BCIP substrate, or by in vivo staining with Annexin V-Alexa488 (*S14*).

Supplemental References

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SUPPLEMENTARY FIGURES

Fig. S1. Imaging setup and Ca^{++} responses after removal of the eye.

(**A**) Anatomical subdivisions of the larval zebrafish tectum. Neuropil is shown in dark green, PVN cell bodies as black ovals in light green layer. (**B**) Schematic representation of dorsal view of a zebrafish larva with the tectum highlighted in green. To confirm that fluorescent signals in the tectum reflect visually induced activity, one eye was removed at 48 hpf and the response to visual stimulation was recorded from each of the two tectal hemispheres at 5 dpf. The LCD screen, displaying the visual stimulus, was positioned in front of the contralateral eye relative to the tectum imaged. In the example shown, response in the left tectum was measured during visual stimulation of the right eye. (**C**) Representative responses to visual stimuli in a *Gal4s1038t*, *UAS:GCaMP1.6* larva, in the ipsilateral and contralateral tectum relative to the enucleated eye. (**D**) Maximum response average in tecta ipsilateral or contralateral to the enucleated eye. Responses to the stimulus are only seen in the tectum connected to the intact eye (ipsilateral). Grey bar indicates the time of visual stimulation. Error bars indicate s. e. m. Abbreviations:

SO, stratum opticum. SFGS, stratum fibrosum et griseum superficiale. SGC, stratum griseum centrale. SAC, stratum album centrale. LCD, liquid crystal display.

Fig. S2. Scatter plot of maximum ΔF/F measured in superficial *vs.* deep layers.

Data from *Gal4s1038t*, *UAS:GCaMP1.6* larvae (*n* = 7) in response to full-field flash show that responses were consistently higher in the superficial layers.

Fig. S3. GABAergic inhibition and filter selectivity: effect of bicuculline on size tuning.

The ratio of deep over superficial in *Gal4s1038t, UAS:GCaMP1.6* was estimated after Bicuculline application in response to a full flash screen, and moving black bars of different size (n=4 larvae). Note that the $ratio_{DEEP/SUP}$ is >1, when GABAergic inhibition is removed.

Fig. S4. SINs form a row of GABAergic cells in the superficial neuropil.

(A-C) DAPI nuclear staining (A) and immunodetection of GCaMP1.6 (GFP antibody) (B) and GABA (C) in the tectum of a *Gal4s1156t*, *UAS:GCaMP1.6* larva at 7 dpf. The white dashed line indicates the neuropil boundary. Scale bar is 50 µm.

Fig. S5. Reelin staining specifically labels SINs in the tectal neuropil.

(**A-D**) Confocal images of the tectum of 7 dpf *Gal4s1156t*, *UAS:GCaMP1.6* larva. Nuclei are counterstained in blue with DAPI (A). GCaMP1.6 immunodetection is shown in green (B), and REELIN immunoreactivity in red (C). White arrows indicate colocalization of REELIN and GCaMP1.6 signals in SINs in merged image (overlap shown as yellow) (D). The white dashed line indicates the neuropil boundary. Scale bar is 50 µm.

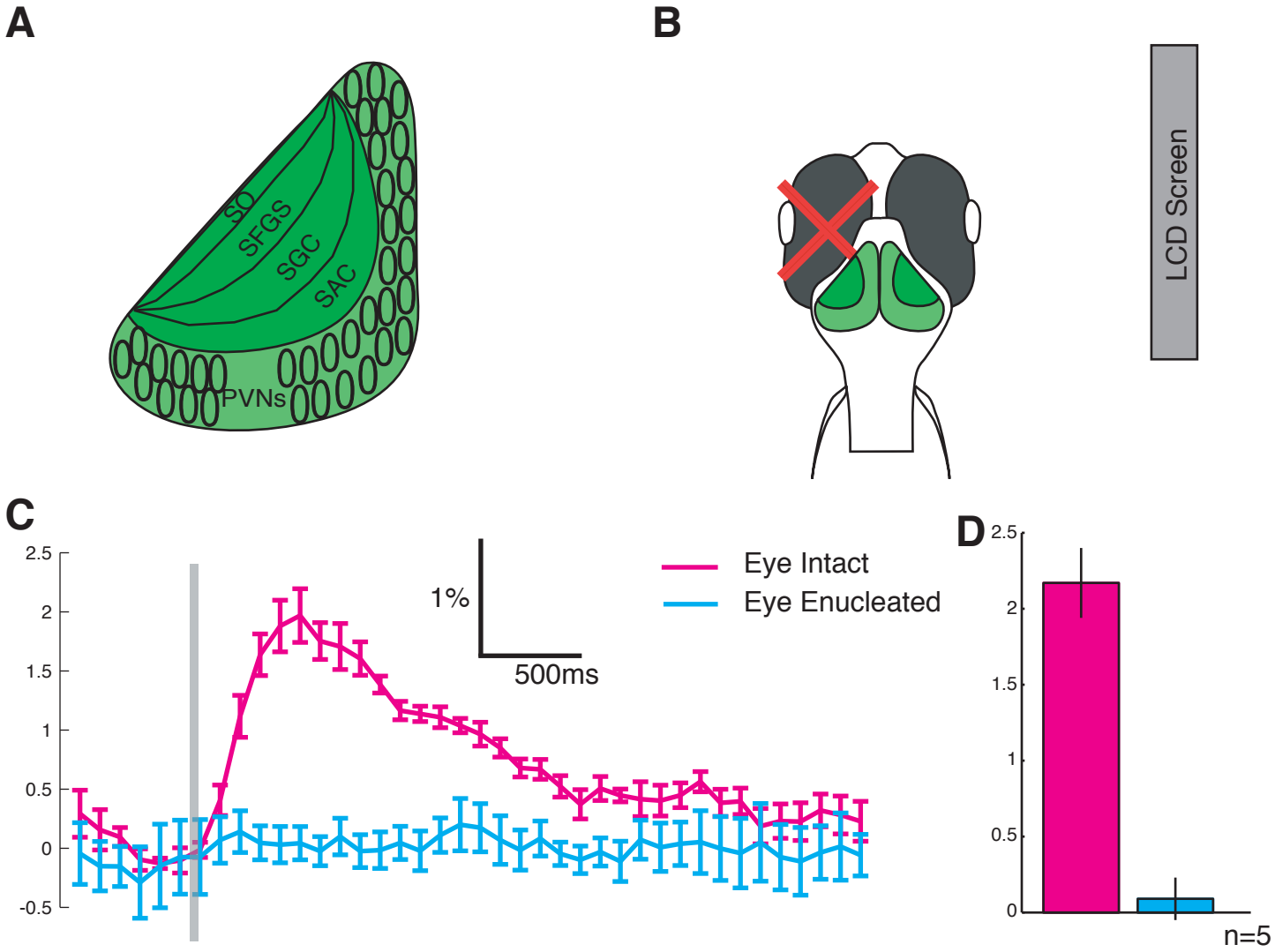
Fig. S6. Optomotor responses are unaffected by genetic silencing of selected tectal neuron subpopulations.

No significant differences in optomotor responses were detected between transgenic larvae expressing TeTxLC-CFP or Kaede in either posterior PVNs (*Gal4s1038t*) or SINs (*Gal4s1156t*), compared to wildtype (CTRL) siblings ($p>0.38$ in all two-way *t*-test comparisons).

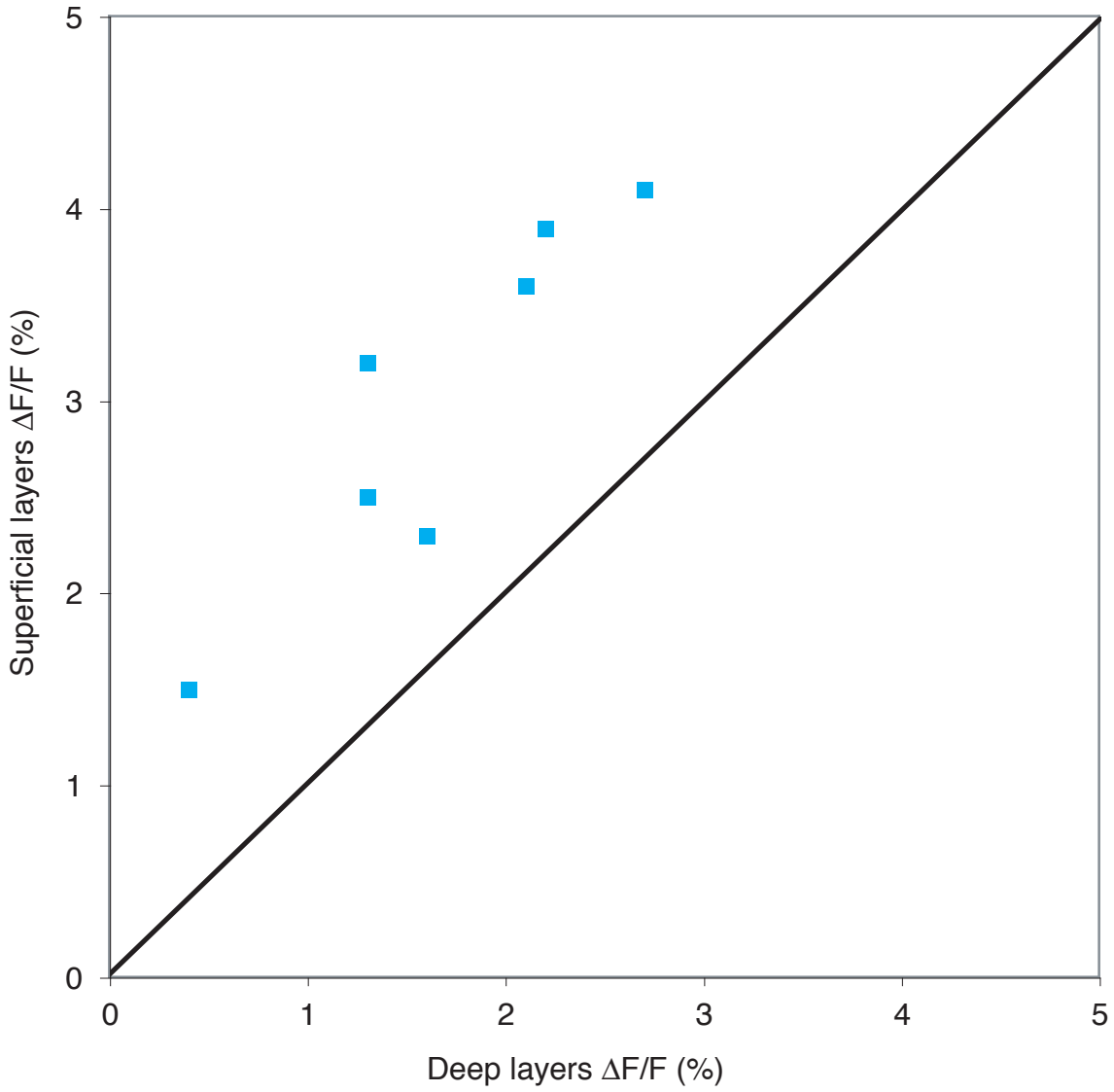
Fig. S7. Possible model of a feed-forward inhibitory circuit operating in the tectum.

Retinal afferents form excitatory synapses (blue) on the dendrites of both SIN and PVIN. The SIN forms an inhibitory synapse (red) onto the dendrite of the PVPN. **Left panel:** Presentation of a large stimulus activates a large number of retinal inputs (indicated by the vertical spikes), which leads to strong activation of the SIN (dark red). As a consequence of inhibitory transmission, the PVIN dendrite is hyperpolarized (red lines). This inhibition shunts direct retinal activation. The PVIN does not propagate the full signal to the PVPN in the deep neuropil layer. The PVPN spike rate is unchanged or increased only slightly (few vertical spikes). **Right panel:** Presentation of a small

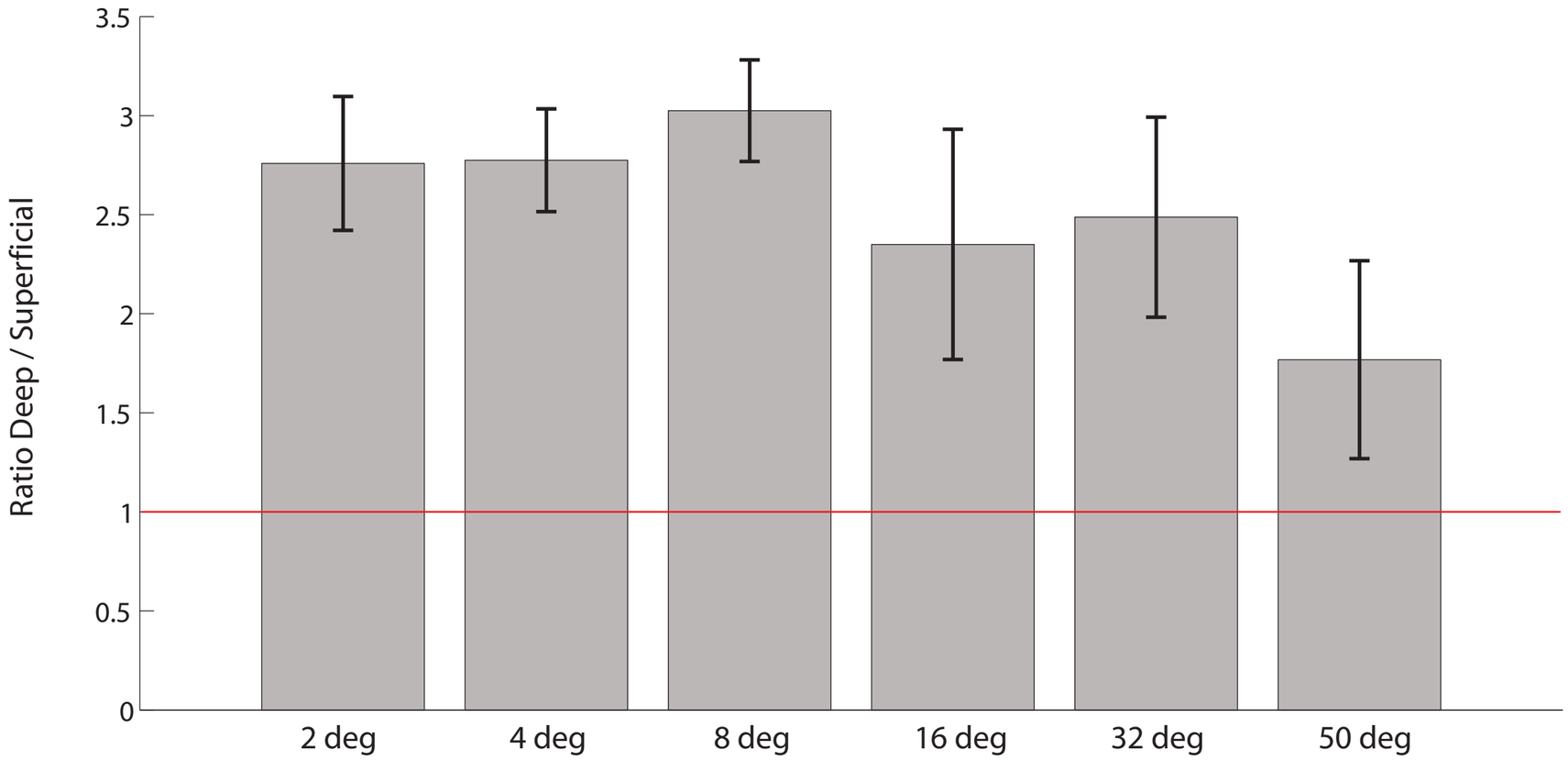
stimulus weakly activates the SIN neuron; only one of the retinal afferents is active (vertical spikes). Direct retinal activation of the PVIN now exceeds inhibition by the SIN. As a result, the PVIN becomes depolarized and activates the PVPN (many vertical spikes). Activity levels are indicated by color intensity. SIN, superficial inhibitory neuron; PVIN, periventricular interneuron; PVPN, periventricular projection neuron.



Del Bene et al. Supplementary Fig. 1



Del Bene et al. Supplementary Fig. 2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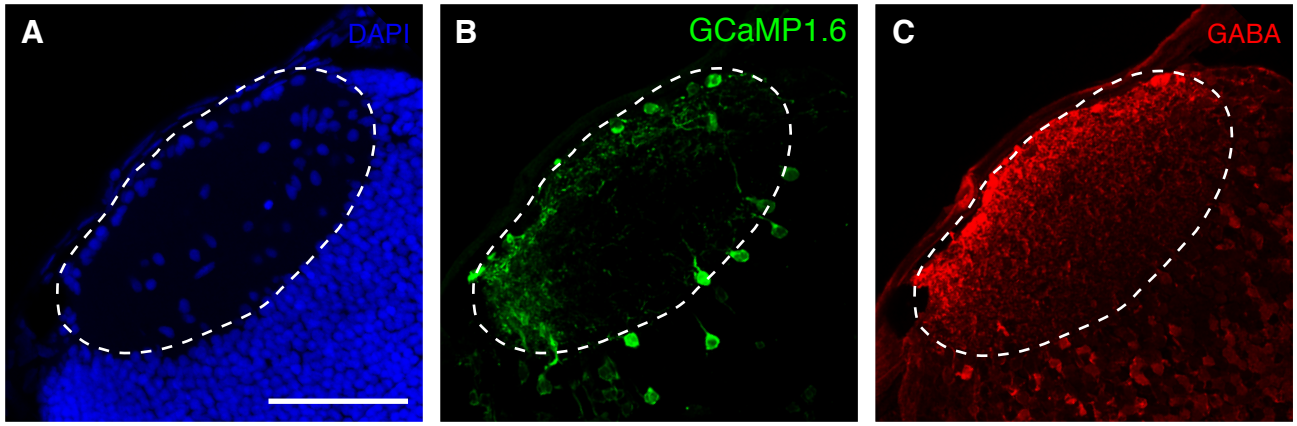
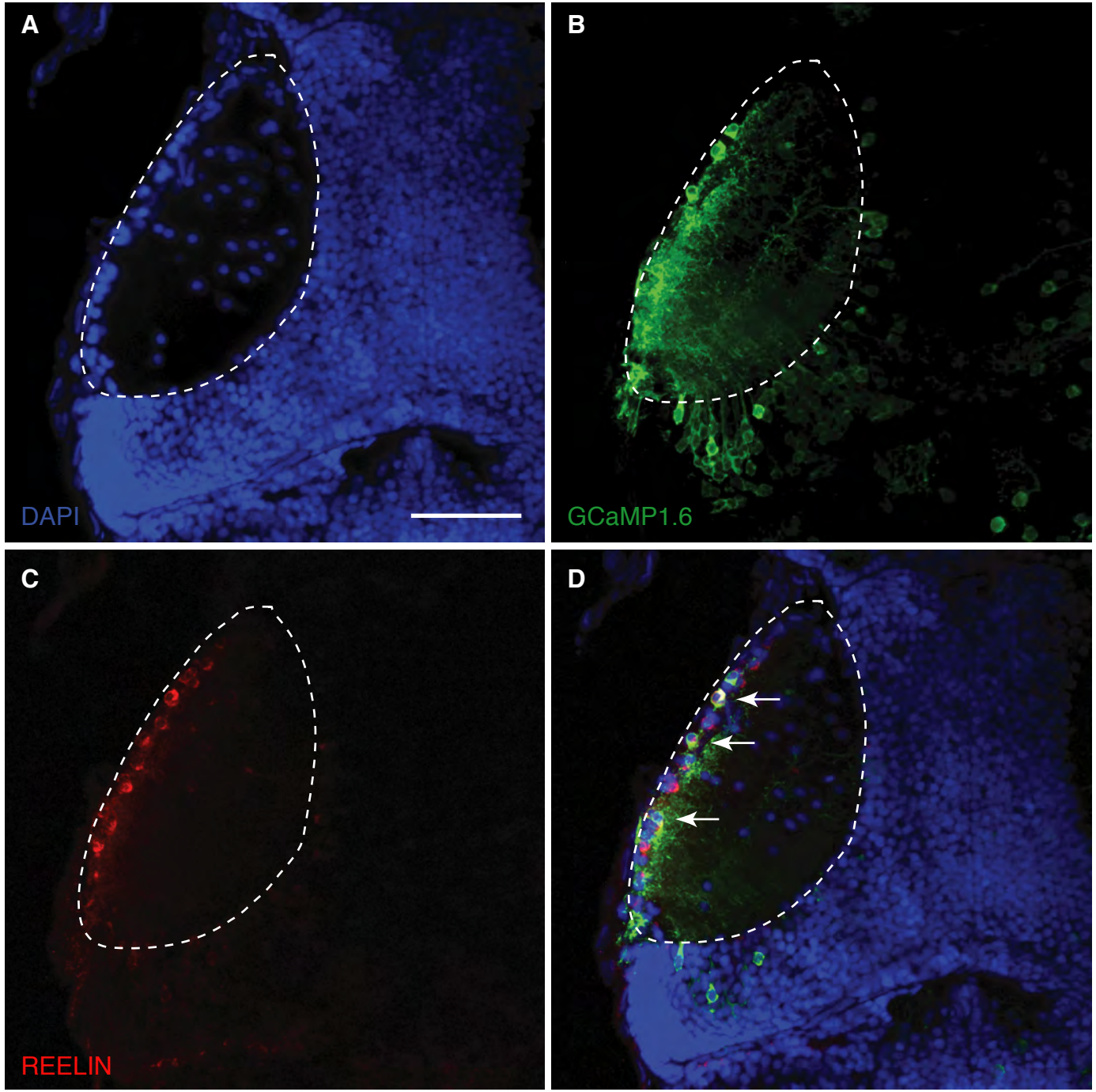
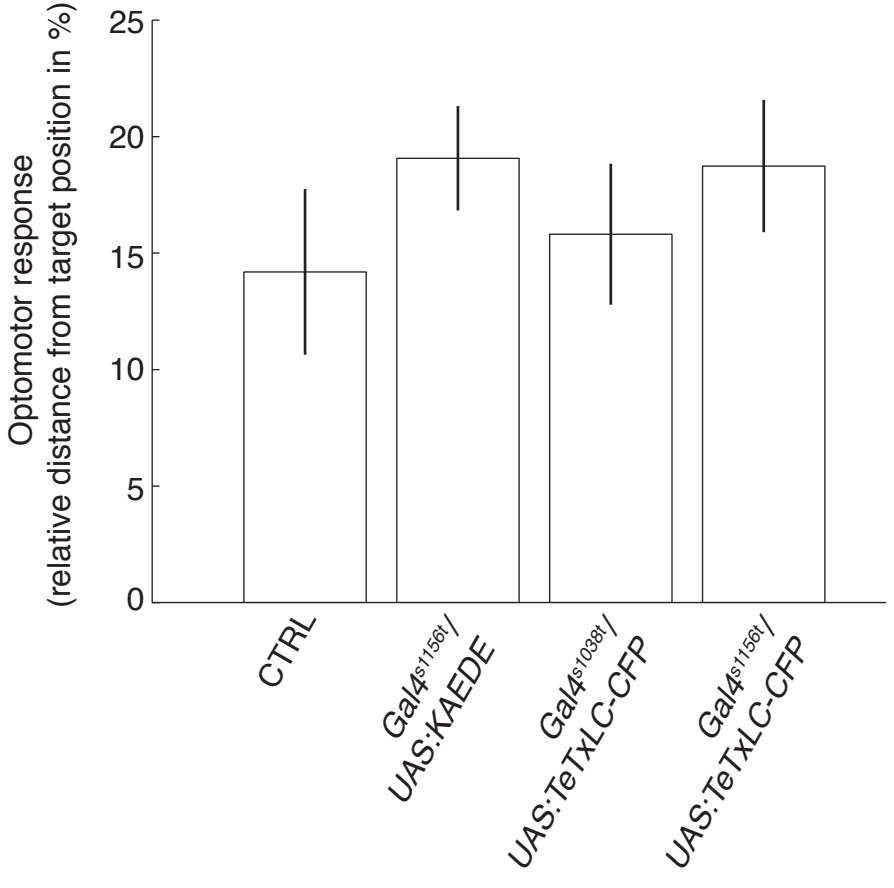


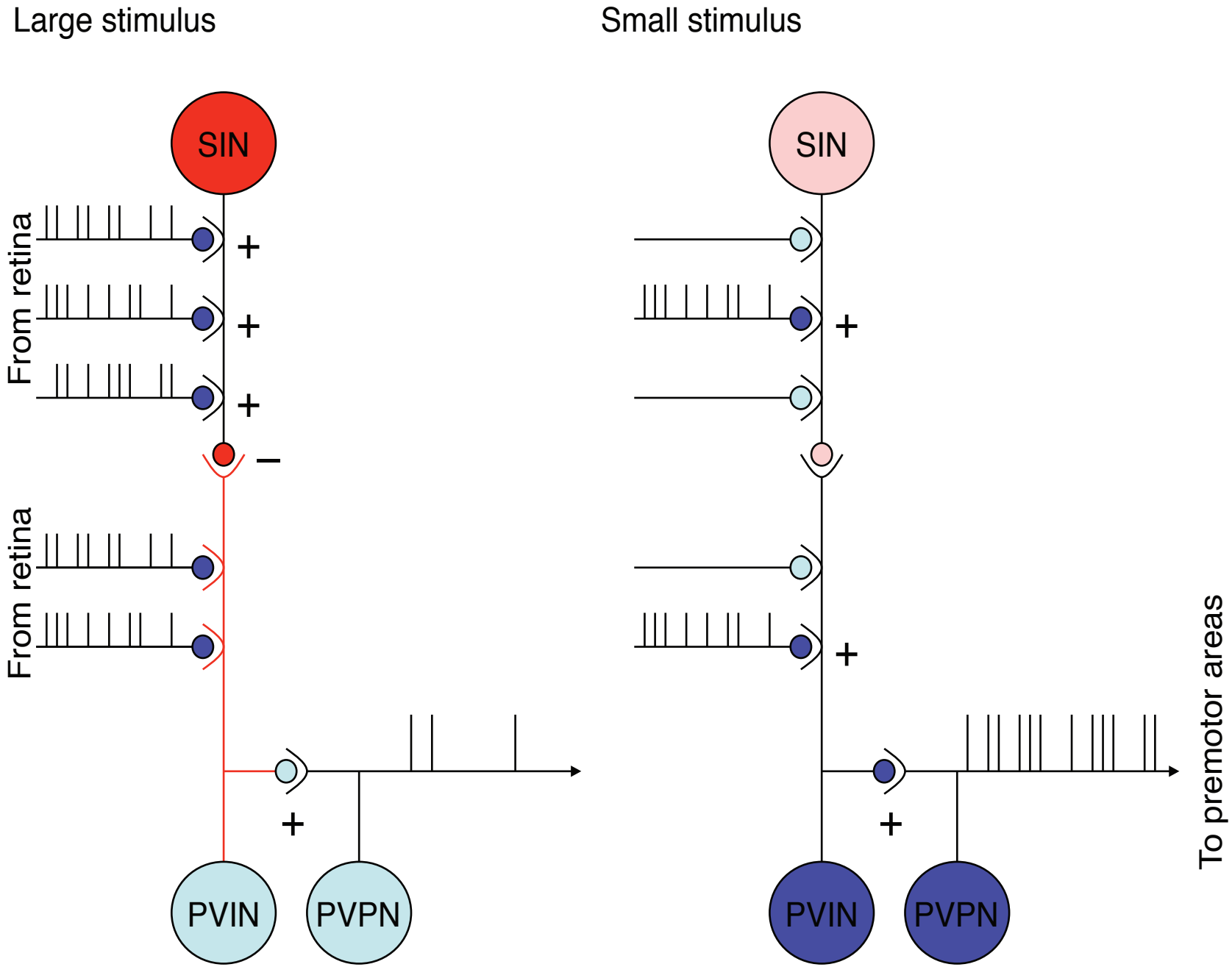
Del Bene et al. Supplementary Fig. S4



Del Bene et al. Supplementary Fig. S5

Del Bene Suppl. Figure S6





Del Bene et al. Supplementary Fig. S7