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## Developmental regulation and activity-dependent maintenance of GABAergic presynaptic inhibition onto rod bipolar cell axonal terminals

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### SUMMARY

Presynaptic inhibition onto axons regulates neuronal output, but how such inhibitory synapses develop and are maintained *in vivo* remains unclear. Axon terminals of glutamatergic retinal rod bipolar cells (RBCs) receive GABA<sub>A</sub> and GABA<sub>C</sub> receptor-mediated synaptic inhibition. We found that perturbing GABAergic or glutamatergic neurotransmission does not prevent GABAergic synaptogenesis onto RBC axons. But, GABA release is necessary for maintaining axonal GABA receptors. This activity-dependent process is receptor subtype specific: GABA<sub>C</sub> receptors are maintained, whereas GABA<sub>A</sub> receptors containing  $\alpha 1$ , but not  $\alpha 3$  subunits, decrease over time in mice with deficient GABA synthesis. GABA<sub>A</sub> receptor distribution on RBC axons is unaffected in GABA<sub>C</sub> receptor knockout mice. Thus, GABA<sub>A</sub> and GABA<sub>C</sub> receptor maintenance are regulated separately. Although immature RBCs elevate their glutamate release when GABA synthesis is impaired, homeostatic mechanisms ensure that the RBC output operates within its normal range after eye-opening, perhaps to regain proper visual processing within the scotopic pathway.

### INTRODUCTION

Presynaptic inhibition onto axonal terminals, commonly provided by GABAergic transmission, regulates neurotransmitter release. GABA receptors on the axon hillock of pyramidal cells (Nusser et al., 1996; Szabadics et al., 2006), on mossy fiber terminals of hippocampal granule cells (Ruiz et al., 2003), on cerebellar parallel fibers (Stell et al., 2007), as well as on retinal bipolar cell axon terminals (Shields et al., 2000; Vardi and Sterling, 1994) serve to modulate action potential firing and neurotransmitter release (Kullmann et

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al., 2005; Luscher et al., 2011). Although much is known about how presynaptic GABAergic inhibition shapes neuronal output, mechanisms that regulate the development and maintenance of such inhibition onto axon terminals are not yet well understood. Here we addressed the role of neurotransmission in the development, maturation and maintenance of GABAergic synapses onto axonal terminals involved in modulation of neurotransmitter release. To do so, we took advantage of a well-characterized circuit in the mammalian retina where glutamate release from axons is regulated by robust presynaptic GABAergic inhibition.

At dim light levels, visual information from rod photoreceptors is conveyed to rod bipolar cells (RBCs), which relay the signal to amacrine cells in the inner retina (reviewed by Wässle, 2004). Specifically, RBCs contact GABAergic A17 amacrine cells (A17s) (Nelson and Kolb, 1985), which provide local feedback inhibition onto the rod bipolar cell axon terminals to modulate their release of glutamate (Chavez et al., 2006; Chun et al., 1993; Hartveit, 1999). In addition, RBC axon terminals also receive GABAergic drive from other widefield amacrine cells (Chavez et al., 2010; McGuire et al., 1984). Unlike other parts of the brain where GABAergic inhibition is mediated mainly by GABA<sub>A</sub> receptors, GABAergic input onto RBC axon terminals involves both GABA<sub>A</sub> and GABA<sub>C</sub> receptor types (Enz et al., 1996; Koulen et al., 1998). GABA<sub>A</sub> and GABA<sub>C</sub> receptors do not co-cluster at the same synaptic site but are expressed at different postsynaptic sites on RBC axon terminals (Fletcher et al., 1998; Koulen et al., 1998). There are also functional differences between the two receptor types: GABA<sub>A</sub> receptors are less sensitive to GABA but have faster kinetics compared to GABA<sub>C</sub> receptors, and thus the two receptor types regulate different aspects of glutamate release from RBCs (Eggers and Lukasiewicz, 2006b; Sagdullaev et al., 2006). The diversity of GABA receptors on RBCs presents an ideal opportunity to investigate how neurotransmission regulates the development and maintenance of not only GABA receptors, but also different GABA receptor types on the same axon terminal.

To examine the role of neurotransmission on the formation and maintenance of axonal GABA receptors, we utilized transgenic mice in which GABAergic or glutamatergic transmission is perturbed throughout the period of circuit assembly. Using electron microscopy, immunohistochemistry and electrophysiology, we assessed whether perturbed neurotransmission affects the structural formation and maintenance of GABA receptor subtypes on RBC axon terminals. Because RBC terminals also receive some glycinergic inhibition (Eggers et al., 2007; Wässle et al., 2009), we further analyzed whether an upregulation of glycine receptors occurred to compensate for the reduction in GABAergic transmission. Furthermore, we asked whether the loss of GABAergic inhibition causes alterations to RBC output beyond that expected solely from disinhibition in the GABA deficient circuit. Because GABA release from A17 amacrine cells normally requires RBC drive due to the reciprocal synaptic arrangement between these two cell types, we determined whether lack of GABAergic transmission also caused developmental changes in the glutamatergic synapses of A17 amacrine cells.

## RESULTS

### Development of GABAergic synapses on RBC axon terminals

GABAergic inhibition onto axon terminals of RBCs is mediated by a variety of amacrine cells, including A17 cells that provide reciprocal feedback inhibition (Figure 1a). GABA is synthesized in amacrine cells by two isoforms of glutamate decarboxylase, GAD67 and GAD65 (Haverkamp and Wässle, 2000; Vardi and Auerbach, 1995). We found that in the sublamina of the inner plexiform layer (IPL) where RBC axonal terminals stratify (sublamina 5, ON-layer), GAD67 immunoreactivity was more abundant compared to

GAD65 (Figure 1b). Quantification of the immunolabeling (see Methods) showed that dendritic processes positive for GAD67 exhibited greater volume overlap with PKC immunoreactive RBC axonal boutons, compared to GAD65 containing processes (Figure 1c), suggesting that GAD67 positive amacrine cell processes provide the majority of GABAergic inhibition onto RBC axon terminals.

To visualize amacrine cell contacts onto RBC terminals during development, we utilized the *GAD67-GFP* transgenic line (Chattopadhyaya et al., 2004), where a fraction of GAD67 positive-amacrine cells (Figure S1a) express GFP. Amacrine cells typically synapse onto bipolar cell axons at enlarged varicosities (Dowling and Boycott, 1966). Using the *GAD67-GFP* transgenic line we visualized large varicosities of GFP labeled amacrine processes contacting RBC boutons at sites immunopositive for GAD67 but not GAD65 (Figure S1b). We further determined when GABAergic contacts are formed onto RBCs that are labeled in the *GAD67-GFP* line by expression of the fluorescent protein tdTomato under the *grm6* promoter (Figure 1d). Appositions between GAD67 positive processes and RBC axonal boutons were already apparent at postnatal day (P) 10 (Figure 1d), several days after axonal differentiation in the bipolar cells (Morgan et al., 2006). At P15, large varicosities were clearly present at sites of contact between amacrine cell dendrites and RBC boutons, which remained evident at P21. We previously found that spontaneous GABAergic inhibitory postsynaptic currents (sIPSCs) can be recorded from RBCs from P10 (Schubert et al., 2008). Thus, the appearance of structural contacts between GABAergic amacrine cells and RBC axon terminals coincides with the emergence of sIPSCs in RBCs. This suggests that functional postsynaptic GABA receptor clusters are already present on RBC terminals before eye-opening (P14).

Previous studies demonstrated that GABA<sub>A</sub> and GABA<sub>C</sub> receptors are present on axon terminals of RBCs across species (Fletcher et al., 1998; Koulen et al., 1998; Koulen et al., 1997; Lukasiewicz, 1996; Enz et al., 1996; Lukasiewicz et al., 2004; McCall et al., 2002). To monitor the appearance of GABA receptor clusters on RBC axonal boutons during circuit assembly, we labeled for GABA<sub>A</sub> and GABA<sub>C</sub> receptors at two developmental time points, before eye-opening (P12) and at maturity (P30). We found abundant GABA<sub>C</sub> receptor clusters on PKC-labeled axon terminals of RBCs as early as P12 and at P30 (Figure 1e, f). To determine the subunit composition of the GABA<sub>A</sub> receptor clusters located at these terminals, we performed immunostaining for GABA<sub>A</sub>  $\alpha$ 1–3 subunits together with PKC.  $\alpha$ 2-containing GABA<sub>A</sub> receptors were not present on RBC axon terminals (Figure S2a), supporting past findings (Fletcher et al., 1998). In contrast, both  $\alpha$ 1 and  $\alpha$ 3-containing GABA<sub>A</sub> receptor clusters were localized on RBC axonal boutons at both ages examined (Figure 1e, f). GABA<sub>A</sub> synapses containing  $\alpha$ 1 or  $\alpha$ 3 subunits did not co-localize with GABA<sub>C</sub> receptors (Figure S2b) as found previously in adult retina (Koulen et al., 1996; Wässle et al., 1998). Furthermore, these three GABA receptor cluster types ( $\alpha$ 1-GABA<sub>A</sub>,  $\alpha$ 3-GABA<sub>A</sub> and GABA<sub>C</sub>) on RBC boutons were each apposed to large *GAD67-GFP* amacrine cell varicosities (Figure S1c). Co-Immunolabeling for GAD67 and GABA receptors revealed that  $93.27 \pm 0.48\%$  of GABA<sub>A</sub> $\alpha$ 1 ( $n=2$  animals),  $93.89 \pm 0.66\%$  of GABA<sub>C</sub> ( $n=2$ ) and  $79.30 \pm 2.04\%$  of GABA<sub>A</sub> $\alpha$ 3 ( $n=3$ ) receptor clusters on RBC terminals were apposed to GAD67 positive terminals. Thus, all three GABA receptor types are present on RBC terminals before eye-opening, suggesting that synapses comprising these receptor types develop concurrently.

### Synaptic contacts between RBCs and amacrine cells form despite reduction of GABAergic or glutamatergic neurotransmission

To determine the role of neurotransmission in the structural development of inhibitory synapses onto RBC axon terminals, we used two transgenic mouse lines (Figure 2a). First, we asked whether suppressing glutamatergic transmission onto amacrine cells that likely

contact RBCs affects the development of amacrine cell synapses onto RBC axons. This was achieved using the *grm6-TeNT* line in which neurotransmission from all ON-bipolar cells including RBCs, is suppressed by expression of the light chain of tetanus toxin (Kerschensteiner et al., 2009). Next, we assessed the outcome when GABA release from amacrine cells is diminished from development onwards. To do so, we crossed *GAD1* conditional knockout mice (Chattopadhyaya et al., 2004) with  $\alpha$ *Pax6-Cre* mice in which cre-recombinase expression is limited to the retina, to generate *GADIKO* mice (Schubert et al., 2010) (Figure S3). In the *GADIKO* retina, the level of GAD67 expression is reduced (Figure S3a), and immunolabeling for GAD67 reveals that loss of GAD67 (in the knockout region) occurs outside a dorsal-ventral strip (the WT-region) in which cre-recombinase is absent (Figure S3b, c; see Marquardt et al., 2001). We use '*GADIKO*' from here on to refer to the knockout regions.

We compared VIAAT (vesicular inhibitory amino acid transporter) immunolabeling across wildtype, *grm6-TeNT* and *GADIKO* retinas to assess whether amacrine cell terminals surrounding RBC axons failed to differentiate when neurotransmission is perturbed. We found no gross changes in the density of VIAAT labeling surrounding P30 RBC axonal terminals in either transgenic line, suggesting that amacrine cell-RBC synapses still formed (Figure 2b). We confirmed that amacrine cell-RBC synapses still formed in the mutant mice, by examining the ultrastructural arrangement of RBC axonal boutons in both *grm6-TeNT* and *GADIKO* retina. In both lines, amacrine cells still synapsed onto RBC boutons, and these synapses were apparent at eye-opening (P15) (n=14 *GADIKO*; n=26 *TeNT*RBC boutons examined) as in wildtype animals (Figure 2c). However, some aspects of the synaptic arrangements differed between *grm6-TeNT* and *GADIKO* retinas. P30 RBC boutons formed dyad synapses at sites containing a single ribbon in the *GADIKO* (n= 20 RBC synapses per genotype) but such arrangements were disrupted in *grm6-TeNT* retinas where sometimes multiple ribbons were apposed to a single postsynaptic density (n= 16 of 26 at P15, and 13 of 23 synaptic sites had >1 ribbon at P30; e.g. Figure 2c), as shown previously (Kerschensteiner et al., 2009). Thus, although the fine arrangement of the RBC presynapse is differentially affected in the *grm6-TeNT* and *GADIKO* retinas, the amacrine cell-RBC synapse forms and is maintained irrespective of whether bipolar or amacrine cell transmission is perturbed.

### Development and maintenance of GABAergic synapses on RBC axon terminals does not rely on glutamatergic transmission

Although amacrine cell synapses are present structurally on RBC terminals in *grm6-TeNT* retinas, it is possible that suppression of glutamatergic drive from bipolar cells leads to alterations in GABA mediated transmission onto RBC axon terminals. We thus performed whole-cell recordings on wildtype and *grm6-TeNT*RBCs and analyzed spontaneous GABAergic inhibitory postsynaptic currents (sIPSCs) (Figure 3a). Spontaneous IPSCs in both genotypes were comparable in mean frequency and amplitude at both P11–13 and P30 indicating that the number of GABAergic synaptic contacts and the size of postsynaptic GABA receptor clusters were not significantly affected by a reduction of glutamatergic input to amacrine cells (Figure 3b). To evoke release of GABA from presynaptic amacrine cells, we puffed AMPA at the axon terminal of RBCs (Chavez et al., 2006) and recorded the evoked sPSCs at 0 mV holding potential (Figure 3c). The mean charge of AMPA-evoked currents was not different in wildtype and *grm6-TeNT*RBCs at both P11–13 and P30 (Figure S4). To distinguish GABA<sub>A</sub> and GABA<sub>C</sub> components of the evoked response, AMPA puffs were repeated in the presence of TPMPA (GABA<sub>C</sub> receptor antagonist) or SR95531 (GABA<sub>A</sub> receptor antagonist) (Figure 3c). Quantification of mean charge and amplitude (data not shown) of the evoked response (total, GABA<sub>A</sub> or GABA<sub>C</sub>) showed no significant differences between RBCs in wildtype and *grm6-TeNT* retinas at P11–13 (Figure

3d) and at P30 (Figure 3e). Together, these results suggest that functional GABAergic synapses are formed normally and maintained even when the bipolar cells fail to transmit effectively to amacrine cells.

### RBC responses to GABA application decline with maturation in the *GAD1* mutant

To assess the functional consequences of reduced GABA synthesis on the formation and maintenance of inhibitory synapses onto RBC axon terminals, we performed whole-cell recordings of these neurons in the *GAD1KO* retina. As expected, spontaneous sIPSCs were rare in the *GAD1KO* at both ages examined (Figure 4a, b). This indicates that deletion of *GAD67* was not accompanied by a compensatory upregulation of the other GABA synthesizing enzyme, *GAD65*. Because synaptic release of GABA in these mutants is greatly impaired, we puffed GABA onto *GAD1KO* RBC axon terminals and recorded the evoked chloride currents in order to assess whether any postsynaptic changes occurred in the *GAD1KO* RBCs. Interestingly, RBC responses to GABA application in the *GAD1KO* were unchanged at P11–13 but were dramatically reduced at P30 (example recordings in Figure 4c). Both mean amplitude and charge of the evoked responses decreased by P30 (Figure 4d). Moreover, the rise time for evoked responses in P30 *GAD1KO* RBCs was slower, whereas their decay time was faster, as compared to control (Figure 4e). Thus, GABAergic transmission plays an important role in the maintenance, although not in the initial formation, of functional GABAergic synapses on RBC axon terminals.

In wildtype animals, RBCs receive little glycinergic input (Eggers et al., 2007). Indeed, immunolabeling for glycine receptors ( $\alpha 1$ - and  $\alpha 2$ -subunit containing receptor clusters) (Ivanova and Muller, 2006; Wässle et al., 2009) showed little glycine receptor expression on RBC axon terminals (Figure S5a, b). However, a severe reduction of GABAergic transmission in the *GAD1KO* may be compensated for by an upregulation of glycine receptor-driven input onto RBC axon terminals. We investigated this possibility by quantifying the expression of glycine receptors containing  $\alpha 1$  or  $\alpha 2$  subunits on RBC axon terminals in the *GAD1KO* retina. But, we did not find any upregulation of these glycine receptor subtypes in the RBC terminals of the *GAD1KO* (Figure S6).

### GABA<sub>A</sub> and GABA<sub>C</sub> receptors on RBC axon terminals are differentially altered in the *GAD1KO*

Because GABA<sub>A</sub> and GABA<sub>C</sub> receptor clusters are found at distinct postsynaptic sites on RBC axon terminals, we next asked whether loss of GABAergic transmission affected both receptor types. Figure 5a shows examples of the GABA-evoked responses of P30 RBCs from a littermate control and a *GAD1KO* animal in which the GABA<sub>A</sub> component is revealed upon blocking the GABA<sub>C</sub> receptor-mediated current. Quantification of the mean amplitude and charge of the evoked GABA<sub>A</sub> responses in RBCs revealed significant reduction in the KO animal (Figure 5b). Similarly, the GABA<sub>C</sub>-evoked responses were isolated for RBCs in the *GAD1KO* and control upon blocking GABA<sub>A</sub> currents (Figure 5c). In contrast to GABA<sub>A</sub>-mediated responses, the mean amplitude of the GABA<sub>C</sub>-mediated response was unchanged (Figure 5d). However, the net charge carried by the GABA<sub>C</sub> currents was significantly reduced in the *GAD1KO* (Figure 5d). This may reflect faster GABA<sub>C</sub>-mediated response kinetics in RBCs from *GAD1KO* compared to littermate control (see Figure 4e).

To correlate these functional changes at P30 with the expression of GABA<sub>A</sub> and GABA<sub>C</sub> receptor types, we immunostained for the  $\alpha 1$  and  $\alpha 3$  subunits of the GABA<sub>A</sub> receptors, and the  $\rho$  subunits of the GABA<sub>C</sub> receptors. We compared the immunolabeling of P30 knockout regions in the *GAD1KO* mutant with corresponding wildtype regions (which provides an ideal control because these regions are from within the same retina) as well as with

littermate control retinas. For GABA<sub>A</sub> receptors, GAD67 immunostaining was used to distinguish knockout regions from wildtype regions in the *GAD1KO* (Figure 6a,b). However, we could not co-label GAD67 and GABA<sub>C</sub> receptors due to species specificity of the antibodies. Instead, we used the GFP signal to identify the knockout regions because GFP is expressed specifically in cells where the *GAD1* exon is excised (Marquardt et al., 2001). Overall, immunoreactivity for  $\alpha 1$ -containing GABA<sub>A</sub> receptors was significantly reduced in the knockout region compared to the wildtype region and the littermate control (Figure 6a). In contrast,  $\alpha 3$ -containing GABA<sub>A</sub> receptor labeling did not appear to have changed in the knockout regions (Figure 6b). Similarly, GABA<sub>C</sub> receptor staining was comparable across regions and genotypes (Figure 6c). Because of the high density of GABA receptor clusters on RBC boutons, it was not always possible to separate individual clusters. Thus, instead of determining the number of receptor puncta, we quantified the percent volume occupied by each receptor subtype on PKC positive RBC boutons (see Methods). We found that the percent volume occupied by  $\alpha 1$ -containing GABA<sub>A</sub> receptors, but not  $\alpha 3$ -containing GABA<sub>A</sub> receptors or GABA<sub>C</sub> receptors, was significantly reduced in the knockout regions (Figure 6). This reduction of GABA<sub>A</sub> $\alpha 1$  clusters in GAD67 deficient regions was corroborated by using another GABA<sub>A</sub> $\alpha 1$  antibody raised in a different species (Figure S6a). To assess whether GABA<sub>A</sub> $\alpha 1$  synthesis levels in *GAD1KO* retina was diminished overall, we performed western blot analysis using P30 retina homogenates from which the dorsal-ventral wedge was removed. The total GABA<sub>A</sub> $\alpha 1$  synthesis levels in the *GAD1KO* mutant was comparable to littermate control samples (Figure S6b, n=2 *GAD1KO* and 2 littermate controls), suggesting that overall synthesis of GABA<sub>A</sub> $\alpha 1$  receptors in GAD67 deficient retina was unchanged. The reduction in immunostaining for GABA<sub>A</sub> $\alpha 1$  in the *GAD1KO* is thus unlikely to be caused by an alteration in receptor synthesis, and is more likely the result of a deficit in the maintenance of GABA<sub>A</sub> $\alpha 1$  clusters on RBC terminals. Because we found little expression of the known inhibitory postsynaptic scaffolding proteins, Neuroligin2 and Gephyrin, at GABA<sub>A</sub> receptors in RBC axon terminals (Figure S5b, c), we decided against analyzing the expression levels of these proteins in *GAD1KO* RBCs. Taken together, our findings demonstrate that GABA<sub>A</sub> and GABA<sub>C</sub> receptors on RBC axon terminals are differentially maintained by GABAergic transmission.

The specific reduction of GABA<sub>A</sub> and not GABA<sub>C</sub> receptor clusters in *GAD1KO* RBCs suggests an independent regulation of the maintenance of these two ionotropic GABA receptor types that coexist on the same axonal terminal. To further test this hypothesis, we immunolabeled  $\alpha 1$ -containing GABA<sub>A</sub> receptor clusters in *GABA<sub>C</sub>KO* mice (McCall et al., 2002)(Figure S7). We found no alteration in the percent volume occupied by  $\alpha 1$ -containing GABA<sub>A</sub> receptors in P30 *GABA<sub>C</sub>KO* RBC terminals (Figure S7). Thus, we conclude that there exists independent regulatory mechanisms for maintaining GABA<sub>A</sub> and GABA<sub>C</sub> receptor clusters on RBC axon terminals.

### Reduced GABAergic drive transiently increases glutamate release from developing RBCs

GABAergic A17 amacrine cells form reciprocal synapses with glutamatergic RBCs. We thus wondered whether there were corresponding changes in the RBC input onto the A17 cells in the *GAD1KO*. We compared RBC input onto A17 cells in *GAD1KO* with wildtype littermate controls at two developmental time-points, P11-P13 and P30, by recording spontaneous excitatory postsynaptic currents (sEPSCs) from A17 amacrine cells. In littermate controls, the mean frequency of sEPSCs in these amacrine cells normally increased with age (Figure 7a,b). In the *GAD1KO*, the mean frequency of sEPSCs from A17 amacrine cells at P11–13 was significantly higher compared to their control littermates (Figure 7a, b). In the KO, there was no further increase in sEPSCs frequency in the A17 cells with maturation (Figure 7a, b), and in fact, the P11–13 sEPSCs were already

comparable to that of P30 controls. These observations together suggest that although the RBC output is initially elevated during development when inhibition is reduced, homeostatic mechanisms ensure that the bipolar cell output operates within its normal range at maturity (Figure 7b).

Is the transient increase in frequency of the sEPSCs from developing A17 cells in the KO simply a result of reduced inhibition onto the RBC axonal terminals? To answer this, we recorded sEPSCs from A17s in P11-P13 *GAD1KO* and littermate control animals in the presence of GABA<sub>A</sub> and GABA<sub>C</sub> receptor blockers, SR95531 and TPMPA respectively (Figure 7c). Even in the presence of GABA receptor blockers, P11-P13 A17 cells showed an increased frequency of sEPSCs in the *GAD1KO* as compared to control (Figure 7c), suggesting that there is an upregulation of glutamatergic drive onto A17 cells in the *GAD1KO*. This upregulation, however, is not the consequence of a loss of GABA<sub>C</sub>-receptor mediated transmission alone, despite GABA<sub>C</sub> receptors carrying the majority of the total charge transfer (Figure 5b,d; Eggers and Lukasiewicz, 2006a; McCall et al., 2002). In the presence of a GABA<sub>A</sub> receptor antagonist, the mean sEPSC frequency of P11–13 A17 cells in the GABA<sub>C</sub> receptor KO is not significantly different from that of littermate controls (Figure S8).

Could the upregulation of glutamatergic drive onto developing A17 cells be due to changes in receptor density on A17 cells? We found that at P11–13, the mean amplitude of the sEPSCs was unchanged for A17s in the *GAD1KO* (Figure 7d,e), indicating that the glutamate receptor density at individual postsynaptic sites is not altered. Also, A17 cell responses to AMPA puffs revealed no differences between *GAD1KO* and control (Figure 7f, g). Thus, the total density (or number) of glutamatergic synapses on A17s is unperturbed in *GAD1KO* animals (Figure 7d-g). This suggests that the increase in A17 sEPSC mean frequency in the *GAD1KO* is not the result of changes in glutamate receptor density on the A17 cell, but more likely due to presynaptic changes, such as the probability of release, in the RBC terminal.

## DISCUSSION

We found that RBC axon terminals receive GABAergic inhibition from GAD67-positive amacrine cells via three distinct GABA receptor subtypes (GABA<sub>A</sub>α1, GABA<sub>A</sub>α3 and GABA<sub>C</sub>). Using mutant mice, we found that GABAergic synapses are still established on RBC axonal terminals when either glutamatergic or GABAergic transmission is perturbed. However, the maintenance of GABA<sub>A</sub>α1 receptor clusters on RBC axonal terminals is selectively disturbed when GABA synthesis is much reduced in the presynaptic amacrine cells (Figure 8). Further, the maintenance of GABA<sub>A</sub>α1 receptor clusters is not dependent on the presence or synaptic drive via GABA<sub>C</sub> receptors. We also discovered that glutamate release from developing RBCs increased in the *GAD1KO* mutant, but not in the *GABA<sub>C</sub>KO* mutant (Figure 8).

### **GABAergic synapse maintenance but not formation on axon terminals is influenced by GABAergic transmission**

How neurotransmission modulates the formation of inhibitory synapses has primarily been addressed for inhibitory synapses onto somata and dendrites of neurons (Chattopadhyaya et al., 2007; Harms and Craig, 2005; Kilman et al., 2002). Blocking global activity in developing pyramidal neurons in culture leads to a decrease in the number and size of inhibitory synapses (Kilman et al., 2002). Here, we assessed the importance of neurotransmission in the development of GABAergic synapses on axon terminals, focusing on amacrine cell-RBC connectivity. Our results demonstrate that presynaptic GABAergic amacrine cells do not require glutamatergic drive from bipolar cells to establish their

inhibitory synapses onto the axons. GABA release onto RBCs as well as GABA receptor density of RBCs are unchanged in the *grm6:TeNT* retinas. This is in contrast to a reduced number of inhibitory synapses and decrease in synaptic vesicle density of terminals contacting the dendrites of spinal cord neurons cultured in the presence of glutamate (non-NMDA) receptor antagonists (Rosato-Siri et al., 2002).

Prior work has demonstrated that GABAergic transmission is not essential for inhibitory synapse formation on dendrites and somas *per se* (Chattopadhyaya et al., 2007; Wojcik et al., 2006; Wu et al., 2012). We found this to also be true for inhibitory synapse formation onto axon terminals as amacrine cell-RBC synapses are evident in the retinal-specific *GADIKO*. Deletion of GAD67 in basket cells of the visual cortex, however, results in fewer perisomatic inhibitory synapses on pyramidal neurons (Chattopadhyaya et al., 2007). This reduction in inhibitory synapse number onto the cell bodies appears to be due to the lack of GABAergic transmission during synaptogenesis, rather than a failure to maintain established synapses. In the retina, we found that reducing GABAergic transmission during development affects the maintenance of GABA receptors on the RBC axons but not the initial formation of these synapses. From previous work (Burrone and Murthy, 2003; Pozo and Goda, 2010; Turrigiano, 2007), we had expected that RBCs in the *GADIKO* might undergo homeostatic adjustment and recruit more GABA receptors to their axons to compensate for reduced GABAergic transmission. Instead, we observed that RBC axon terminals lose GABA receptors at maturity when presynaptic GABA release is reduced chronically.

#### Activity dependent maintenance of axonal GABA receptors is receptor subtype specific

To date, most studies focusing on the activity-dependent maintenance of GABA<sub>A</sub> receptors in neurons have assessed the distribution of the entire GABA<sub>A</sub> receptor population, irrespective of their subunit composition. This is because in most parts of the nervous system, GABA<sub>A</sub> receptors can comprise mixed  $\alpha$ -subunits together with  $\beta$ - and  $\gamma$ - subunits (Fritschy and Mohler, 1995; Kasugai et al., 2010). However, in the mammalian retina, three distinct subtypes of GABA<sub>A</sub> receptors can be distinguished by the presence of specific  $\alpha$ -subunits ( $\alpha 1$ - $\alpha 3$ ) localized at non-overlapping synapses (Koulen et al., 1996; Wässle et al., 1998). On mouse RBC axon terminals, we identified two types of GABA<sub>A</sub> receptor synapses, containing either the  $\alpha 1$ -subunit or the  $\alpha 3$ -subunit. Both these GABA<sub>A</sub> receptor types were apposed to GAD67-positive processes but, functionally, they could provide GABA<sub>A</sub> receptor-mediated inhibition with different time courses, because  $\alpha 1$ -containing GABA<sub>A</sub> receptors exhibit faster response kinetics compared to  $\alpha 3$ -containing receptors (Gingrich et al., 1995; Ortinski et al., 2004; Vicini et al., 2001). Surprisingly, we found that reduced GABAergic neurotransmission selectively regulated the maintenance of GABA<sub>A</sub> $\alpha 1$  but not GABA<sub>A</sub> $\alpha 3$  receptor clusters.

Reducing GABAergic transmission also differentially affected the maintenance of the two ionotropic GABA receptor types (GABA<sub>A</sub> and GABA<sub>C</sub>) on RBC axons. While the amplitude of the GABA<sub>A</sub> receptor component of the GABA-evoked response was decreased at P30 in the *GADIKO*, there was no change in amplitude of the GABA<sub>C</sub> component. Our immunostaining for GABA<sub>A</sub> and GABA<sub>C</sub> receptors supported this differential reduction in GABA<sub>A</sub> versus GABA<sub>C</sub> receptors on RBC axons. What might be the differences in GABA<sub>A</sub> and GABA<sub>C</sub> receptors that could account for this differential outcome in the *GADIKO*? GABA<sub>C</sub> receptors have a higher affinity for GABA compared to GABA<sub>A</sub> receptors (Feigenspan and Bormann, 1994). Perhaps these receptors require only low levels of GABA for their maintenance. GABA<sub>A</sub> and GABA<sub>C</sub> receptors do not colocalize at the same postsynapse (Fletcher et al., 1998; Koulen et al., 1998). Thus, it could also be that these two GABA receptor types are differentially positioned on the RBC terminal relative to the GABA release site, such that GABA<sub>A</sub> receptors normally 'sense' higher GABA levels



compared to GABA<sub>C</sub> receptors, and thus need a substantial amount of GABA in the synaptic cleft for their maintenance. We further showed that loss of GABA<sub>C</sub> receptors *per se* does not affect the maintenance of GABA<sub>A</sub>α1 receptors in the RBC terminals. We found no down-regulation of GABA<sub>A</sub> receptors in the *GABA<sub>C</sub>KO* retina. Furthermore, in the *GABA<sub>C</sub>KO* retina, the function of glycine receptors (Eggers and Lukasiewicz, 2006a) on RBCs axon terminals is not affected. Accordingly, in the *GADIKO* we found no upregulation of glycine receptor clusters on RBC axon terminals. Taken together, our observations highlight independent mechanisms for regulating the distribution and density of distinct inhibitory receptor types (GABA receptors versus glycine receptors, GABA<sub>A</sub> versus GABA<sub>C</sub> receptors, GABA<sub>A</sub>α1 versus GABA<sub>A</sub>α3 receptor types) on the same RBC axon terminal.

What underlies the eventual reduction of α1-containing GABA<sub>A</sub> receptors in the *GADIKO*? The role of activity in inhibitory receptor accumulation has been addressed in spinal cord cultures, where blocking neuronal activity by TTX application prevented glycine receptor accumulation (Kirsch and Betz, 1998). However, our physiological recordings suggest that GABA receptors accrue normally on RBC axons initially (P11–13), and total GABA<sub>A</sub>α1 synthesis in adult (P30) *GADIKO* retina also appeared unimpaired. Another possibility is a failure to stabilize GABA<sub>A</sub>α1 receptor clusters after they have formed at synapses. Tracking movements of the inhibitory postsynaptic scaffold protein gephyrin in dissociated spinal cord neurons previously demonstrated activity-dependent stabilizations of the gephyrin-fluorescent protein conjugates at synaptic vs extrasynaptic locations (Hanus et al., 2006). The phenotypic alterations we observed in density of α1-containing GABA<sub>A</sub> receptors on RBC axons in the *GADIKO* could be a result of failed stabilizations of GABA<sub>A</sub> receptors at this synapse. This could lead to greater receptor internalization and subsequent degradation detectable as reduced receptor volume occupancy. Indeed, blocking neuronal activity has been shown previously to induce ubiquitination and degradation of GABA<sub>A</sub> receptors (Luscher et al., 2011). However, because Neurologin2 and Gephyrin do not appear to be at all GABA<sub>A</sub>α1 receptor clusters on the RBCs, future work is necessary to identify the postsynaptic scaffold proteins at the RBC axonal GABA receptors before we can systematically investigate the molecular mechanisms underlying the loss of GABA<sub>A</sub>α1 receptor clusters on RBC axon terminals in the *GADI* mutant.

### **RBCs adjust their output homeostatically in response to reduced GABAergic drive**

We found that RBCs increase their output onto A17 amacrine cells with maturation. Our study shows that this output is structurally and functionally modified by alterations in neurotransmission during development and circuit maturation. When glutamate release from RBCs is suppressed by TeNT expression in these cells, more ribbons are often recruited to RBC output sites. When GABAergic transmission is impaired, glutamate release from developing RBC terminals is increased beyond that expected solely from disinhibition in the GABA deficient circuit, perhaps as an attempt to facilitate GABA release from the ‘silenced’ A17 amacrine cells with which it shares a reciprocal synapse. Interestingly, this increase in glutamate release in developing RBCs in *GADIKO* retinas does not appear to evoke a change in glutamate receptor density on the A17 amacrine cell. The lack of a complementary change in the A17 cell is unexpected for two reasons: (i) the RBC-A17 synapse is bidirectional, and (ii) in other systems, down-regulation of postsynaptic glutamate receptors occurs when there is enhanced presynaptic glutamate release due to homeostatic mechanisms coming into play (Burrone and Murthy, 2003; Pozo and Goda, 2010; Turrigiano, 2007). In the *GADIKO*, RBC output further undergoes homeostatic regulation with maturation to ‘cap’ or limit its output with circuit maturation, perhaps to maintain normal processing in the scotopic pathway involving the other postsynaptic partner, the AII amacrine cell (Bloomfield and Dacheux, 2001). This later homeostatic regulation of the

RBC output parallels the loss of GABA<sub>A</sub> receptors on the RBCs, but these events are unlikely to be related. This is because the progressive loss of GABA receptors does not affect inhibition on the RBCs as there is little GABA release in the *GADIKO*. Our results highlight divergent mechanisms behind the regulation of output from RBC axon terminals and GABA receptor maintenance on these terminals. Our current findings also underscore the importance of assessing changes in GABA receptors on axons in addition to the somata and dendrites of neurons for understanding neurodevelopmental disorders such as schizophrenia where GABAergic transmission is perturbed (Lewis et al., 2005; Wassef et al., 2003).

## METHODS

### Transgenic mouse lines

Several transgenic mouse lines were used in this study. In the *GAD67-GFP (G42)* line, the *GAD67* promoter drives GFP expression in a fraction of GABAergic amacrine cells (Chattopadhyaya et al., 2004). In the *grm6-tdtomato* mouse line, the red fluorescent protein *tdtomato* is expressed specifically by ON-bipolar cell under the mGluR6 promoter (Kerschensteiner et al., 2009). In *GAD1/lox/lox* mice (Chattopadhyaya et al., 2007), exon 2 of *GAD1*, the gene encoding GAD67 (Bu et al., 1992), is flanked by loxP sites. To obtain retina-specific excision of exon 2, this line was bred to the  $\alpha$ -*Cre* line, in which Cre-recombinase expression is regulated by the alpha enhancer of the Pax6 promoter (Marquardt et al., 2001). The excision of exon 2 in cells expressing Cre-recombinase results in a frameshift mutation of *GAD1* (Chattopadhyaya et al., 2007). We refer to this double transgenic line as *GADIKO*. To abolish glutamatergic transmission in RBCs, we used *grm6-TeNT* transgenic in which the light chain of tetanus toxin is expressed specifically in ON bipolar cells (Kerschensteiner et al., 2009). To eliminate GABA<sub>C</sub> receptors from the retina, the gene encoding GABA<sub>C</sub>p1 subunit was inactivated (McCall et al., 2002); we refer to these mice as *GABA<sub>C</sub>KO*.

### Immunohistochemistry

Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Washington. All procedures were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice deeply anaesthetized with Isoflurane were decapitated and enucleated. The cornea was punctured with a 30 gauge needle and the retinas removed in cold oxygenated mouse artificial cerebrospinal fluid (mACSF, pH 7.4) containing (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl<sub>2</sub>, 1.3 MgCl<sub>2</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 11 glucose, and 20 HEPES. For vibratome sectioning, the retina was fixed for 20 min in 4% paraformaldehyde in mACSF (pH 7.4). For fixed flat-mount preparations, retinas were isolated and mounted retinal ganglion cell side up on black membrane filters (Millipore, HABP013). The retina and filter paper were then immersed in 4% paraformaldehyde in mACSF (pH 7.4) for either 15 mins (for GABA<sub>A</sub> $\alpha$ 1 and GABA<sub>A</sub> $\alpha$ 3 immunolabeling) or 30 mins (for GABA<sub>C</sub> labeling). After fixation, the tissue was washed in 0.1 M phosphate-buffered saline (PBS, pH 7.4), preincubated in PBS containing 5% normal donkey serum (NDS) and 0.5% Triton X-100 and incubated with primary antibodies in the same solution. For retinal whole-mounts, primary antibody incubation was performed over 3 nights. Secondary antibody incubation was carried out in PBS and retinas were subsequently mounted in Vectashield (Vector Labs). Immunolabeling was performed using antibodies directed against PKC (rabbit polyclonal, 1:1000, Chemicon or mouse monoclonal, 1:1000, Sigma), GAD67 (mouse monoclonal, 1:1000, Chemicon), GAD65 (rabbit polyclonal, 1:1000, Chemicon), VIAAT (guinea pig polyclonal, 1:1000, Synaptic Systems), GFP (chicken polyclonal, 1:1000, Abcam), Glycine receptor  $\alpha$ 1-subunit (mouse monoclonal mAb2b, 1:500, Synaptic systems, Glycine receptor  $\alpha$ 2-subunit (goat polyclonal, 1:300,

Santa Cruz), Gephyrin (mouse monoclonal 3B11, 1:1000, Synaptic Systems), Neuroligin2 (rabbit polyclonal, 1:4000, kindly provided by F. Varoqueaux and N. Brose), GABA<sub>C</sub> receptor subunit (rabbit polyclonal, 1:500, kindly provided by R. Enz), GABA<sub>A</sub>α1 receptor subunit (polyclonal guinea-pig, 1:5000, kindly provided by J.M. Fritschy), GABA<sub>A</sub>α1 receptor subunit (polyclonal rabbit, 1:2000, kindly provided by J.M. Fritschy), GABA<sub>A</sub>α2 receptor subunit (polyclonal guinea-pig, 1:2000, kindly provided by J.M. Fritschy), and GABA<sub>A</sub>α3 receptor subunit (polyclonal guinea-pig, 1:3000, kindly provided by J.M. Fritschy). Secondary antibodies utilized were either anti-isotypic Alexa Fluor conjugates (1:1000, Invitrogen) or DyLight conjugates (1:1000, Jackson ImmunoResearch).

### Imaging and Analysis

Image stacks were acquired on a Olympus FV1000 laser scanning confocal microscope. Fixed tissue was imaged using a 1.35 NA 60X oil immersion objective at a voxel size of 0.069–0.069–0.3 μm or 0.051–0.051–0.3 μm (x-y-z) for images used in quantification. To visualize RBC – amacrine cell contacts at different developmental time points, we crossed the *GAD67-GFP* and *grm6-tdtomato* mouse lines and imaged the retinas using a custom-built 2-photon microscope and an Olympus 60X water objective. Each optical plane was averaged 3–4 times. Raw image stacks were processed using MetaMorph (Universal Imaging) and Amira (Mercury Computer Systems). For volume estimation, the PKC signal of RBC boutons was masked in 3 dimensions using the ‘label field’ function of Amira. Subsequently this PKC mask was multiplied with the GAD or GABA receptor channel to isolate signal specifically within the RBC boutons. Next, a constant threshold for image stacks from the same retina (including WT-KO region comparison across the retina), was applied to detect the volume occupied by the signal using the ‘label voxel’ function of Amira. The % of signal volume as compared to PKC mask volume was estimated by the ‘tissue statistics’ function of Amira. The specificity of GABA receptor antibodies utilized in this study was tested by performing colocalization analysis and assessing the extent of random associations (Soto et al., 2011). In vertical retinal sections, we found an 87% apposition of GABA<sub>A</sub>α3 receptor clusters with the presynaptic marker VIAAT.

### Electron Microscopy

Retinas were fixed by eye-cup immersion in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 3 hours. The tissue was then washed in buffer, fixed further in 1% osmium tetroxide in cacodylate buffer for an hour prior to *en bloc* staining with 1% uranyl acetate. The tissue was then dehydrated in graded ethanol series, embedded in araldite resin, sectioned and stained with 5% lead citrate.

### Western Blot

Retinas from *GADIKO* and littermate control mice (2 retinas pooled per animal) were prepared on ice in homogenizing buffer (50 mM TRIS, 150 mM NaCl, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS) containing protease inhibitor cocktail. Samples (30 μg/ml per lane) were run by SDS-PAGE (10% resolving gel), blotted onto nitrocellulose membranes, incubated with antibodies, and visualized with ECL. Primary antibodies utilized: GAD67 (mouse monoclonal, 1:2000, Chemicon), GABA<sub>A</sub>α1 receptor subunit (polyclonal rabbit, 1:2000, kindly provided by J.M. Fritschy) and Actin (mouse monoclonal, 1:3000, Chemicon). For preparation of samples to compare GABA<sub>A</sub>α1 levels from retinal homogenates, the dorsal-ventral wedge of the retina was not included for both *GADIKO* and littermate control samples.

## Preparation of mouse retinal slices for whole-cell recording

The preparation of retinal slices has been described in detail by Eggers and Lukasiewicz (Eggers and Lukasiewicz, 2006a). In brief, the eyecup was incubated for 20 min in mACSF containing 0.5 mg/ml hyaluronidase (Sigma) to remove any remaining vitreous. The retinas were then put onto filter paper (Millipore, HABP013) with the photoreceptor layer facing up. Vertical retinal slices of 200  $\mu\text{m}$  thickness were cut using a standard technique (Werblin, 1978) and subsequently stored in carbogenated mACSF at room temperature.

## Whole-cell recordings and data analysis

Whole-cell recordings were made from RBCs (in *GADIKO* and *grm6-TeNT*) and A17s (in *GADIKO* and *GABA<sub>C</sub>KO*) in retinal slices as previously described (Eggers and Lukasiewicz, 2006a; Schubert et al., 2008). The resistance of the electrodes with standard solutions usually ranged between 6 and 8 M $\Omega$ . Liquid junction potentials of 15 mV were corrected before measurements with the pipette offset function. Series resistance (mean:  $67.6 \pm 3.7$  M $\Omega$ , n=18) and capacitance of pipettes as well as cell capacitance were not compensated. Seal resistances > 1 G $\Omega$  were routinely obtained. RBCs were voltage-clamped at 0 mV, the reversal potential for cation-mediated currents through ionotropic glutamate receptors to record GABA-evoked currents in RBCs, and GABAergic spontaneous postsynaptic currents (sIPSCs). For all RBC recordings, glycinergic input and network activity was blocked by strychnine (0.5  $\mu\text{M}$ ) and TTX (1  $\mu\text{M}$ ), respectively. For A17 amacrine cells, excitatory postsynaptic currents through ionotropic glutamate receptors (sEPSCs) were recorded at a holding potential of -60 mV, the reversal potential for chloride-mediated currents set by the internal solution (Schubert et al., 2008). Action potentials in A17s were blocked by including QX-314 (2 mM) in the intracellular solution. For recording sEPSCs from P11-P13 A17s in the presence of GABA receptor blockers, TPMPA (50  $\mu\text{M}$ ) and SR95531 (5  $\mu\text{M}$ ) was included in the extracellular mACSF solution. The holding current was monitored during the experiment, and recordings with shifting holding currents were aborted. All experiments were carried out at room temperature (20–22°C) and under room light conditions.

Data acquisition was performed using an Axopatch 200B amplifier with an acquisition frequency of 10 kHz using the Clampex software (Axon Instruments, Foster City, CA). Signals were 2 kHz Bessel filtered. Data were further processed using a routine written in Igor Pro 6 (Wavemetrics, Portland, OR) and visualized with Origin software (Microcal, Northampton, MA). To quantify parameters of spontaneous synaptic events, the mean frequency  $\pm$  S.E.M and the mean peak amplitude  $\pm$  S.E.M of the events were determined. To quantify evoked currents, peak amplitude and total charge transfer (as complete area under the curve), both relative to the pre-application baseline current, were determined and averaged across cells. In addition, we characterized the kinetics of the evoked current by determining the time points from the exponential fits where the traces reached 20% and 80% of the peak amplitude, and calculated the differences, t<sub>20–80</sub> rise and t<sub>80–20</sub> decay.

## Solution and drugs

The extracellular solution used for recording (mACSF) contained (in mM): 122.5 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub> and 20 glucose, adjusted to pH 7.4 with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The intracellular solution contained (in mM): 120 cesium gluconate, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 Na-Hepes, 11 EGTA, 10 TEA-Cl and was adjusted to pH 7.2 with CsOH. Under our experimental conditions, the calculated chloride equilibrium potential was -59.3 mV. To block GABA<sub>C</sub> receptors and GABA<sub>A</sub> receptors, (1,2,5,6-tetrahydropyridine-4yl) methyphosphinic acid (TPMPA, 50  $\mu\text{M}$ ) and SR-95531 (5  $\mu\text{M}$ ) were used, respectively. All drugs were purchased from Sigma, St.Louis, MO. Solutions in the recording chamber were exchanged using a gravity-driven superfusion system previously

described (Lukasiewicz and Roeder, 1995). GABA receptor and glutamate receptor agonists, GABA (200  $\mu\text{M}$ ) and AMPA (100  $\mu\text{M}$ ), were dissolved in mACSF with 0.005% sulforhodamine B and applied with a puff pipette (5–7 M $\Omega$ ) using a Picospritzer system. The puffing direction as well as the duration of the 300 ms puff were chosen such that the axonal terminal of the patched RBC was completely covered by the puff, visualized by including sulforhodamine B in the puffer pipette.

### Statistics

For comparisons across data sets, the Wilcoxon rank-sum test was used in all cases. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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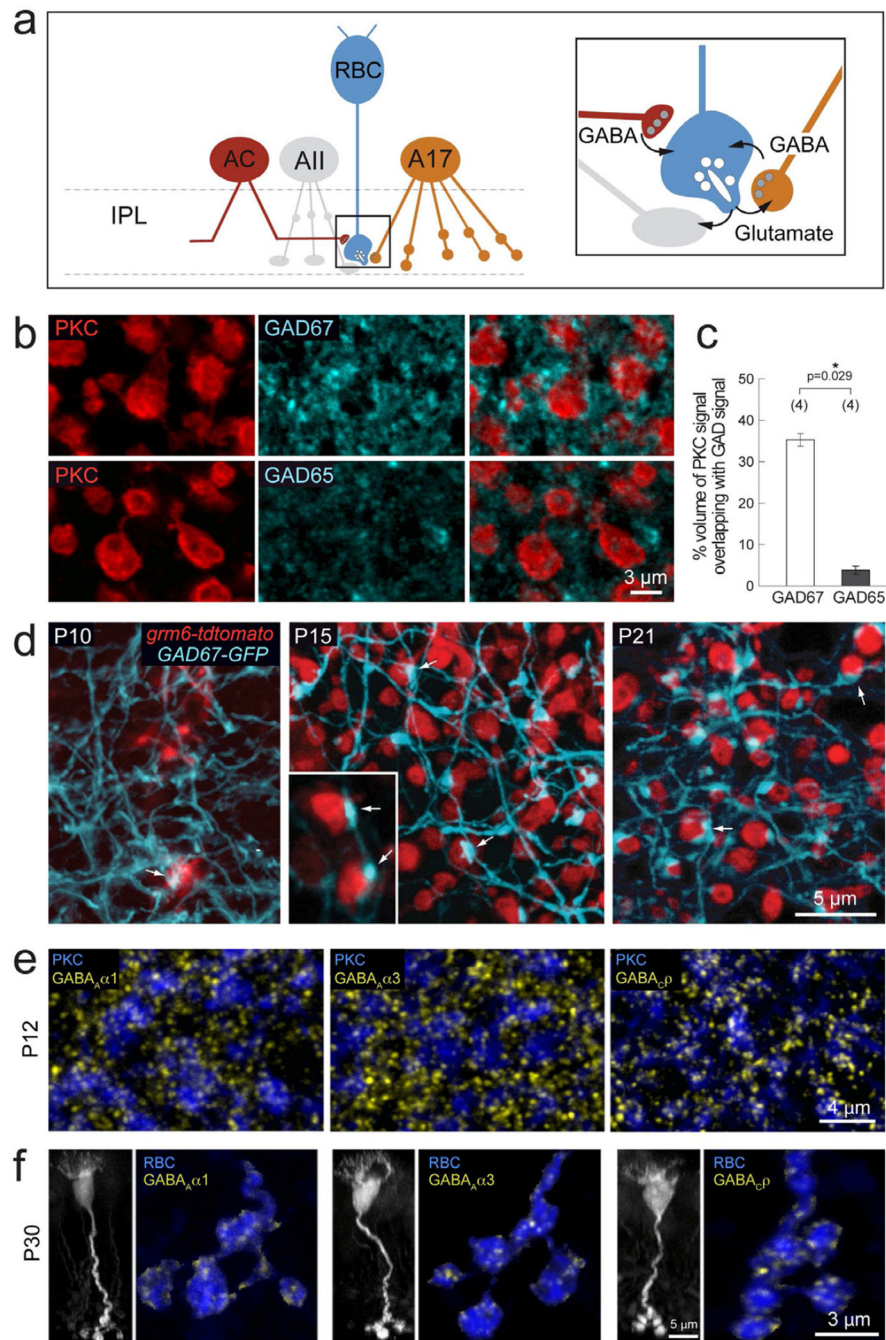
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### Highlights

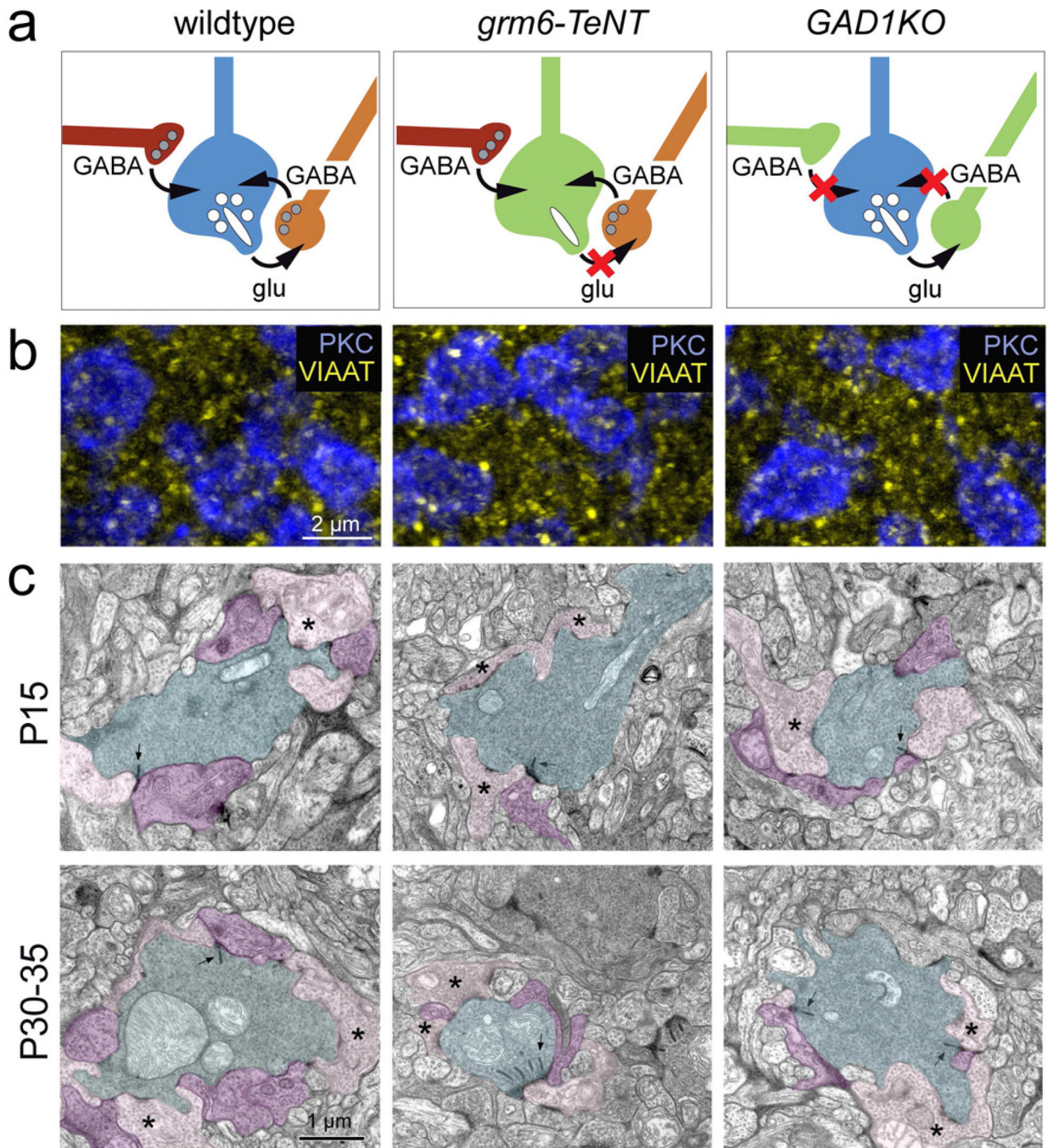
1. GABAergic but not glutamatergic transmission maintains axonal GABA receptors.
2. GABA controls maintenance of axonal GABA<sub>A</sub>α1 but not GABA<sub>A</sub>α3 or GABA<sub>C</sub> receptors.
3. GABA<sub>C</sub> receptor loss does not perturb RBC GABA<sub>A</sub> receptor distribution.
4. RBCs homeostatically regain normal output despite loss of presynaptic inhibition.



**Figure 1. Development of GABAergic synapses onto RBC axon terminals**

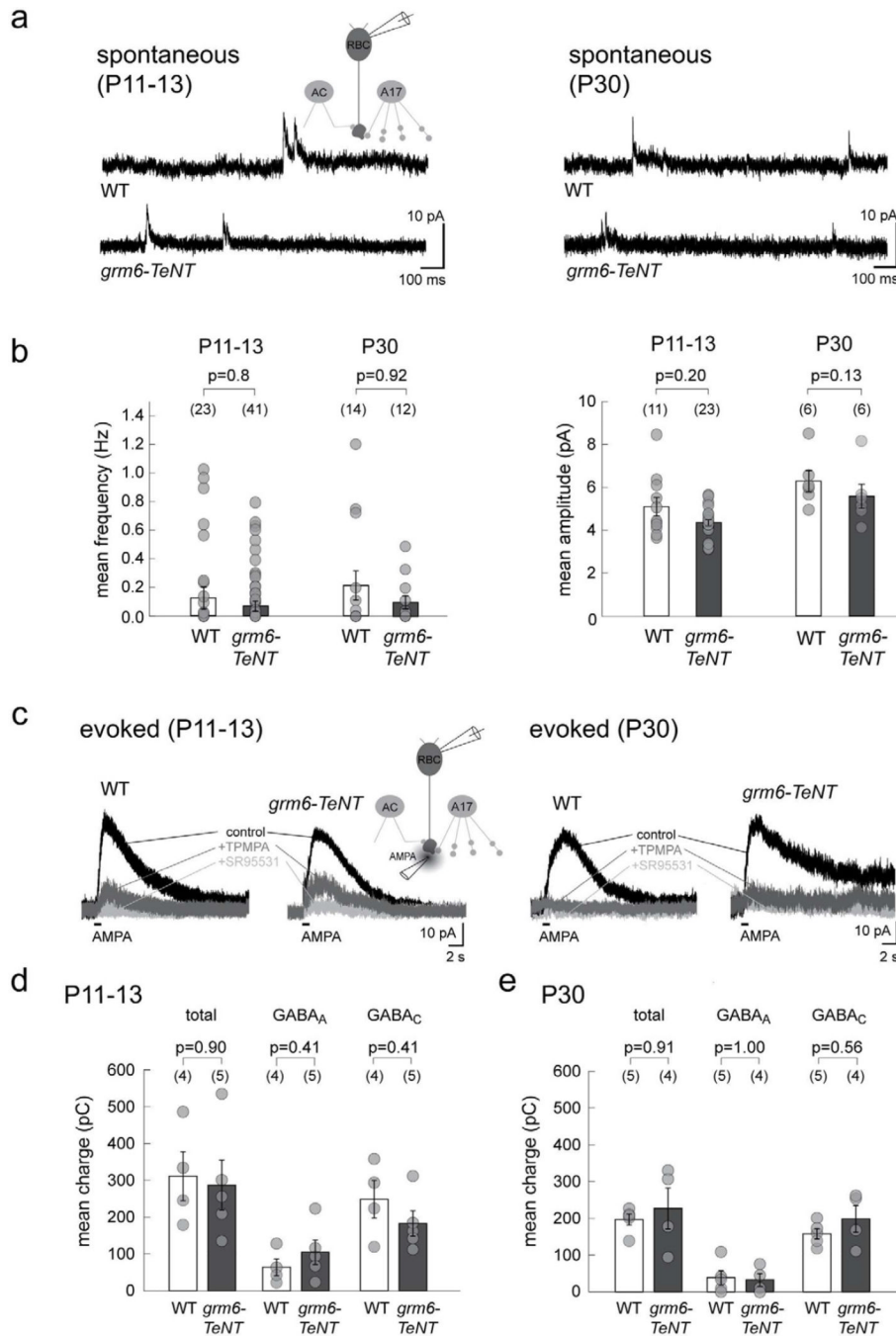
(a) Schematic showing RBC connections with amacrine cells in retina IPL. RBCs (blue) provide excitatory glutamatergic drive (glu) onto glycinergic AII (grey) and GABAergic A17 (orange) amacrine cells. In turn, they receive feedback inhibition from A17 cells and additional GABAergic input from other as yet unidentified amacrine cells (AC, red). (b) PKC labeled RBC terminals (red) colabeled with GAD67 or GAD65 (cyan), reveal more abundant immunoreactivity of GAD67 as compared to GAD65 in the IPL lamina where RBC axons stratify. (c) Quantification of % volume of GAD signal overlapping with PKC immunoreactivity revealed a significantly higher % overlap with GAD67 positive processes

as compared to GAD65. Asterisk marks significant difference. **(d)** Development of contacts (arrows) between RBCs (red) and GAD67-GFP positive amacrine processes (cyan) in the *grm6-tdtomato*×*GAD67-GFP* double transgenic line. By P15, large varicosities can be seen at sites of contact between RBC axon terminals and GAD67-GFP amacrine processes. **(e)** Axonal terminals of P12 PKC-labeled RBCs (blue) express GABA<sub>A</sub>α1, GABA<sub>A</sub>α3, and GABA<sub>C</sub>P receptor clusters (yellow), indicating the presence of these three GABAergic postsynapses on RBC terminals before eye-opening. **(f)** P30 RBCs in the *grm6-tdtomato* transgenic line (left, vertical view, grayscale). Axonal terminals of individual P30 RBCs (horizontal view, blue) express all three GABA receptor cluster types: GABA<sub>A</sub>α1, GABA<sub>A</sub>α3, and GABA<sub>C</sub>P (yellow),



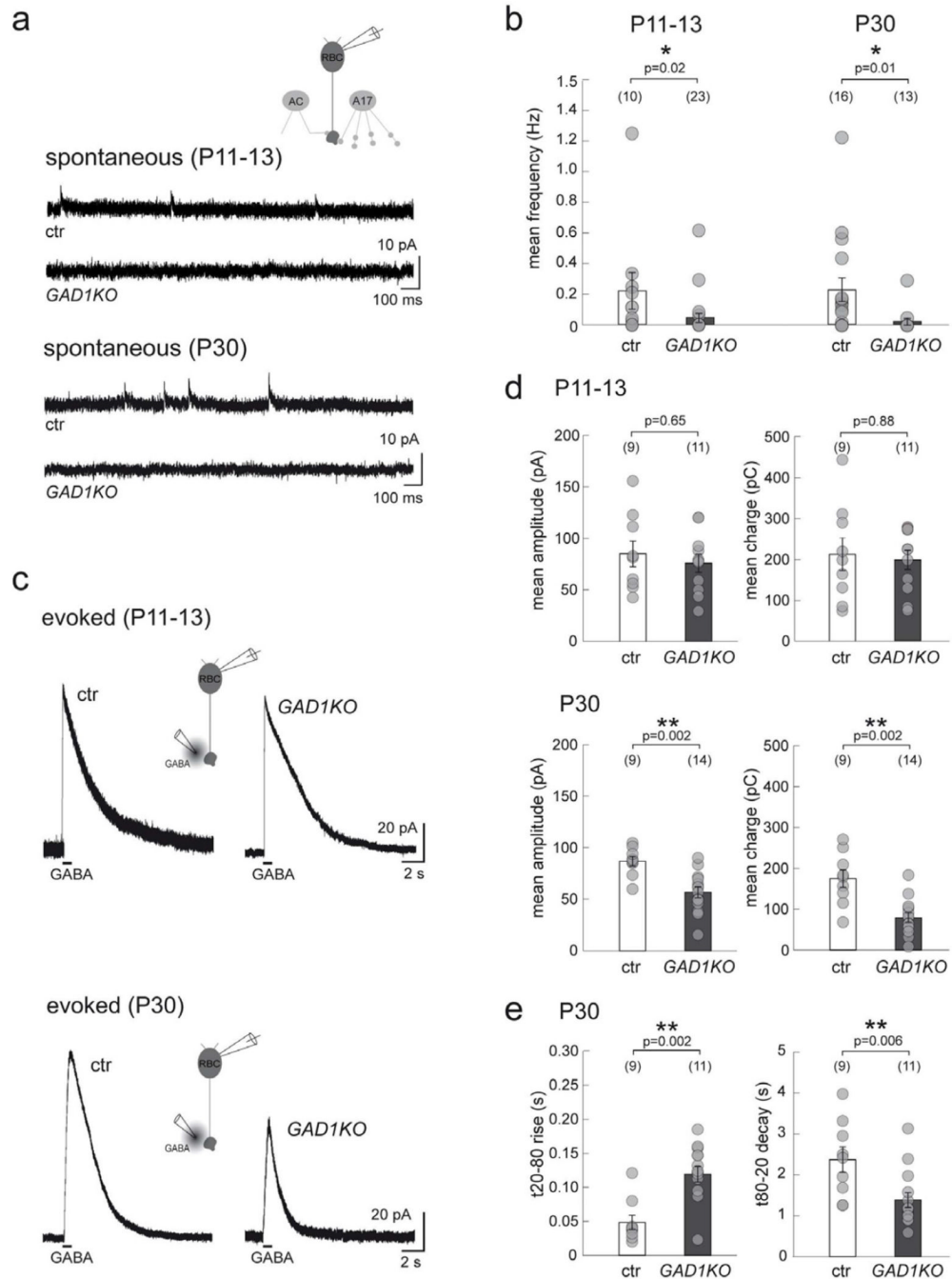
**Figure 2. Inhibitory synapses onto RBC axons in the *grm6-TeNT* and *GAD1KO* retina**  
**(a)** Schematic showing RBC axon terminal circuitry in wildtype and transgenic lines with suppressed glutamatergic transmission from ON-bipolar cells (*grm6-TeNT*) and reduced GABAergic transmission from amacrine cells (*GAD1KO*). **(b)** Immunolabeling for VIAAT (yellow) at the level of PKC positive RBC boutons (blue) in P30 wildtype, *grm6-TeNT* and *GAD1KO* retina. **(c)** Electron micrographs showing dyad synapses between RBC boutons (cyan) and AII (purple) and A17 (pink) amacrine cell processes in the wildtype (WT), *grm6-TeNT* and *GAD1KO* retinæ at P15 and P30–35. Presynaptic ribbons are indicated by

arrows. Asterisks mark examples of A17 contact. Note that RBCs in the P30 *grm6-TeNT* retina have multiple ribbons at a single dyad synapse.



**Figure 3. Spontaneous and GABA-evoked currents of RBCs in the *grm6-TeNT* retina**  
**(a)** Spontaneous GABAergic currents recorded in *grm6-TeNT* and wildtype (WT) RBCs at P11–13 and P30. **(b)** Quantification revealed no significant differences in either the mean frequency or mean amplitude of GABAergic sIPSCs from *grm6-TeNT* RBCs as compared to WT. **(c)** Example traces showing chloride-mediated outward currents in RBCs evoked by AMPA puffs in *grm6-TeNT* and WT RBCs at both ages. The GABA<sub>C</sub> component was isolated by application of TPMPA, and residual GABA<sub>A</sub>-mediated current blocked by SR95531. Scatter plots show that the mean charge transfer of the total, GABA<sub>A</sub>-mediated and GABA<sub>C</sub>-mediated currents are comparable in *grm6-TeNT* RBCs and WT at P11–13 **(d)**

and P30 (**e**). Numbers in brackets in b, d, and e represents number of cells. Error bars represent S.E.M.

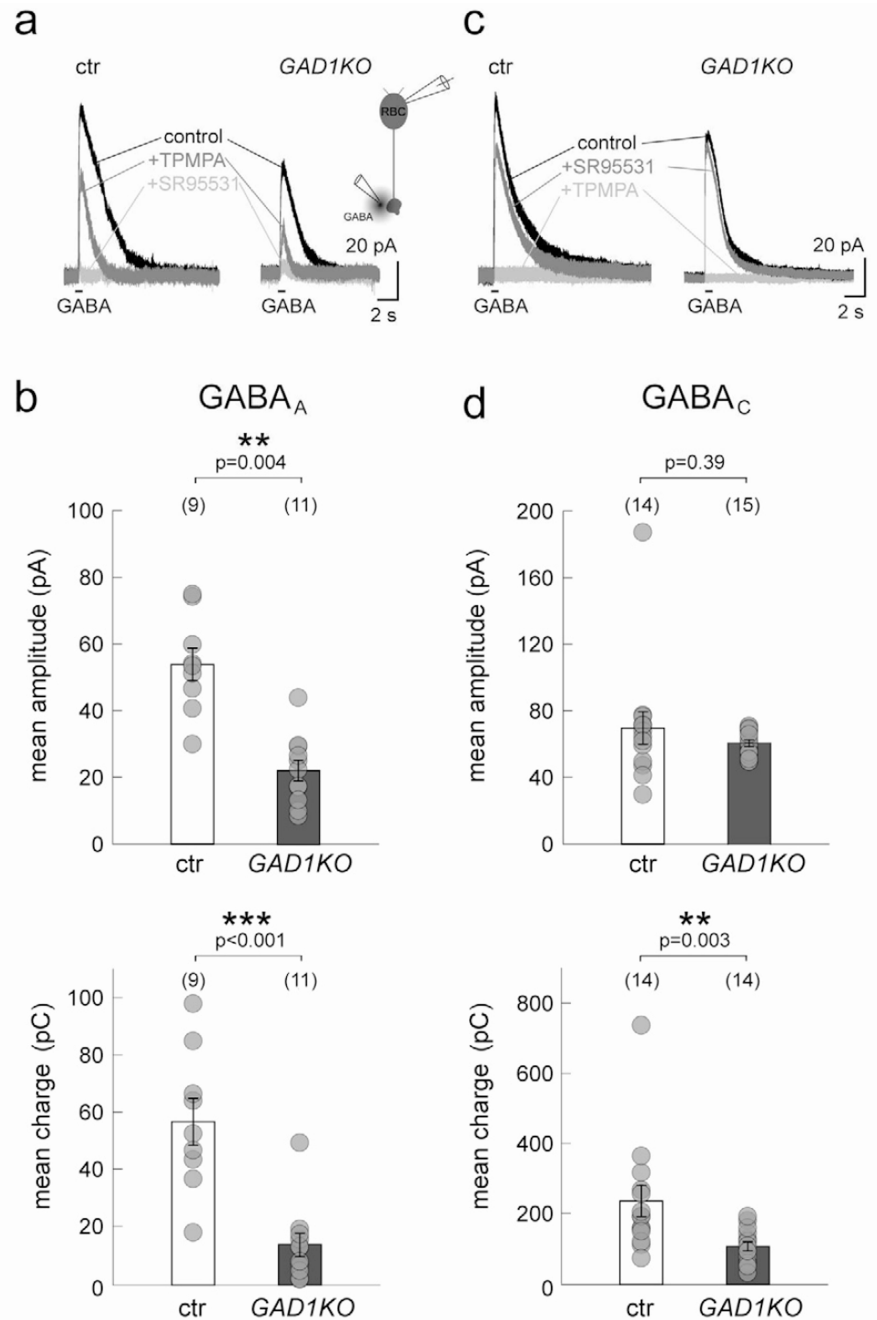


**Figure 4. Spontaneous and GABA-evoked currents of RBCs in *GAD1KO* retina**

(a) Spontaneous GABAergic IPSCs at RBC axon terminals recorded from *GAD1KO* and littermate control (ctr) at P11–13 and P30. (b) Scatter plots show that the mean frequency of these sIPSCs are reduced in *GAD1KO* as compared to ctr. (c) Puffs of GABA evoke similar chloride-mediated outward currents in RBCs from ctr and *GAD1KO* at P11–13, which are markedly reduced for *GAD1KO* RBCs at P30. (d) Quantification reveals a significant reduction for both the mean peak amplitude and charge of GABA-evoked currents from *GAD1KO* RBCs at P30. (e) In addition, the rise time for the P30 evoked response from *GAD1KO* RBCs is longer while the decay is faster as compared to control. Numbers in

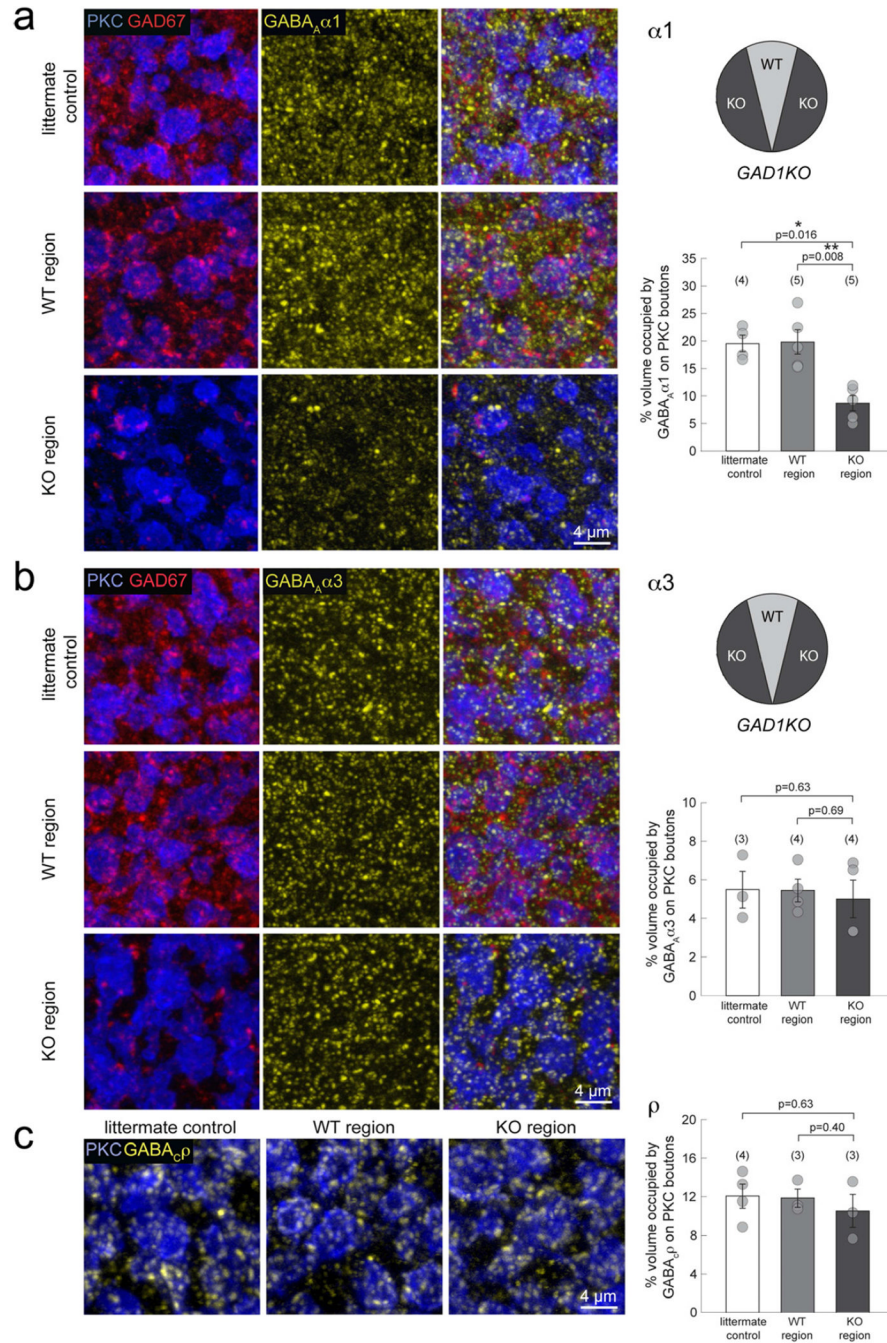


brackets in b, d, and e represents number of cells. Asterisks mark significant difference. Error bars represent S.E.M.



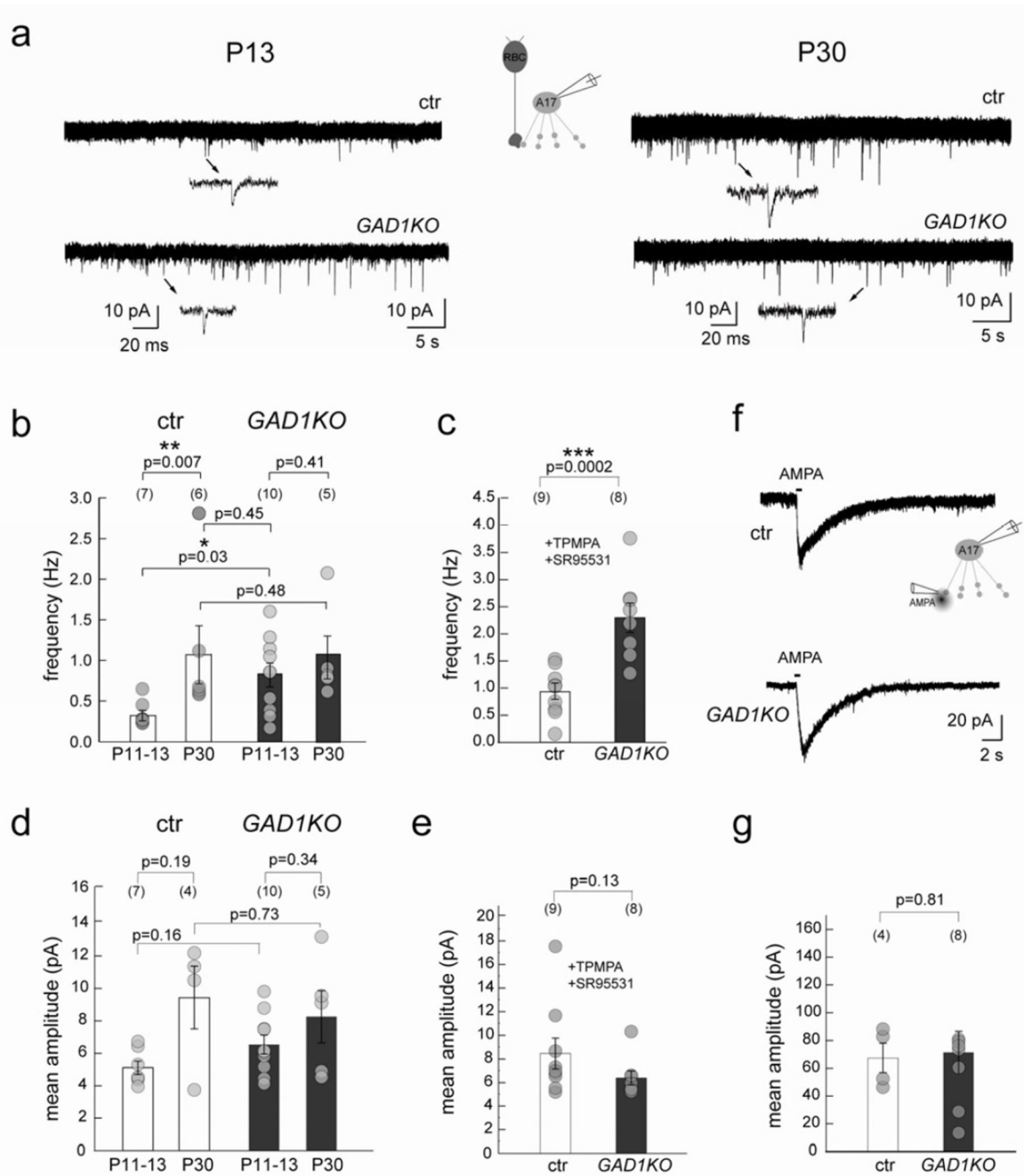
**Figure 5. GABA<sub>A</sub> and GABA<sub>C</sub> receptor-mediated currents from P30 RBCs in *GAD1KO* retina**  
**(a)** Example traces of P30 *GAD1KO* and littermate control (ctr) RBC responses to GABA puffs. TPMPA was applied to isolate the GABA<sub>A</sub>-mediated component. Further addition of SR95531 blocked all evoked response. **(b)** Scatter plots show that mean peak amplitude and charge of GABA<sub>A</sub>-mediated currents in *GAD1KO* RBCs are significantly reduced as compared to ctr. **(c)** Application of SR95531 alone isolates the GABA<sub>C</sub>-mediated component of evoked responses in RBCs. **(d)** Quantification of GABA<sub>C</sub>-mediated currents indicates a significant reduction of the mean charge in *GAD1KO* RBCs as compared to ctr. However, the mean amplitude remained unchanged. Numbers in brackets in b and d

represents number of cells. Asterisks mark significant difference. Error bars represent S.E.M.



**Figure 6. GABA<sub>A</sub> receptor subsets on mature RBC boutons in *GAD1KO* retina**  
**(a)** Triple immunolabeling of PKC immunopositive RBCs (blue), GAD67 (red) and GABA<sub>A</sub>α1 subunits (yellow), revealed a visible reduction of α1-containing GABA<sub>A</sub> receptor clusters in the KO region of *GAD1KO* retina, compared to the WT region or littermate control. Quantification of the % volume occupied by GABA<sub>A</sub>α1 clusters on PKC positive boutons confirmed a significant reduction for the KO region. **(b)** Immunostaining for the GABA<sub>A</sub>α3 receptor subunit revealed no significant differences in the % volume occupied by these receptor clusters on KO region RBC boutons. **(c)** Immunolabeling and % volume occupancy of GABA<sub>C</sub>ρ receptor subunits were comparable across littermate control,

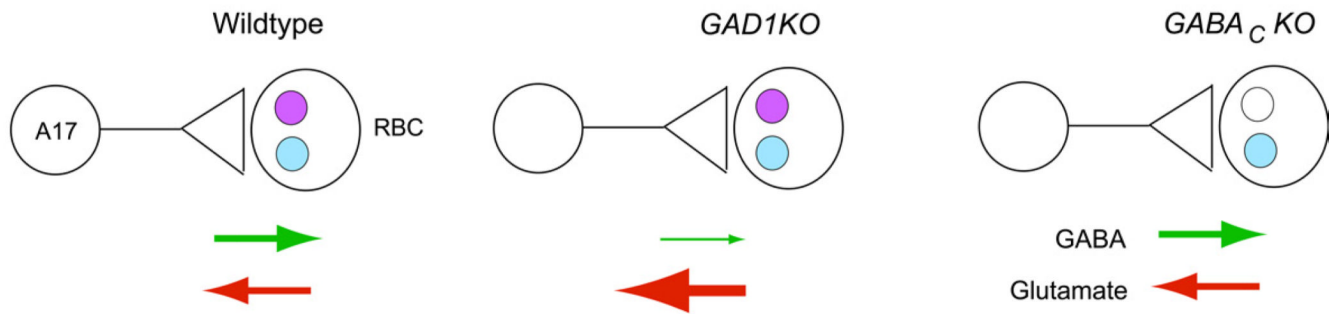
WT region and KO region. Numbers in brackets represent number of animals. Asterisks mark significant difference. Error bars represent S.E.M.



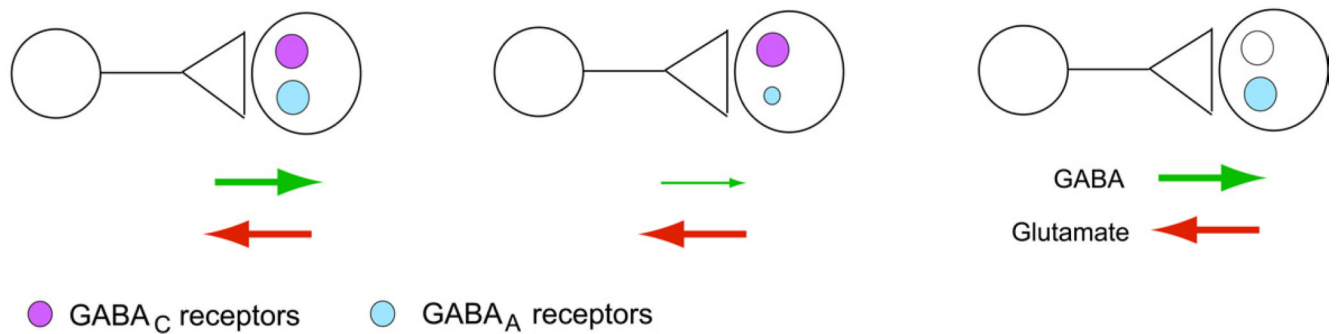
**Figure 7. RBC output is transiently increased in *GAD1KO* retina during circuit development**  
**(a)** sEPSCs recorded from A17 amacrine cells at P13 and P30 in *GAD1KO* and littermate control retina shows an increase during normal development (ctr). **(b)** The sEPSC frequency was increased for A17 amacrine cells in *GAD1KO* as compared to ctr early in development at P13, but was comparable to control at P30. **(c)** Mean frequency of sEPSCs from P11–13 A17 amacrine cells in *GAD1KO* and ctr in the presence of GABA receptor antagonists TPMPA and SR95531, also showed increased frequency in the *GAD1KO*. **(d)** Mean sEPSC amplitudes recorded in A17s from littermate control and *GAD1KO* at P11–13 and P30 revealed no differences. **(e)** Mean amplitude of sEPSCs recorded from P11–P13 A17

amacrine cells in *GADIKO* and ctr in the presence of GABA receptor antagonists TPMPA and SR95531 showed no difference. **(f)** Example traces showing AMPA puff evoked cation-mediated inward currents from A17 cells clamped at  $-60$  mV evoked by AMPA puffs in ctr and *GADIKO* at P11–13. Scatter plots show that the mean amplitude **(g)** is comparable between genotypes. Numbers in brackets represents number of cells. Asterisk marks significant difference. Error bars represent S.E.M.

## Before eye-opening



## After eye-opening



**Figure 8. Summary of the alterations in RBC-A17 synapses in GABAergic transmission-defective mutants before and after eye-opening**

Schematic illustrating the A17-RBC synapse before and after eye-opening in the wildtype, *GAD1KO* and *GABA<sub>C</sub>KO* retina. GABA and glutamate release is similar between wildtype and *GABA<sub>C</sub>KO* (see also Eggers and Lukasiewicz, 2006a), but is altered in the *GAD1KO*. There is a transient upregulation of glutamate release from developing RBCs in the *GAD1KO* before eye-opening. However, after eye-opening, glutamate release from RBCs in the *GAD1KO* becomes comparable to wildtype. GABA<sub>A</sub> and not GABA<sub>C</sub> receptors are selectively reduced at maturity in the *GAD1KO*. This reduction of GABA<sub>A</sub> receptors on RBCs does not occur in *GABA<sub>C</sub>* receptor mutants.