Neurotransmission plays contrasting roles in the maturation of inhibitory synapses on axons and dendrites of retinal bipolar cells

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Neuronal output is modulated by inhibition onto both dendrites and axons. It is unknown whether inhibitory synapses at these two cellular compartments of an individual neuron are regulated coordinately or separately during in vivo development. Because neurotransmission influences synapse maturation and circuit development, we determined how loss of inhibition affects the expression of diverse types of inhibitory receptors on the axon and dendrites of mouse retinal bipolar cells. We found that axonal GABA but not glycine receptor expression depends on neurotransmission. Importantly, axonal and dendritic GABA receptors comprise distinct subunit compositions that are regulated differentially by GABA release: Axonal GABA receptors are down-regulated but dendritic receptors are up-regulated in the absence of inhibition. The homeostatic increase in GABA receptors on bipolar cell dendrites is pathway-specific: Cone but not rod bipolar cell dendrites maintain an up-regulation of receptors in the transmission deficient mutants. Furthermore, the bipolar cell GABA receptor alterations are a consequence of impaired vesicular GABA release from amacrine but not horizontal interneurons. Thus, inhibitory neurotransmission regulates in vivo postsynaptic maturation of inhibitory synapses with contrasting modes of action specific to synapse type and location.

GABA receptor | retina | synaptic inhibition | axon-dendrite

Interneurons of the CNS control neuronal excitability through release of γ-amino butyric acid (GABA) and glycine. How inhibition modifies neuronal output depends largely on the types of presynaptic interneurons making synapses onto a postsynaptic cell, and the location and densities of these synapses (1–3). Moreover, inhibitory receptor types with distinct transmitter affinities and kinetics present on the axon or dendrites of an individual neuron can critically shape its output (3–6). Although much is known about how different inhibitory synapses shape the spatiotemporal activity patterns of mature neurons, it is less clear what factors regulate the expression of inhibitory receptors at these synapses during development in vivo. Is the expression of distinct inhibitory receptor types within a cellular compartment (axon or dendrite) regulated coordinately or independently? Conversely, is the expression of the same receptor type at different cellular compartments of an individual neuron regulated by common or separate factors?

To answer these questions, we assessed expression of inhibitory receptors on the axon and dendrites of individual glutamatergic retinal neurons in mice with genetically suppressed inhibition. We generated retina-specific knockouts of the vesicular inhibitory amino acid transporter (VIAAT), which mediates uptake of GABA or glycine into synaptic vesicles (7, 8). We perturbed inhibition because it has been found previously to influence pre- and postsynaptic maturation of GABAergic synapses (9–12). However, whether inhibitory receptor expression at the “input” and “output” compartments of an individual neuron is coordinately regulated by activity remains unknown. We focused on retinal bipolar cells (BCs) because of the rich variety of inhibitory synapses found on these neurons. Moreover, the many types of BCs enabled us to determine whether inhibitory transmission plays a uniform or diverse role in regulating inhibitory synapses across cell types that signal in parallel. We compared the postsynaptic maturation of GABA- and glycineergic synapses on cone BCs (CBCs) versus rod BCs (RBCs) that operate at different light levels. Among CBCs, we analyzed both ON and OFF BC types, which depolarize or hyperpolarize to light increments, respectively (13).

Results

In the mouse retina, GABA (α1–3, β1–3 with γ2 or an auxiliary subunit), GABAC (p1–3 subunits), and glycine receptors (α1–4 with a β subunit) (5, 14, 15) are present at nonoverlapping synapses. However, the complement of inhibitory receptor types and their relative expressions on the axons and dendrites of individual BCs has not been compared across BC types. We thus generated transgenic mouse lines to visualize ON and OFF BCs. We previously showed that ON BCs, especially type 6 CBCs and RBCs, express tdTomato in Gmtn-tdTomato mice (ref. 16; SI Appendix, Fig. S1A). To label OFF CBCs, we cloned and used

Significance

Neuronal output is modulated by inhibition onto axons and dendrites by diverse inhibitory synapses comprising distinct receptor subunits. Factors that regulate the in vivo maturation of these synapses across cell compartments are not well understood. We discovered that axonal GABA receptors are down-regulated whereas dendritic GABA receptors are up-regulated on retinal bipolar cells in the absence of vesicular GABA release. Deleting the γ2 subunit of GABA receptors specifically in bipolar cells only alters axonal GABA receptor expression, suggesting that axonal and dendritic GABA receptors have distinct subunit compositions that are regulated independently. Moreover, vesicular GABA release from presynaptic amacrine but not horizontal interneurons is important. Thus, regulation of inhibitory synapse maturation across the bipolar cell is input-type, receptor-type, and cell-compartment-type specific.

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that GABAA receptors are abundant on axon terminals of BCs (4, 19). Moreover, we discovered that although the axons of different BC types express distinct combinations of GABA and glycine receptors, their dendrites all have GABAAα1 receptors.

Perturbing GABA Release Exerts Cell-Type-Specific Effects on Bipolar Cell Morphology and Cone Connectivity. VIAAT is expressed by both mouse horizontal cells and amacrine cells (21). To assess the importance of GABA and glycine release on the formation and maintenance of inhibitory synapses on BCs, we perturbed vesicular release of inhibitory neurotransmitters specifically in the retina by abolishing VIAAT expression using a Pax6-Cre (22) mice (VIAAT KO). In the KO, VIAAT immunoreactivity is virtually eliminated from both the outer and inner plexiform layer (OPL and IPL respectively, SI Appendix, Fig. S34). To confirm the lack of inhibitory neurotransmission in the VIAAT KO, we performed whole-cell voltage clamp recordings from OFF BCs (SI Appendix, Fig. S3B). Indeed, OFF BCs in the VIAAT KO retina displayed a dramatically reduced frequency of spontaneous inhibitory postsynaptic currents (sIPSCs, SI Appendix, Fig. S3B), compared with BCs of the same type in littermate controls (control). Light-evoked inhibitory responses of OFF CBCs were also abolished in the VIAAT KO retina (SI Appendix, Fig. S3C).

Because GABA can act as a trophic signal to influence morphogenesis (23, 24), we examined the axonal and dendritic morphologies of BCs in the VIAAT KO retina before eye-opening and at maturity (Fig. 2). In the mature retina (postnatal day P30), the axonal and dendritic arbors of all BC types examined were unaffected in the VIAAT KO, with the exception that type 1 CBC dendritic arbors in the KO were smaller than normal (Fig. 2 A and B). Before eye-opening (P12), some BCs in the VIAAT KO retina exhibited transient differences in arbor sizes compared with controls (Fig. 2B). However, unlike in

the Vax1 promoter to drive expression of cerulean fluorescent protein. In one Vax1-cerulean mouse line, we found sparse labeling of OFF CBCs, particularly type 1 and 2 CBCs (SI Appendix, Fig. S1A). Previous electrophysiological studies suggest that GABAα1 receptors are abundant on axon terminals of BCs (4, 19). Using subunit-specific antibodies, we determined the expression of GABAα1 subunits on the axons and dendrites of type 1 and 2 OFF CBCs, type 6 ON CBCs, and RBCs (Fig. 1 and SI Appendix, Fig. S2). For all BCs examined, we found GABAα1 subunit-containing GABAα1 receptors (GABAα1) at both axon terminals and dendrites. We quantified receptor expression by calculating the % of the volume of the axon terminal or dendritic arbor occupied by immunolabeled receptors, and called the measure % occupancy (SI Appendix, Fig. S1 C–E and SI Materials and Methods). GABAα1 receptors were more abundant at BC axons relative to dendrites (Fig. 1C). GABAα3 receptor expression was low compared with GABAα1 expression at both ON and OFF BC axon terminals (SI Appendix, Fig. S2B). We did not immunolabel for GABAα2 subunits because they are not localized to BCs (15). As found previously (4, 19, 20), our immunolabeling analysis showed that ON, but not OFF BC axon terminals express substantial amounts of GABAα1 receptors (Fig. 1 B and D). Also OFF but not ON BC axons express abundant α1-subunit-containing glycine receptors (GlyRα1), with type 2 CBC axons expressing more GlyRα1 compared with type 1 CBCs (P = 0.0035, Fig. 1 B and E). Our immunolabeling thus reveals expression patterns of inhibitory receptors across BC axon types consistent with previous electrophysiology. Moreover, we discovered that although the axons of

**Fig. 1.** Inhibitory receptor types expressed on the axons and dendrites of mouse retinal bipolar cells. (A) α1-subunit-containing GABAα1 (GABAα1) receptors (white) on the axons and dendrites (red) of types 1, 2, and 6 cone bipolar cells and rod bipolar cells (RBCs). Insets show raw images of receptor immunolabeling (yellow) for axonal or dendritic processes within the boxed region. (B) ρ-subunit-containing GABAα1 receptors (GABAα1) and α1-subunit-containing glycine receptors, GlyRα1, (white) on the axon terminals of OFF (type 1 and 2) and ON (type 6 and rod) bipolar cells (red). Axon terminals of ON rather than OFF bipolar cells robustly express GABAα1 receptors, whereas OFF bipolar cells abundantly express GlyRα1. (C) GABAα1 occupancy (%) at the axons and dendrites (Dend) of bipolar cells (see SI Appendix, SI Materials and Methods and text for definition of % occupancy). (D and E) GABAα1 and GlyRα1 receptor occupancy (%) in bipolar cell axon terminals. All error bars represent SEM. Number of cells is shown in parentheses; for all cell types, n > 3 animals. ***P < 0.001.

**Fig. 2.** Loss of VIAAT influences the morphology of ON and OFF bipolar cells in a cell-type-specific manner. (A) Types 1, 2, and 6 cone bipolar cells and rod bipolar cells (RBCs) in mature VIAAT knockout (KO) and littermate control (Ctrl) retinas. En face view of the dendrites and axon terminals of individual bipolar cells are displayed above and below the side view of the cell, respectively. Arrow points to the smaller dendritic arbor of a type 1 OFF cone bipolar cell in KO retina. (B) Quantification of the axonal (top plots) and dendritic (bottom plots) volumes of immature (postnatal day 12, P12) and mature (P30) bipolar cells in KO (red) and Ctrl (black) retinas. (C, Upper) Colabeling for cone arrestin (green) and dendrites of types 1 and 2 (magenta) OFF bipolar cells in adult retina. (Lower) Quantification of the number of cones contacted by type 1 and type 2 OFF bipolar cells in KO (red) and Ctrl (black) retinas. All error bars represent SEM. Number of cells in parentheses; for all cell types, n = 3 animals. Statistics for different cell types shown for Ctrl versus KO comparisons at each age. *P < 0.05; **P < 0.01.
control retina, type 1 CBCs in the VIAAT KO did not increase their dendritic arbor size with maturation (control cells: \( P = 0.007, P12 \) versus P30; KO: \( P = 0.75, P12 \) versus P30; Fig. 2 B and C). Type 1 CBCs contacted fewer cone photoreceptors, as might be expected from their reduced dendritic territory (Fig. 3, Fig. 2C). Because type 1 dendritic arbors were normal in size early in development (Fig. 2B), the failure to contact more cones with maturation may be due to slowed dendritic growth and/or a failure to stabilize elaborating dendrites. Type 1 axon terminal sizes were normal in adult VIAAT KO retina (Fig. 2B), suggesting that their smaller dendritic arbors are not the result of a general impairment to cell growth. Together, these observations indicate a cell-type-specific role for VIAAT-mediated transmitter release in regulating the development and synaptic connectivity of BC dendrites.

GABA Receptor Expression on Axons and Dendrites of maturing Bipolar Cells Is Regulated Separately, and by Vesicular GABA Release from One Presynaptic Input Type. Does loss of VIAAT-mediated transmitter release perturb BC GABA or glycinergic synapse development in a receptor-type-specific and/or BC-type-specific manner? Could cell-compartment-specific alterations in receptor distribution occur on the axon and dendrites of BCs in VIAAT KO retina? To answer these questions, we analyzed GlyR\( \alpha_1 \) receptors on OFF BC (type 1 and 2) axons, GABA\(_{\alpha_1}\) receptors on ON BC (type 6 and RBC) axons, and GABA\(_{\alpha_1}\) receptors on the axon and dendrites of all four BC types in VIAAT KO and control retinas (Fig. 3). GlyR\( \alpha_1 \) receptor expression on the axon terminals of OFF BCs was unaffected in the P30 VIAAT KO retina (Fig. 3 A and B). In contrast, GABA\(_{\alpha_1}\) receptors were reduced on type 6 CBC and RBC axon terminals in VIAAT KO retina at this age (Fig. 3 C and D). Similarly, GABA\(_{\alpha_1}\) receptor expression was diminished on the axon terminals of P30 RBCs and the CBC types studied in the VIAAT KO retina (Fig. 3 E and F, Top). However, CBC dendrites showed increased GABA\(_{\alpha_1}\) receptor expression in the P30 VIAAT KO retina (Fig. 3 E and F). In contrast, RBC dendrites in the KO maintained GABA\(_{\alpha_1}\) receptors at levels comparable to control (Fig. 3 E and F). Thus, impaired vesicular release of inhibitory neurotransmitters GABA and glycine in the retina causes disparate effects on GABA and glycine receptor distribution across BC types and cell compartments.

The changes we observed in GABA receptor expression in the mature VIAAT KO retina could arise from a failure to localize receptors early in development and/or an impairment of their maintenance thereafter. To distinguish between these possibilities, we examined BC GABA receptor expression in the VIAAT KO retina at two developmental time points: P12, before eye-opening and P16 after eye-opening (Fig. 3 D and F and SI Appendix, Fig. S4). Axonal GABA\(_{\alpha_1}\) receptors on ON BCs were reduced in P16 VIAAT KO retina compared with controls (Fig. 3 D and SI Appendix, Fig. S4). Similarly, axonal expression of GABA\(_{\alpha_1}\) receptors was reduced by P16 for all BC types studied in VIAAT KO compared with control (Fig. 3 F and SI Appendix, Fig. S4). In control animals, axonal GABA\(_{\alpha_1}\) receptor expression increased significantly from P12 to P16 (\( P < 0.03 \) for all four BC types), but this developmental increase did not occur in the VIAAT KO. In fact, in the KO, axonal GABA\(_{\alpha_1}\) receptor expression decreased in type 1 and 6 CBCs and RBCs between P12 and P16 (\( P < 0.009 \) for the three BC types), whereas axonal receptor expression did not change between P12 and P16 in type 2 CBCs (\( P = 0.128 \)). Together, these observations indicate that the initial accumulation of GABA receptors on BC axons is largely unaffected in the VIAAT KO, but the further localization and maintenance of these receptors after eye-opening is disrupted by the absence of vesicular GABA release.

GABA\(_{\alpha_1}\) receptor expression on BC dendrites in VIAAT KO retina also increased relative to control at P16 (Fig. 3 F and SI Appendix, Fig. S4). At P16, dendritic expression of GABA\(_{\alpha_1}\) in the KO retina increased above normal values for the CBCs and RBCs, but remained elevated in the mature retina only at CBC but not RBC dendrites (P30, Fig. 3F). Thus, the RBC dendritic compartment has the capacity to reset its GABA\(_{\alpha_1}\) expression to wild-type levels by maturity.

To confirm that alterations in GABA\(_{\alpha_1}\) receptor expression reflect functional changes, we recorded GABA-evoked responses from adult retinal BCs by puffing GABA onto their axon terminals or dendrites (Fig. 4). Indeed, puffing GABA onto axons of RBCs in the KO elicited a much-reduced response compared with cells in control retina (Fig. 4D). Because RBC terminals have both GABA\(_{\alpha_1}\) and GABA\(_{\beta_2}\) receptors, we isolated the GABA\(_{\alpha_1}\) component by blocking GABA\(_{\beta_2}\) receptors with TPMPA. The GABA\(_{\alpha_1}\)-evoked response was significantly reduced at RBC terminals in VIAAT KO retinas (Fig. 4D), corroborating the immunolabeling results. Consistent with the GABA\(_{\alpha_1}\) immunostaining, the GABA\(_{\alpha_1}\) component of the RBC terminal was also significantly reduced in the VIAAT KO (mean peak amplitudes: control = 100 ± 11 pA, KO = 48 ± 9 pA, \( P < 0.007 \)). GABA application at RBC dendrites generated equivalent responses in KO and control retinas, also consistent with the immunostaining (Fig. 4B, Left). TPMPA did not reduce the dendritic GABA-evoked PSC amplitude in RBCs in the KO retina (Fig. 4B, Right), suggesting that RBC dendritic receptors are predominantly GABA\(_{\alpha_1}\) receptors. In keeping with these observations, puffing GABA onto dendrites of isolated mouse RBCs elicits GABA\(_{\alpha_1}\)- and not GABA\(_{\beta_2}\)-mediated currents (25).
In contrast to RBC responses, OFF CBC dendritic responses to puffed GABA were enhanced in the VIAAT KO retina compared with control (Fig. 4C, Left). The OFF CBC dendritic response in KO retina is mediated primarily by GABA<sub>A</sub> receptors (Fig. 4C, Right).

Collectively, our immunolabeling and electrophysiological experiments demonstrate that GABA<sub>B</sub> receptors at ON BC terminals and GABA<sub>A</sub>,γ<sub>1</sub> receptors at ON and OFF BC axons are sensitive to loss of vesicular transmitter release. In addition, GABA<sub>A</sub>,γ<sub>1</sub> receptors at BC dendrites increase in CBCs but not RBCs when vesicular release of GABA is impaired, suggesting rod/cone pathway-specific alterations in the outer retina.

Compartment (axon versus dendrite) specific alterations of GABA<sub>A</sub>,γ<sub>1</sub> expression on BCs raised an important question: Does loss of VIAAT in both major inhibitory presynaptic cell classes, amacrine and horizontal interneurons, contribute to the GABA<sub>A</sub>,γ<sub>1</sub> expression changes? To answer this question, we examined GABA<sub>A</sub>,γ<sub>1</sub> immunoreactivity in retinas in which VIAAT was selectively eliminated from horizontal cells (HC VIAAT KO, Fig. 5). The pan-VIAAT KO line, generated by crossing with the αδεψ-cre line, showed the expected overall reduction of GABA<sub>A</sub>,γ<sub>1</sub> immunoreactivity in the IPL and elevation of the signal in the OPL relative to control (Fig. 5A). In the HC VIAAT KO, however, GABA<sub>A</sub>,γ<sub>1</sub> labeling in the IPL and OPL appeared normal (Fig. 5B and C). Thus, GABA<sub>A</sub>,γ<sub>1</sub> expression in the IPL and OPL depends on vesicular GABA release from amacrine cells but not horizontal cells. Our results, however, cannot exclude the possibility that nonvesicular release of GABA from horizontal cells could contribute to GABA<sub>A</sub>,γ<sub>1</sub> regulation.

**Discussion**

Our observations corroborate previous findings showing that inhibitory neurotransmission is not essential for synapse formation but is necessary for synaptic maturation (8–11, 29). However, our findings here add significantly to our current understanding of differential regulation of axonal and dendritic GABA<sub>A</sub>,γ<sub>1</sub> subunits which may depend on coassembly with GABA<sub>A</sub>,γ<sub>2</sub> subunit. GABA<sub>A</sub>,γ<sub>1</sub> expression on BC axons and dendrites may be regulated differentially if GABA<sub>A</sub> receptors in these separate compartments have different subunit compositions. Alternatively, GABA<sub>A</sub> receptors on the axons and dendrites could have the same subunit composition, but their regulation by activity could depend on their subcellular location. To distinguish between these possibilities, we immunolabeled adult wild-type retina for the γ<sub>2</sub> subunit of the GABA<sub>A</sub> receptor (GABA<sub>A</sub>,γ<sub>2</sub>) known to be abundant in the retina (15), and localized at the majority of CNS GABAergic synapses (26). We found that type 6 BC axon terminals robustly express GABA<sub>A</sub>,γ<sub>2</sub>, but surprisingly, their dendrites are deficient in this subunit (SI Appendix, Fig. S5A). In fact there was very little GABA<sub>A</sub>,γ<sub>2</sub> immunoreactivity in the OPL (SI Appendix, Fig. S5A). Using cross-correlation analysis, we confirmed that GABA<sub>A</sub>,γ<sub>1</sub> and γ<sub>2</sub> immunolabeling overlapped highly at type 6 CBC axon terminals (SI Appendix, Fig. S5 B and C).

These observations raise the possibility that GABA<sub>A</sub>,γ<sub>1</sub> receptors on BC axon and dendrites may not have the same subunit composition. This also makes the prediction that loss of axonal GABA<sub>A</sub>,γ<sub>1</sub> in the VIAAT KO retina should be accompanied by a loss of axonal GABA<sub>A</sub>,γ<sub>2</sub>. Indeed, we found that axonal GABA<sub>A</sub>,γ<sub>2</sub> in type 6 CBCs was reduced in VIAAT KO retina compared with control but dendritic GABA<sub>A</sub>,γ<sub>2</sub> expression was not altered (Fig. 6 A and B). We focused on type 6 CBCs because their morphology remains unaltered in the VIAAT KO (Fig. 2). To further examine whether GABA<sub>A</sub>,γ<sub>2</sub> coassembles with axonal but not dendritic GABA<sub>A</sub>,γ<sub>1</sub>, we genetically deleted GABA<sub>A</sub>,γ<sub>2</sub> specifically in ON BCs by crossing GABA<sub>A</sub>,γ<sub>2</sub> conditional KO mice (27) with a transgenic line we created in which Cre-recombinase is expressed by ON BCs shortly after their differentiation (Gmr6-Cre mice; Fig. 6C, SI Appendix, Fig. S6 and SI Materials and Methods; ref. 28). As expected, we found that a loss of GABA<sub>A</sub>,γ<sub>2</sub> in type 6 BCs caused a reduction in axonal but not dendritic GABA<sub>A</sub>,γ<sub>1</sub> receptors (Fig. 6 C and D). Together, these observations demonstrate that axonal but not dendritic GABA<sub>A</sub>,γ<sub>1</sub> receptors on BCs coassemble with the γ<sub>2</sub> subunit, and lend support to the possibility that GABA<sub>A</sub> receptors with distinct compositions, rather than the same receptors at separate cell compartments, are differentially regulated by vesicular GABA release.
from amacrine but not horizontal cells regulates BC GABA release.

VIAAT KO retinas, we discovered that vesicular GABA release
dendrite of an individual neuron. Comparing pan-VIAAT and HC-
from separate presynaptic GABAergic cell types on the axon and
GABA release in regulating the maturation of synaptic contact
Our study further uncovered distinct requirements for vesicular

cortical neurons in the visual cortex is reduced whereas input
from basket interneurons onto dendrites of Layer 4 pyramidal
connections, the effects of neurotransmission on GABAergic
in the density of glycine receptors (8). Even among GABAergic
cord cultures from VIAAT KO animals also show no alteration
though GABAA receptors on the same axons are reduced. Spinal
sion on OFF CBC terminals is unchanged in VIAAT KOs even
presynaptic input type. We found that glycine receptor expres-
specific GABAA receptor levels in BCs, how-
also be observed in visually deprived mice where metabotropic glutamate receptors on cone but not rod BC dendir-
are up-regulated (39).

What factors could be responsible for the distinct activity-
dependent changes of receptor expression in BC axons and den-
drites? Although contact with different amacrine subtypes could
be responsible for the opposite outcomes, it is also possible that
“postsynaptic” factors play a role. We found that GABAA re-
ceptors on the axons but not dendrites of BCs are composed of
GABA\textsubscript{\textgamma}2 subunit. Thus, differences in receptor subunit com-
position may explain why axonal and dendritic GABA\textalpha receptors
of the same BC are regulated in opposite directions by GABA
release. Our observations also raise the possibility that GABA\textalpha receptors with \textalpha y subunit composition could be highly sus-
ceptible to perturbed neurotransmission. The \textgamma y subunit is nec-
cessary for synaptic localization of GABA\textalpha receptors (3). Thus,
\textgamma y receptors may be sensitive to loss of GABA release because
they are clustered directly opposite transmitter release sites,
whereas receptors further away may be less susceptible. GABA\textalpha receptors on BC axons are localized at synapses (11), but the
location of BC dendritic GABA\textalpha receptors relative to GABA release sites in the OPL is not yet known. It is also possible that
regardless of their location, \textalpha y-subunit-containing GABA\textalpha receptors at all synapses require presynaptic GABA release for
their maintenance. If so, one may find that such receptors on other
neurons or at other cellular compartments exhibit the same
form of regulation by GABAergic transmission.

A final factor to consider is that the effects of GABA release on
synaptic development or maintenance could be “dose dependent.” For example, reducing GABA synthesis by deleting one of the
GABA synthetic enzymes, \textit{GAD}1, from basket interneurons decreases synapse size and density, as well as axonal arbor size (9). In
contrast, complete blockade of inhibitory transmission (VIAAT KO) yields opposite effects comprising overproliferation of small
cells and overgrowth of the axonal arbor (10). We also found that in retinas lacking \textit{GAD}1, GABA\textalpha but not GABA\textgamma receptors on BC dendrites are up-regulated (35) at nonoverlapping sites (33, 34) but opposite the same presynaptic A17 amacrine cell bouton (35) are similarly down-
regulated in the VIAAT KO. Together, these observations imply
that activity-dependent regulation of inhibitory receptors is input type-specific.

Activity Differentially Controls \textalpha y-Subunit Containing GABA\textalpha Receptor Expression on Bipolar Cell Axons and Dendrites. A differential effect on axonal and dendritic GABA\textalpha receptors has been observed in
hippocampal cell cultures in which chronic depolarization increased the GABA\textalpha receptor mobility in the axon initial seg-
ment but not in dendrites (36). We found that in vivo GABA\textalpha expression at input and output compartments of individual BCs are altered differently in the absence of GABA-mediated trans-
mission. In VIAAT KO retina, cone bipolar axonal GABA\textalpha receptors are lost whereas dendritic GABA\textalpha receptors are up-regulated. The homeostatic increase of dendritic GABA\textalpha receptors in the absence of GABA release is reminiscent of the increase in glutamate receptors found in other brain regions when excitatory transmission is suppressed or blocked (37, 38). Homeostatic regulation of dendritic GABA\textalpha receptors is lost whereas dendritic GABA\textalpha is up-regulated. The home-
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Activity-Dependent Regulation of Inhibitory Receptor Expression Varies with Presynaptic Input Type. The diversity of inhibitory connections onto retinal BCs enabled us to investigate the dep-

cence of postsynaptic expression of inhibitory receptors on presynaptic input type. We found that glycine receptor expres-
sion on OFF CBC terminals is unchanged in VIAAT KO s even though GABA\textalpha receptors on the same axons are reduced. Spinal
cord cultures from VIAAT KO animals also show no alteration in the density of glycine receptors (8). Even among GABAergic
connections, the effects of neurotransmission on GABAergic synapse development and maturation have been found to vary
with input types (30–32). After visual deprivation, transmission from basket interneurons onto dendrites of Layer 4 pyramidal
cortical neurons in the visual cortex is reduced whereas input from regular spiking nonpyramidal interneuron is increased (31).

Our study further uncovered distinct requirements for vesicular GABA release in regulating the maturation of synaptic contact
from separate presynaptic GABAergic cell types on the axon and
dendrite of an individual neuron. Comparing pan-VIAAT and HC-
VIAAT KO retinas, we discovered that vesicular GABA release from amacrine but not horizontal cells regulates BC GABA rece-
ceptors. Moreover, even among amacrine cells, expression of

GABA\textalpha receptors at amacrine synapses within the IPL versus the
OPL are regulated in distinct ways. In the VIAAT KO, GABA\textalpha receptors at synapses onto BC axons are down-regulated whereas its expression on the dendrites is up-regulated. Furthermore, different ion-
tropic GABA receptor types (GABA\textalpha and GABA\textgamma) cluster
at nonoverlapping sites (33, 34) but opposite the same presynaptic A17 amacrine cell bouton (35) are similarly down-
regulated in the VIAAT KO. Together, these observations imply
that activity-dependent regulation of inhibitory receptors is input type-specific.

Activity Differentially Controls \textalpha y-Subunit Containing GABA\textalpha Receptor Expression on Bipolar Cell Axons and Dendrites. A differential effect on axonal and dendritic GABA\textalpha receptors has been observed in


Fig. 6. Bipolar cell axonal and dendritic GABA\textalpha receptors have different subunit compositions. (A) \textalpha y-subunit staining GABA\textalpha receptors (red) at synaptic immunolabeling (yellow) in VIAAT knockout (KO) and littermate control (Ctrl) retinas in the same field as the labeled bipolar cell (BC). GABA\textalpha receptors (white) on a type 6 cone BC (red). (B) GABA\textgamma2 receptor occupancy on the axonal and dendritic arbors of type 6 BCs in VIAAT KO and Ctrl. (C) GABA\textalpha receptor occupancy (yellow) in Ctrl retinas and retinas with ON BC
specific GABA\textalpha7 deletion (KO). (C) GABA\textalpha7 conditional knockout (Cre/Grm6-Cre mice). Examples of GABA\textalpha7 receptors (white) on type 6 BC axons and dendrites (red). (D) Quantification of GABA\textalpha7 receptor occupancy (%) on type 6 BCs in KO and Ctrl. Error bars represent SEM. Number of
cells is shown in parentheses; all genotypes, n > 3 animals. **p < 0.001.
(E) Schematic summarizing compartment-specific changes in the expression of BC axonal and dendritic GABA\textalpha7 receptors in pan-VIAAT KO (both
central retina (HC) and amacrine cell (AC) affected), HC-specific VIAAT KO (HC VIAAT KO), and BC-specific \textgamma y KO.
GABA in the GAD1 mutant could thus be sufficient to maintain GABA\(_{\alpha 1}\) receptors, but already trigger loss of GABA\(_{\alpha 4}\) receptors.

In summary, our study has distinguished the role of vesicular release of inhibitory transmitters per se from the effects produced by overall changes in network activity on the postsynaptic expression of inhibitory receptors in vivo specifically in pre-synaptic input type, and varies with receptor subunit composition, cellular compartment, and the levels of transmitter release. Although complex, such diverse roles for inhibitory transmitter release provide a rich platform from which pre- and postsynaptic mechanisms can be selected to control the maturation and maintenance of distinct inhibitory connections within the network.

Materials and Methods

Transgenic Mouse Lines. 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. Vsx1-creerulean, Grm6-ttdTomato, pax6-Cre/VIAAT KO, Csx57-Cre/VIAAT KO, and Ai9/ GABA\(_{\alpha 2}\) conditional KO/Grm6-Cre mouse lines were used in this study. Detailed information on the transgenic lines is provided in SI Appendix, SI Materials and Methods.

Immunolabeling. Retinas were isolated in cold oxygenated mouse artificial cerebrospinal fluid and fixed in 4% (wt/vol) paraformaldehyde. Primary antibodies were directed against: VIAAT, GFP, Synaptotagmin-2, GlyR.

Functional Recordings and Data Analysis. Retinal slices (200 μm thick) were prepared from wild-type and GABA receptor KO mice and control mice as described (41). Detailed information on functional recordings and analyses is provided in SI Appendix, SI Materials and Methods.

Statistics. As the data passed the normality test, for control-KO comparisons, a two-tailed unpaired T test was used. For comparing cone contact numbers, Wilcoxon–Mann–Whitney rank sum test was used. *P < 0.05; **P < 0.01; ***P < 0.001.

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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 (Slc32a1tm1Lowl/J) and crossed with the αPax6-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 (Slc32a1tm1Lowl/J).

To eliminate GABA_\Aγ2 from bipolar cells, the GABA_\Aγ2 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 Sall, Agel, Ascl and PacI restriction sites was cloned into the Sall 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_Aγ2 conditional KO/Grm6-Cre mice were further crossed into the Ai9 reporter (Jackson Laboratory, B6.Cg-Gt(ROSA)26Sor tm9(CAG-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 4°C. 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
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$_{A}$α1 receptor subunit (polyclonal guinea-pig, 1:5000, kindly provided by J.M. Fritschy), anti-GABA$_{A}$α3 receptor subunit (polyclonal guinea-pig, 1:3000, kindly provided by J.M. Fritschy), anti-GABA$_{A}$γ2 (rabbit polyclonal, 1:500, Abcam), anti-GABA$_{C}$ρ 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.
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 90° to its original orientation. We found only $0.15 \pm 0.06\%$ (mean ±SEM) GABA$_{A_{\alpha 1}}$ 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 $\alpha 1$ and $\gamma 2$ subunits. To identify the contribution of random overlap of signals between channels, one channel was flipped 180° relative to the other and the correlation coefficient recalculated.

To quantify the expression of GABA$_{A_{\alpha 1}}$ 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 $\sim 32^\circ$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 $\sim 8$ 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<sub>α</sub> (GABA<sub>α</sub>α1) 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<sub>α</sub>α1 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<sub>α</sub>α1 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$\alpha_1$ 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<sub>A</sub> receptors on the axons and dendrites of adult mouse bipolar cells.

**A:** α1-subunit-containing GABA<sub>A</sub> (GABA<sub>Aα1</sub>) 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<sub>A</sub> (GABA<sub>Aα3</sub>) 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: $\alpha_1$-subunit-containing glycine receptors (GlyR$\alpha_1$, 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: $\rho$-subunit-containing $\text{GABA}_C$ 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 $\text{GABA}_C$ receptors on the axon terminals of ON bipolar cells in VIAAT KO retina is evident at P16.

C: $\alpha_1$-subunit-containing $\text{GABA}_A$ ($\text{GABA}_A\alpha_1$) 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 $\text{GABA}_A\alpha_1$ receptor expression within bipolar cell axons and dendrites occurs around eye-opening.
Supplementary Figure 5
Supplementary Figure 5: Distribution of GABA_{A\gamma 2} subunit in retinal bipolar cells.

A: \gamma 2-subunit-containing GABA_{A} (GABA_{A\gamma 2}) receptor immunolabeling (yellow) on Type 6 ON cone bipolar cell axon terminal and dendrites (red). Very little GABA_{A\gamma 2} signal can be observed in the layer where the dendrites of Type 6 bipolar cells arborize. Accordingly, little GABA_{A\gamma 2} signal was found within the bipolar cell dendritic mask in contrast to abundant GABA_{A\gamma 2} signal within the axon terminal.

B: Type 6 (T6, blue) axon terminal co-labeled with \alpha 1-subunit-containing GABA_{A} (GABA_{A\alpha 1}) receptors (green) and GABA_{A\gamma 2} (red). GABA_{A\gamma 2} and GABA_{A\alpha 1} are both prominently expressed at the axonal compartment.

C: Pixel intensity correlation of GABA_{A\gamma 2} and GABA_{A\alpha 1} immunolabeling within the axonal compartments. The correlation coefficient (CC) for GABA_{A\gamma 2} and GABA_{A\alpha 1} is much higher than when the channels are rotated 180° relative to each other (CC_{Rot}) thereby indicating non-random association between the GABA_{A\gamma 2} and GABA_{A\alpha 1} signals. All error bars represent standard error of the mean. Number of cells = 5 from > 3 animals.
Supplementary Figure 6
Supplementary Figure 6: GABA$_{\alpha \gamma 2}$ subunit deleted from ON retinal bipolar cells in Ai9/GABA$_{\alpha \gamma 2}$ conditional KO/Grm6-Cre transgenic mice.

Sections from Grm6-Cre/Ai9 mice in Control (Ctrl, top panel) and GABA$_{\alpha \gamma 2}$ conditional knockout (KO) (γ2 bipolar cell-KO, bottom panel) labeled for γ2-subunit-containing GABA$_{\alpha}$ (GABA$_{\alpha \gamma 2}$) 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$_{\alpha \gamma 2}$ labeling in the IPL together with ON bipolar cell terminals (Ai9: magenta). GABA$_{\alpha \gamma 2}$ is eliminated from ON bipolar cells expressing Cre recombinase in γ2 bipolar cell KO retina.
References for Supplementary information


