SUPPLEMENTARY MATERIAL & METHODS

Transgenic mouse lines

Vsx1-cerulean and *Grm6-tdTomato* lines were used to visualize and identify OFF and ON bipolar cells in mouse retina, respectively. In the *Grm6-tdTomato* line, the metabotropic glutamate receptor (mGluR6) promoter drives expression of the red fluorescent protein tdTomato in ON bipolar cells (1). In the *Vsx1-cerulean* line, the promoter for *Vsx1* (a homeobox gene required for the late differentiation and function of OFF bipolar cells) (2) was used to drive expression of a cyan fluorescent protein (cerulean) in OFF bipolar cells. A 3.3 kb PCR fragment with the 5' upstream genomic region of murine *Vsx1* was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) to obtain the *Vsx1* promoter. Cerulean was further cloned downstream of the *Vsx1* promoter. In one of the two *Vsx-Cerulean* lines sparse populations of OFF bipolar cells could be visualized. This was the line used for the study. To suppress inhibitory transmission in the retina, VIAAT conditional KO animals were obtained from Jackson Laboratory (*Slc32a1*^{tm1Lowl}/J) and crossed with the α*Pax6*-Cre line in which Cre recombinase expression is regulated by the *Pax6* promoter (3). These VIAAT KOs were further crossed to *Vsx1-cerulean* or *Grm6-tdTomato* line to label bipolar cells in the mutant background. To eliminate VIAAT specifically from horizontal cells, connexin57 (*Cx57*)-Cre mice were generated by homologous recombination between the protein-coding sequence of *Cx57* gene (*Gja10*) and an 'improved' Cre recombinase gene, such that specific expression occurs only in horizontal cells (generated and provided by Arlene Hirano, Jim Boulter and Nicholas Brecha, UCLA). To generate the horizontal cell VIAAT conditional KO animals, the *Cx57*-Cre mouse line was crossed with the floxed VIAAT line (*Slc32a1*^{tm1Lowl}/J).

To eliminate GABA$_A\gamma$2 from bipolar cells, the *GABA$_A\gamma$2* conditional KO mouse (B. Luscher) (4) was crossed with the *Grm6*-Cre line generated by cloning Cre into the mGluR6 promoter. Cre was PCR amplified from the pTurbo-Cre plasmid with 5' NotI and 3' PacI and NotI restriction

sites added. A linker containing SalI, AgeI, AscI and PacI restriction sites was cloned into the SalI site of mGluR6-pSKII+ to enable release of the *mGluR6*-Cre transgene. NotI digested Cre PCR product was cloned into NotI cut mGluR6-5' linker. The mGluR6-Cre transgene was released from the vector backbone with PacI and gel purified with QiaexII (Qiagen). The purified transgene was injected into the pronucleus of B6/CBA hybrids using standard methods. To label Cre expressing bipolar cells, *$GABA_A\gamma 2$* conditional KO/*Grm6*-Cre mice were further crossed into the Ai9 reporter (Jackson Laboratory, B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J) background such that Cre expressing cells are labeled with tdTomato.

Immunohistochemistry

All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of Washington and University of California, Los Angeles. Unless specified otherwise, all experiments were carried out on 4-6 week old animals. Animals were deeply euthanized with Isoflurane, decapitated and enucleated. Retinas were isolated in cold oxygenated mouse artificial cerebrospinal fluid (mACSF, pH 7.4) containing (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1 NaH_2PO_4, 11 glucose, and 20 HEPES. For vibratome sectioning, eye-cups were fixed for 15 mins in 4% paraformaldehyde in phosphate buffer (PBS), embedded in agarose (Sigma, low gelling temperature) and sectioned at 100 μm. For whole-mount immunoabeling, retinas were flattened on a filter paper (Millipore, HABP013) and fixed for 15 mins in 4% paraformaldehyde. After rinses in PBS, the retinas were pre-incubated in blocking solution containing 5% donkey serum and 0.5 % Triton X-100 and then incubated with primary antibodies over 3 nights at 4ºC. Secondary antibody incubation was carried out overnight in PBS using anti-isotypic Alexa Fluor (1:1000, Invitrogen) or DyLight conjugates (1:1000, Jackson Immunoresearch). Primary antibodies utilized in this study were: anti-VIAAT (rabbit polyclonal, 1:1000, Synaptic

Systems), anti-GFP (chicken polyclonal, 1:1000, Abcam), anti-Synaptotagmin-2 (mouse monoclonal, 1:1000, znp-1, Zebrafish International Resource Center), anti-glycine receptor α1-subunit (mouse monoclonal mAb2b, 1:500, Synaptic Systems), anti-$GABA_A\alpha$1 receptor subunit (polyclonal guinea-pig, 1:5000, kindly provided by J.M. Fritschy), anti-$GABA_A\alpha$3 receptor subunit (polyclonal guinea-pig, 1:3000, kindly provided by J.M. Fritschy), anti-$GABA_A\gamma$2 (rabbit polyclonal, 1:500, Abcam), anti-$GABA_C\rho$ receptor subunit (rabbit polyclonal, 1:500, kindly provided by R. Enz), anti-RFP (mouse monoclonal, 1:1000, Abcam), anti-DsRed (rabbit polyclonal, 1:1000, Clontech), anti-PKC (mouse monoclonal, 1:1000, Sigma) and anti-cone arrestin (rabbit polyclonal, 1:1000, Millipore).

Image acquisition and analysis

Samples were imaged using an Olympus FV 1000 laser scanning confocal microscope with a 1.35 NA 60X oil immersion objective, at a voxel size of 0.05-0.05-0.3 µm (x-y-z). Image stacks were further processed using MetaMorph (Molecular Devices) and Amira (FEI Visualization Sciences Group) software.

% Receptor occupancy was calculated by dividing the volume occupied by immunostained receptors by the total volume of the axon terminal or dendrite multiplied by 100. To estimate receptor volume occupancy in a bipolar process, the bipolar process was first masked in 3D using the Labelfield function in Amira. The axonal process was masked up to the point of the axonal shaft, whereas for the dendritic arbor the soma was excluded. The receptor signal within the bipolar process was then isolated using the Arithmetic function in Amira and a threshold applied to the receptor signal to eliminate background pixels and enable the detection of receptor pixels above the background. To select the threshold, all pixels in the receptor channel were plotted against the gray value and the curve fitted with a gamma function to estimate the value of the noise peak and the standard deviations from the peak

(Fig. S1F). Thereafter a threshold of 4 standard deviations above the noise peak was selected and applied to detect the volume of the receptor pixels in the bipolar process. To estimate random associations between the labeled cell and the immunostained receptors, we calculated the percentage of receptor volume occupancy upon flipping the receptor channel 90º to its original orientation. We found only 0.15 ± 0.06 % (mean ±SEM) $GABA_A\alpha1$ occupancy at Type 6 CBC dendrites (n = 4 cells from 3 animals), far less than the observed data.

A Matlab routine (corrcoef) was used to calculate the correlation coefficient between the intensity of pixels composing the image stacks acquired from regions co-immunostained for $\alpha1$ and $\gamma2$ subunits. To identify the contribution of random overlap of signals between channels, one channel was flipped 180º relative to the other and the correlation coefficient recalculated.

To quantify the expression of $GABA_A\alpha1$ receptors in the OPL and IPL of the horizontal cell specific VIAAT KO, pixels above a threshold set to remove background fluorescence (see Fig. S1) within a single optical plane were counted and divided by the total number of pixels in the same field. A total of 4-5 image planes per retina were sampled from 6 retinas, 3 animals.

Functional recordings and data analysis

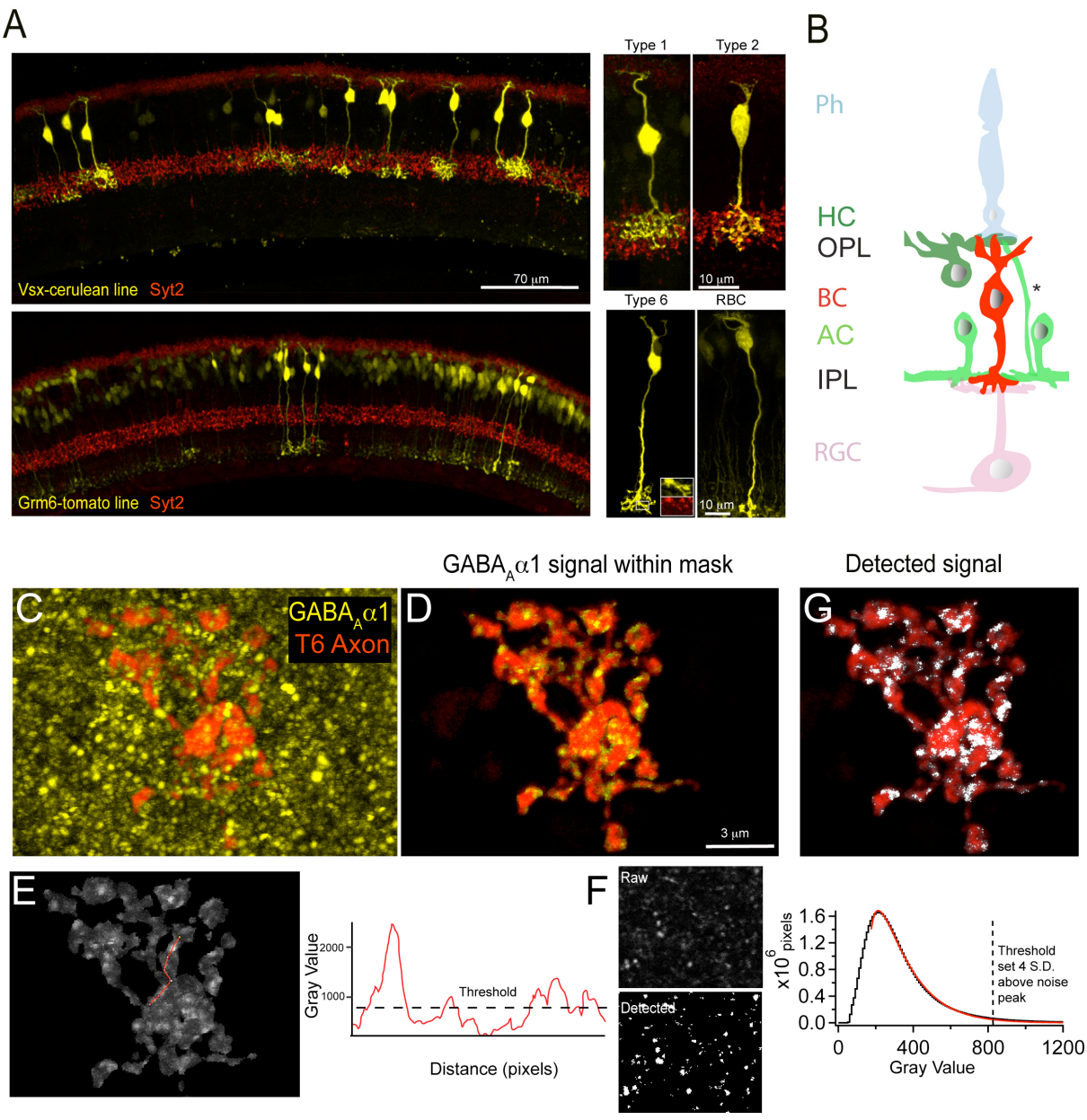
Retinal slices (200 µm thick) were prepared from dark-adapted VIAAT KO and control mice. Isolated retinas were stored in oxygenated (95% O2/5% CO2) Ames medium (Sigma) at ~32°C–34°C. The retinas were embedded in agarose and sliced as previously described (5). Once under the microscope, slice preparations were perfused by Ames solution at a rate of ~8 mL/min. Retinal bipolar cells were visualized and targeted for whole-cell recordings using

infrared light (>950 nm). Light stimulus in the form of full-field illumination (diameter, 500 µm) was delivered to the tissue from LEDs with peak spectral outputs at 470 nm.

Voltage-clamp recordings were obtained using pipettes (10–14 MΩ) filled with an intracellular solution containing (in mM) the following: 105 Cs methanesulfonate, 10 TEA-Cl, 20 HEPES, 10 EGTA, 2 QX-314, 5 Mg-ATP, 0.5 Tris-GTP, and 0.1 Alexa-555 hydrazide (~280 mOsm; pH ~7.2 with KOH). (1,2,5,6-Tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA, 50 µM; Tocris, Bristol, United Kingdom) was added to the perfusion solution as indicated in Figure 4. To isolate excitatory or inhibitory synaptic input in voltage-clamp recordings, cells were held at the estimated reversal potential for inhibitory or excitatory input of ~−60 mV and ~+10 mV. Absolute voltage values were not corrected for liquid junction potentials (−8.5 mV).

Puffed agents were applied using a Picospritzer II (General Valve) connected to a patch pipette (resistance, ~5–7 MΩ). GABA (200 µM) was dissolved in Hepes-buffered Ames medium with 0.1 mM Alexa-488 hydrazide and applied with the puff pipette. The puffing direction and the duration of the 500 ms puff were chosen such that the dendritic arbor or the axonal terminal of the voltage-clamped bipolar cell was completely covered by the puff.

Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded at ~+10mV and analysed using a combination of in-built event-detection routine in the software Axograph X (AxoGraph Scientific, Sydney, Australia) together with self-written routines in Matlab (Mathworks, USA). To quantify evoked currents, peak amplitude relative to the pre-application or pre-stimulus baseline current, were determined and averaged across cells.



Supplementary Figure 1

Supplementary Figure 1: Transgenic lines and image analysis routine used to identify inhibitory receptors on individual ON and OFF bipolar cells.

A: *(Left)* OFF and ON retinal bipolar cells can be specifically labeled in *Vsx1-Cerulean* (top panel, yellow) and *Grm6-tdTomato* (bottom panel, yellow) lines respectively. *(Right)* Individual bipolar cells can be further identified depending on their axonal stratification (Type 1 and 2 stratify in the same lamina) and further by co-labeling with Synaptotagmin-2 (Syt2, red) present at Type 2 and Type 6 bipolar cell terminals (6). Rod bipolar cell (RBC) terminals can be easily recognized by their distinct morphology and characteristic axonal bouton morphology (1, 7).

B: Schematic of the mouse retinal circuit. Bipolar cells (BC) integrate input from photoreceptors (Ph) at the outer plexiform layer (OPL) and provide output to retinal ganglion cells (RGC) at the inner plexiform layer (IPL). BC dendrites receive inhibitory input from horizontal cells (HC) and from interplexiform processes (marked with asterisk) of amacrine cells (AC). Diverse AC types provide inhibition onto the axon terminals of BCs.

C: Image of α1-subunit-containing $GABA_A$ ($GABA_A\alpha1$) receptor immunolabeling (yellow) on the axon terminal of a Type 6 ON cone bipolar cell (T6, red).

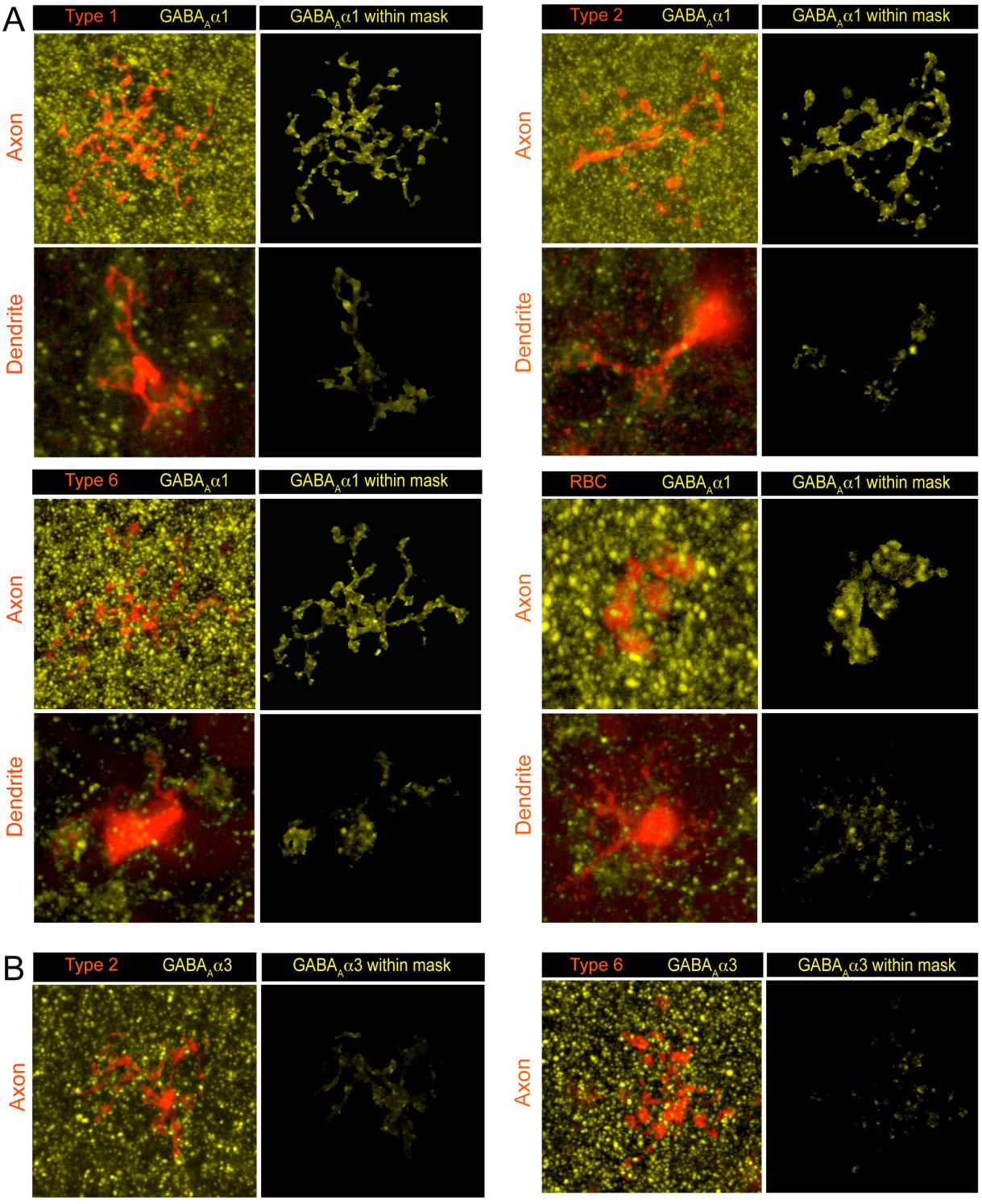
D: The axon terminal of the bipolar cell (red) was masked in 3D and the $GABA_A\alpha1$ receptor signal within the mask was isolated (yellow).

E: Only pixel values above a threshold are considered as representing true receptor expression. Intensities (pixel values) of the receptor labeling within the bipolar cell volume plotted along a line (red line) show peaks for each receptor puncta. The dashed line represents the threshold applied for receptor detection.

F: $GABA_A\alpha1$ receptor signal before (top) and after (bottom) the application of a threshold for pixel detection. To generate a conservative threshold that can be reliably applied, all the pixels of the image were plotted against the gray values (right) and the curve fitted with a gamma function (red curve). The mode of the curve represents the noise peak and a

threshold 4 standard deviations above the noise peak was selected for bipolar cell receptor analyses across conditions.

G: $GABA_A\alpha1$ receptor pixels within the bipolar cell (red) are represented by white pixels. These white pixels are obtained after setting a threshold to omit background fluorescence (see E-F).

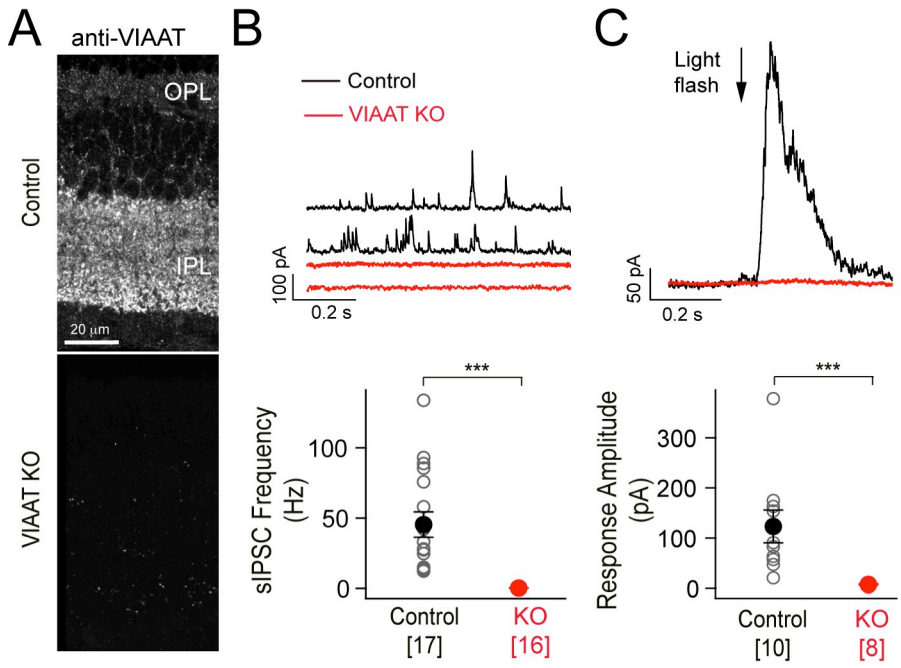


Supplementary Figure 2

Supplementary Figure 2: $GABA_A$ receptors on the axons and dendrites of adult mouse bipolar cells.

A: α1-subunit-containing $GABA_A$ ($GABA_A\alpha1$) receptor immunolabeling (yellow) within the axon terminals and dendrites (red) of Type 1 and 2 OFF cone bipolar cells, Type 6 ON cone bipolar cell and rod bipolar cell (RBC). Both raw images of immunolabeling within the field and receptor labeling digitally isolated within the bipolar cell mask are displayed. Same field of view and cells shown in Figure 1A.

B: α3-subunit-containing $GABA_A$ ($GABA_A\alpha3$) receptor immunolabeling (yellow) within the axon terminals (red) of Type 2 OFF and Type 6 ON cone bipolar cells. Raw images of immunolabeling within the field and receptors within the bipolar cell mask are displayed.



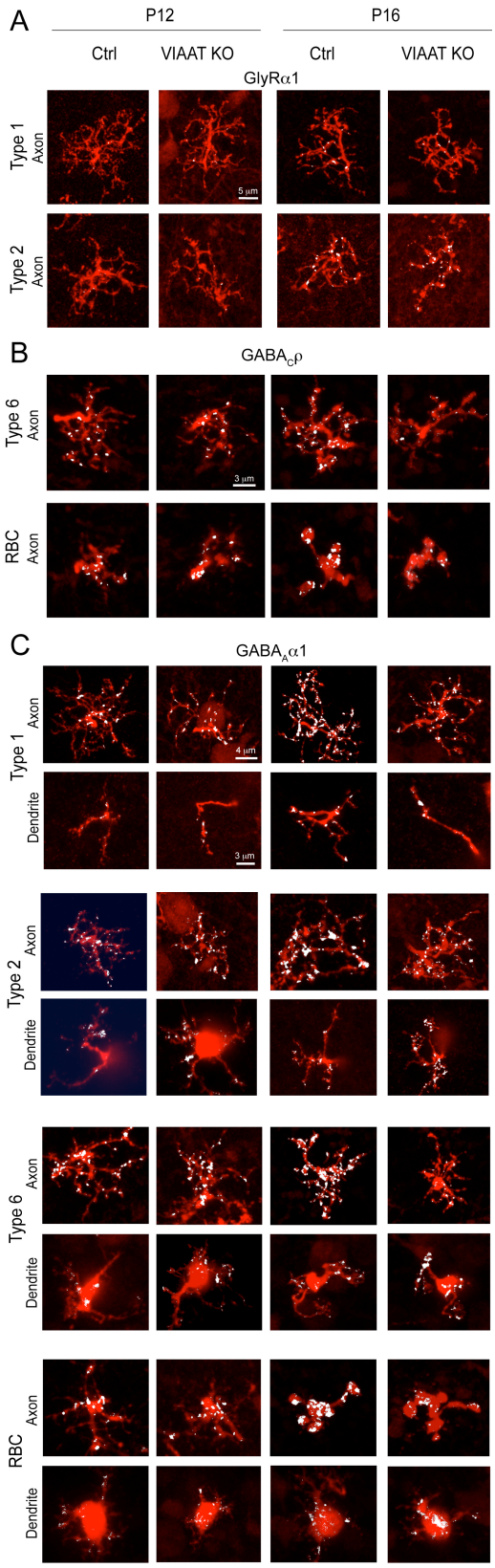
Supplementary Figure 3

Supplementary Figure 3: Inhibitory neurotransmission is suppressed in VIAAT deficient retina.

A: Expression of vesicular inhibitory amino acid transporter (VIAAT) in outer and inner plexiform layers (OPL and IPL respectively) of VIAAT knockout (KO) and littermate control (Control) retina.

B: (*Top*) Spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from OFF bipolar cells that were whole-cell voltage clamped at 0 mV in Control (black trace) and VIAAT KO (red) retina. (*Bottom*) sIPSC frequency is severely reduced for OFF bipolar cells in KO retina.

C: (*Top*) Light-driven outward current of a VIAAT KO and a Control OFF bipolar cell evoked by a 10 ms light flash (40 rhodopsin isomerization/Rod/s) delivered while the cells were held at 0 mV (excitatory reversal potential). (*Bottom*) Quantification of the peak amplitude of the light-evoked inhibitory response of OFF bipolar cells from KO and Control retina. All error bars represent standard error of the mean. Number of cells in parentheses; for all recordings, $n > 5$ animals. *** $p<0.001$.



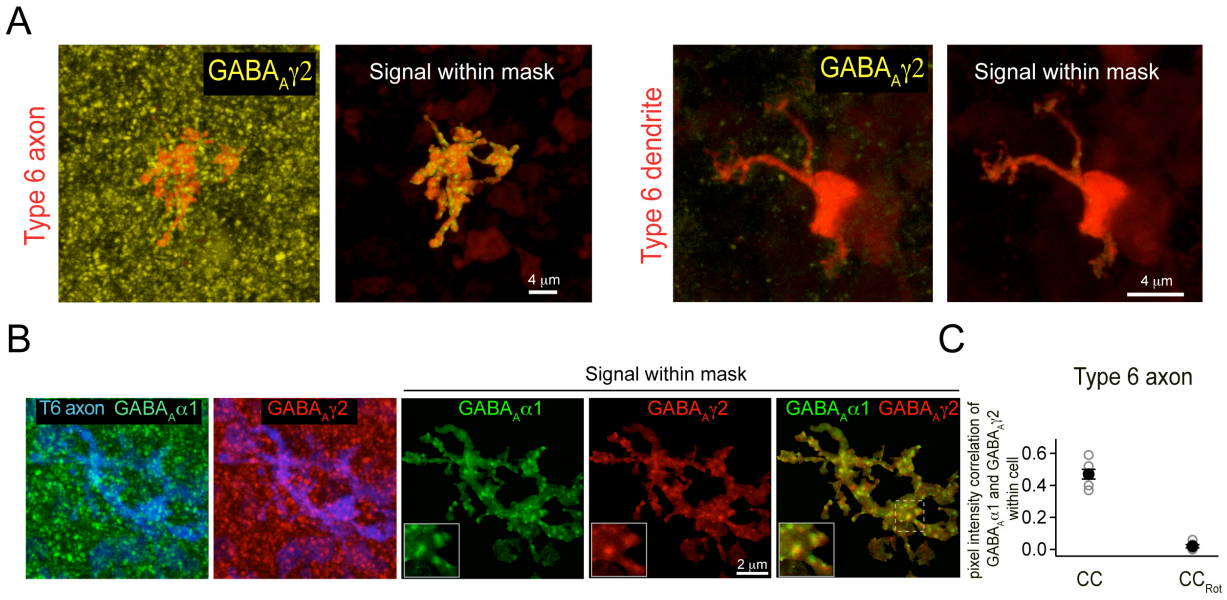
Supplementary Figure 4

Supplementary Figure 4: Alterations of bipolar cell GABA receptors in VIAAT KO retina occurs around eye-opening.

A: α1-subunit-containing glycine receptors (GlyRα1, white) on the axon terminals (red) of Type 1 and 2 OFF cone bipolar cells in VIAAT knockout (KO) and littermate control (Ctrl) retina. Bipolar cell terminals depicted at postnatal day 12 (P12), before eye-opening and at P16, after eye-opening.

B: ρ-subunit-containing $GABA_C$ receptors (white) on the axon terminals (red) of Type 6 ON cone bipolar and rod bipolar cells (RBC) in P12 and P16 KO and Ctrl retinas. Reduction of $GABA_C$ receptors on the axon terminals of ON bipolar cells in VIAAT KO retina is evident at P16.

C: α1-subunit-containing $GABA_A$ ($GABA_A$α1) receptors (white) on cone (Type 1, Type 2 and Type 6) and rod bipolar cell axon terminals and dendrites (red) in P12 and P16 VIAAT knockout (KO) and littermate control (Ctrl) retinas. Alteration in $GABA_A$α1 receptor expression within bipolar cell axons and dendrites occurs around eye-opening.



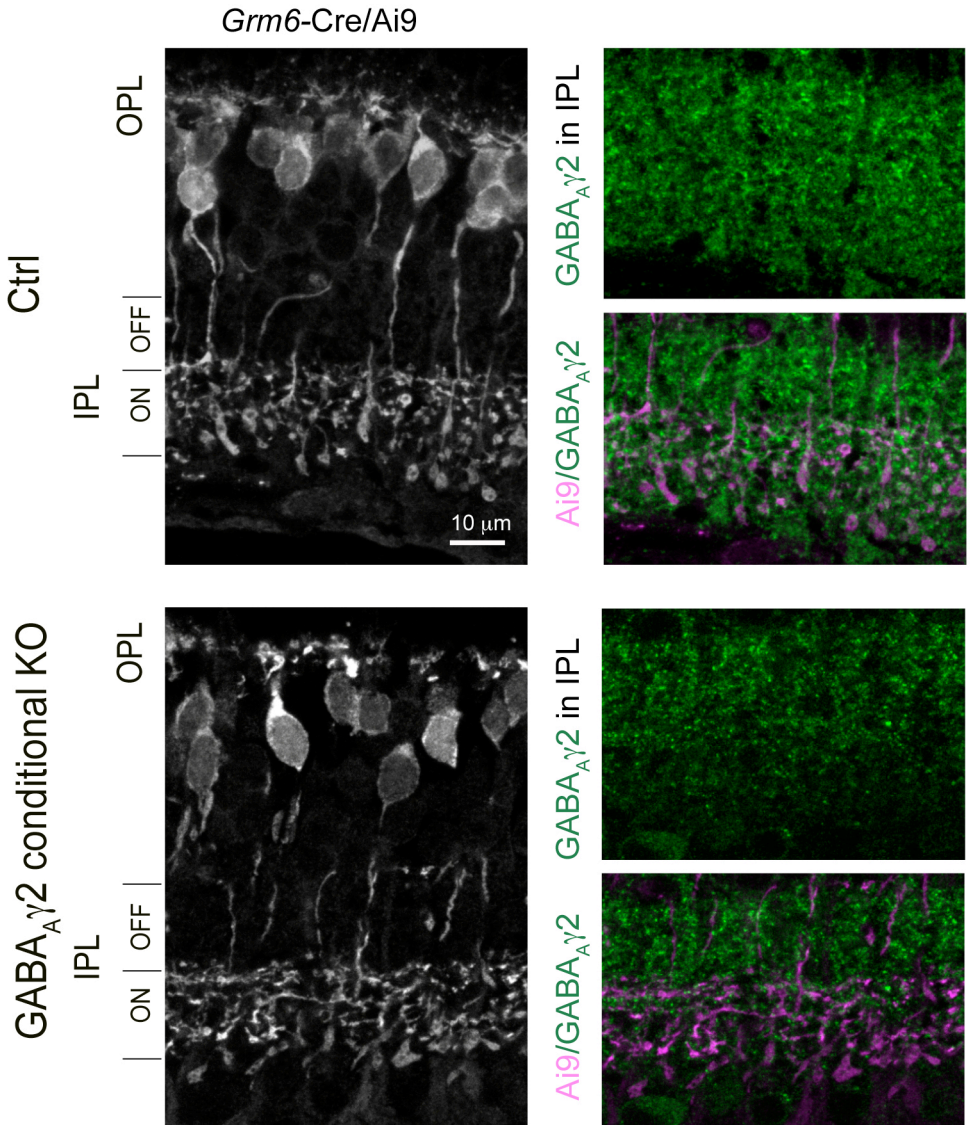
Supplementary Figure 5

Supplementary Figure 5: Distribution of $GABA_A\gamma2$ subunit in retinal bipolar cells.

A: $\gamma2$-subunit-containing $GABA_A$ ($GABA_A\gamma2$) receptor immunolabeling (yellow) on Type 6 ON cone bipolar cell axon terminal and dendrites (red). Very little $GABA_A\gamma2$ signal can be observed in the layer where the dendrites of Type 6 bipolar cells arborize. Accordingly, little $GABA_A\gamma2$ signal was found within the bipolar cell dendritic mask in contrast to abundant $GABA_A\gamma2$ signal within the axon terminal.

B: Type 6 (T6, blue) axon terminal co-labeled with $\alpha1$-subunit-containing $GABA_A$ ($GABA_A\alpha1$) receptors (green) and $GABA_A\gamma2$ (red). $GABA_A\gamma2$ and $GABA_A\alpha1$ are both prominently expressed at the axonal compartment.

C: Pixel intensity correlation of $GABA_A\gamma2$ and $GABA_A\alpha1$ immunolabeling within the axonal compartments. The correlation coefficient (CC) for $GABA_A\gamma2$ and $GABA_A\alpha1$ is much higher than when the channels are rotated 180º relative to each other (CC_{Rot}) thereby indicating non-random association between the $GABA_A\gamma2$ and $GABA_A\alpha1$ signals. All error bars represent standard error of the mean. Number of cells = 5 from > 3 animals.



Supplementary Figure 6

Supplementary Figure 6: $GABA_A\gamma2$ subunit deleted from ON retinal bipolar cells in Ai9/ *$GABA_A\gamma2$* conditional KO/*Grm6*-Cre transgenic mice.

Sections from *Grm6*-Cre/Ai9 mice in Control (Ctrl, top panel) and *$GABA_A\gamma2$* conditional knockout (KO) (γ2 bipolar cell-KO, bottom panel) labeled for γ2-subunit-containing $GABA_A$ ($GABA_A\gamma2$) receptors (green). *Left* ON bipolar cells are labeled with *Grm6*-Cre/Ai9 (grayscale) in both lines. OPL: outer plexiform layer, IPL: inner plexiform layer, ON, OFF: ON and OFF sublaminae in the IPL. *Right* $GABA_A\gamma2$ labeling in the IPL together with ON bipolar cell terminals (Ai9: magenta). $GABA_A\gamma2$ is eliminated from ON bipolar cells expressing Cre recombinase in γ2 bipolar cell KO retina.

References for Supplementary information

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