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In vivo electroretinographic studies of the role of $GABA_C$ receptors in retinal signal processing

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Abstract

All three classes of receptors for the inhibitory neurotransmitter GABA (GABAR) are expressed in the retina. This study investigated roles of GABAR, especially $GABA_CR$ (GABA(A)-ρ), in retinal signaling *in vivo* by studying effects on the mouse electroretinogram (ERG) of genetic deletion of $GABA_CR$ versus pharmacological blockade using receptor antagonists. Brief full-field flash ERGs were recorded from anesthetized $GABA_CR^{-/-}$ mice, and WT C57BL/6 (B6) mice, before and after intravitreal injection of $GABA_CR$ antagonists, TPMPA, 3-APMPA, or the more recently developed 2-AEMP; $GABA_AR$ antagonist, SR95531; $GABA_BR$ antagonist, CGP, and agonist, baclofen. Intravitreal injections of TPMPA and SR95531 were also made in Brown Norway rats. The effect of 2-AEMP on GABA- induced current was tested directly in isolated rat rod bipolar cells, and 2-AEMP was found to preferentially block $GABA_CR$ in those cells. Maximum amplitudes of dark (DA) and light-adapted (LA) ERG b-waves were reduced in $GABA_CR^{-/-}$ mice, compared to B6 mice, by 30–60%; a-waves were unaltered and oscillatory potential amplitudes were increased. In B6 mice, after injection of TPMPA (also in rats), 3-APMPA or 2-AEMP, ERGs became similar to ERGs of $GABA_CR^{-/-}$ mice. Blockade of

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$GABA_AR$s and $GABA_BR$s, or agonism of $GABA_BR$s did not alter B6 DA b-wave amplitude. The negative scotopic threshold response (nSTR) was slightly less sensitive in $GABA_CR^{-/-}$ than in B6 mice, and unaltered by 2-AEMP. However, amplitudes of nSTR and photopic negative response (PhNR), both of which originate from inner retina, were enhanced by TPMPA and 3-APMPA, each of which has $GABA_B$ agonist properties, and further increased by baclofen. The finding that genetic deletion of $GABA_CR$, the $GABA_CR$ antagonist 2-AEMP, and other antagonists all reduced ERG b-wave amplitude, supports a role for $GABA_CR$ in determining the maximum response amplitude of bipolar cells contributing to the b-wave. $GABA_CR$ antagonists differed in their effects on nSTR and PhNR; antagonists with $GABA_B$ agonist properties enhanced light-driven responses whereas 2-AEMP did not.

Keywords

electroretinogram; GABA; GABA receptors; retina; retinal signaling

Introduction

GABA (γ-aminobutyric acid) is a major inhibitory neurotransmitter in the central nervous system, including in the retina (Lukasiewicz et al. 2004). Three classes of retinal GABA receptors have been described: ionotropic $GABA_A$ and $GABA_C$ (also known as GABA(A)-ρ) receptors that are linked to Cl^- ion channels, and metabotropic $GABA_B$ receptors that are coupled to G-proteins and work through second messenger systems to modulate K^+ or Ca^{2+} channels (Enz and Cutting 1998).

In mammals, $GABA_A$ receptors ($GABA_AR$s) are expressed throughout the retina including on bipolar cell dendrites in the outer plexiform layer (OPL) and terminals of bipolar cells, processes of amacrine and ganglion cells in the inner plexiform layer (IPL). $GABA_C$ receptors ($GABA_CR$) are more localized to bipolar cells than $GABA_AR$, mainly to axon terminals, but also have been observed in dendrites of bipolar cells in mouse retina (Haverkamp and Wässle 2000; McCall et al. 2002). The presence of $GABA_CR$ in photoreceptors and horizontal cells is unresolved in mice. $GABA_CR$-gated current has been reported in horizontal cells in white perch (Qian and Dowling 1993, 1994), but $GABA_CR$ antibody immunoreactivity has not been detected in photoreceptors in mammalian retina (Enz et al. 1996; Ogurusu et al. 1997) or in horizontal cells (Koulen et al. 1998a). $GABA_B$ receptor immunoreactivity has been observed presynaptically in amacrine and retinal ganglion cells, and in the processes of horizontal cells in mouse and rat retina (Koulen et al. 1998b; Zhang et al. 1998). Retinal Müller cells have $GABA_AR$s, but not $GABA_CR$ or $GABA_BR$, and they have GABA transporters that remove GABA from the extracellular space (Newman and Reichenbach 1996).

GABA is released from amacrine cells in the retina and also has been reported to be released from horizontal cells (Deniz et al. 2011). GABA-mediated feedback and feed-forward inhibition are critical to normal processing of visual signals in the inner retina. Across species, the retinal GABA- induced current mediated by $GABA_AR$s is a fast transient response that quickly desensitizes, while the $GABA_C$ component is slow, sustained, and

desensitizes more slowly (Lukasiewicz and Shields 1998). $GABA_CR$ also mediates a spontaneous tonic current, which is regulated by GAT-1 GABA transporters (Jones and Palmer 2009; (Ichinose and Lukasiewicz 2002).

The electroretinogram (ERG) is a mass potential representing the summed activity of all retinal cells. In the dark-adapted (DA) ERG, responses to weak stimuli called the positive and negative scotopic threshold response (pSTR and nSTR) are related to activation of inner retinal neurons, i.e. the amacrine cells and/or ganglion cells, and mediated by Müller glia currents (Frishman and Steinberg 1989; Saszik et al. 2002; Sieving et al. 1986). The initial negative a-wave of the DA-ERG is mainly associated with photoreceptor activity, but includes postreceptoral contributions (Hood and Birch 1990; Robson et al. 2003). The positive going b-wave originates primarily from the rod bipolar cells in scotopic ERG (Robson and Frishman 1995, 1998; Robson et al. 2004). Small waves superimposed on the leading edge of b-wave are oscillatory potentials (OPs) which reflect high- frequency activity of inner retinal circuits (Wachtmeister 1998). In the light-adapted (LA) ERG, the a-wave originates mainly from cone photoreceptors and Off pathway neurons (Bush and Sieving 1994; Robson et al. 2003). The b-wave originates from the activity of On- and Off-bipolar cells and is shaped by horizontal cell feedback onto cones (Sieving et al. 1994).

The ERG can be used to study the functional role of GABA receptors in the retina *in vivo.* Kapousta-Bruneau (2000) reported that a $GABA_C$ receptor antagonist, 3-APA (3-aminopropylphosphonic acid, 500 μM) (Vien et al. 2002) reduced b-wave maximum amplitude, and enhanced the negative scotopic threshold response (nSTR) and oscillatory potentials (OPs) of the dark-adapted ERG recorded from rat retina. Dong and Hare (2002) also found that a $GABA_CR$ antagonist TPMPA (1,2,5,6-tetrahydropyridine-4-yl-methylphosphinic acid) (Ragozzino et al. 1996) reduced b- wave amplitude in rabbit retina. However, McCall et al. (2002) reported that $GABA_CR^{-/-}$ mice lacking expression of both ρ1 and ρ2 subunits of $GABA_CR$ in the retina did not show reduced b-wave amplitudes, but did have enhanced OPs in the DA ERG, compared to those of wild type mice. More recently, Herrmann et al. 2011 observed reduced b- wave amplitudes in mice lacking functional $GABA_cR$. They suggested that $GABA_CR$ participates in modulating rod-driven bipolar cell responses by a mechanism involving tonic GABA-induced Cl^- current, and that this current sets the resting membrane potential to a more hyperpolarized level than would occur in the absence of active $GABA_CR$, thereby allowing a greater range over which the cells can depolarize. Because assessment of the b-wave is commonly used in clinical diagnostic procedures, a clear understanding of its origins should benefit clinical applications as well as basic research.

$GABA_C$ receptor function in retinal signal processing is not fully understood, and new receptor effectors could be useful for future studies. Chowdhury et al. (2007) reported the synthesis of 2-aminoethyl methylphosphonate (2-AEMP, chemical structure in Figure 1 along with the structure of GABA and of other known $GABA_CR$ antagonists) and found this GABA analog to be a competitive antagonist to homomeric ρ1 $GABA_C$ receptors expressed in Xenopus oocytes. Further work showed competitive antagonism of 2-AEMP in neuroblastoma cells transfected with human GABA ρ1 subunit (Xie et al. 2011). To date, however, this relatively new antagonist has not been tested *in vivo*.

One aim of the present study was to investigate the role of traditionally inhibitory GABARs in retinal visual signal processing *in vivo* by studying their effects on ERGs, with a focus on $GABA_CR$ in shaping responses originating from bipolar cells in both rod and cone pathways. Another aim was to test the more recently synthesized $GABA_CR$ antagonist 2-AEMP by comparing its effect with the effect of $GABA_CR^{-/-}$, and of other known $GABA_CR$ antagonists on the mouse ERG. Preliminary results of this study have been reported in abstract form (Wang et al., *Invest Ophthalmol Vis Sci* 2009: E-Abstract 2179, Wang et al., *Invest Ophthalmol Vis Sci* 2011: E-Abstract 1603, Xie et al., *Soc Neurosci Abstr* 34:608.10).

Methods

Animals

Subjects were adult C57BL6 mice, 2 – 6 months old (Simonsen Labs, USA; Jackson Labs, USA, n=76), to be referred as C57BL6/J (B6) mice, $GABA_CR^{-/-}$ mice, 2 – 3 months old (n = 12; from Dr. Maureen McCall, back crossed with B6 mice for over nine generations), and adult Brown Norway (BN) rats (n = 5, Charles River Laboratories, Inc.), 7 weeks to 6 months of age. Rats used for the isolated bipolar cell experiments at University of Illinois at Chicago (UIC) were Sprague-Dawley (SD) albino rats, male and female, ages approximately 8–16 weeks. The $GABA_C\rho1$ gene was inactivated in the $GABA_CR^{-/-}$ mice, and electrophysiological and immunohistochemical studies in $GABA_CR^{-/-}$ mice reported by McCall et al. 2002 showed no remaining function or expression of $GABA_CR$ in the retina, whereas overall retina morphology was intact (McCall et al. 2002). McCall et al. also showed that the ERGs of WT and (+/−) mice were similar to those of B6, which was the background for the $GABA_CR^{-/-}$ mice. In the present study, B6 mice were used as control (wildtype: WT) mice, and for studies using GABA receptor antagonists and agonists as well. All mice and rats were housed in a room with a 12h light on and 12h light off cycle. All experimental and animal care procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council, adhered to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Houston or, for the SD rat experiments, the University of Illinois at Chicago.

Cell isolation and patch-clamp recording

Solitary bipolar cells were isolated from the rat retina according to published protocols (Qian and Dowling 1995). Rats were euthanized by carbon dioxide administration, eyes were enucleated and hemisected, and the retinas gently removed from the posterior eyecup and immersed for 40 min in a modified Ames media (supplemented with 0.88 g/l NaCl, 2.36 g/l HEPES, and 10,000 units/l penicillin/streptomycin, pH 7.4) containing 2 mg/ml papain (EMD Biosciences, San Diego, CA) and 1 mg/ml of L-cysteine (Sigma, St. Louis, MO). After several brief washes, the tissue was triturated through a sterile pipette, and aliquots of the supernatant containing dissociated cells were placed in culture dishes containing the modified Ames medium. The cells were maintained at room temperature for up to 8 h, and, prior to recording, the culture medium was replaced with an extracellular solution consisting

(in mM) of NaCl (120), KCl (5), $NaHCO_3$ (25), $CaCl_2$ (2), $MgCl_2$ (1), HEPES (10), and dextrose (10), pH 7.4. Whole-cell membrane currents were obtained with a patch pipette filled with an intracellular solution containing (in mM): CsCl (130), KCl (4), $CaCl_2$ (1), $MgCl_2$ (2), EGTA (11), HEPES (10), MgATP (1), NaGTP (0.2), pH 7.4. A Perfusion Fast-Step system (Warner Instrument Co., Hamden, CT) was used to deliver drugs onto the cell. The solution changing rate, measured by liquid junction potential, was 25 ms. Rod bipolar cells were identified by their characteristic morphology. Cells were held at −60 mV, and responses elicited by GABA (Sigma-Aldrich) were recorded with an Axopatch 200B amplifier controlled by pCLAMP software (Axon Instrument, Union City, CA); data were analyzed and plotted using Origin software (Microcal, Northampton, MA). GABA receptor antagonists included TPMPA (Tocris Biosciencs), $GABA_AR$ antagonist bicuculline (Tocris Biosciences) and $GABA_AR$ agonist 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP, Tocris Biosciences).

ERG recording

Dark-adapted (DA) and light-adapted (LA) full-field flash ERGs were recorded differentially between the two eyes, before and after intravitreal injection of pharmacologic agents, with DTL fiber electrodes placed across the center of the cornea in each eye under a contact lens (Saszik et al. 2002). Animals were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (7 mg/kg), and anesthesia was maintained with subcutaneous injections of ketamine (20 mg/kg) and xylazine (1 mg/kg) every 20 – 30 minutes. Pupils were fully dilated with topical atropine 0.5% and phenylephrine 2.5%. ERGs were recorded after the response amplitude had reached full growth and had stabilized (Mojumder and Wensel 2010). Body temperature was maintained between 36.5 and 37°C with a thermostatically controlled blanket (CWE Inc.). For DA-ERG, animals were prepared for recording under red illumination (LED, λ>620 nm).

Visual stimulation

After overnight dark-adaptation, DA-ERGs were recorded in response to brief (<4 ms) full-field flashes provided by LEDs (λ_{max} 462 nm) ranging from −6 to 1.6 log sc td s. The interval between the flashes was adjusted from 1.5 sec at the lowest flash energy to 6 sec at the highest flash energy. LA- ERGs were recorded using brief full-field flashes (<4 ms) or 200 ms flashes on a rod-suppressing background of 2.6 log sc td, after light-adapting for 45 min. The stimuli were provided by LEDs (λ_{max} 513 nm) for energies of 0.3 to 1.9 log sc td (Espion ColorDome stimulator, Diagnosys LLC, Lowell MA) with an interval of 1 sec and by a xenon flash tube for higher strength flashes (2.0–3.5 log sc td s) with a flash interval of 6 sec. Responses were averaged over 20 trials for weak stimuli, fewer for stronger stimuli.

Intravitreal injection and dose selection

Intravitreal injections were made using a Hamilton microsyringe (Hamilton Company, Reno, NV, USA) with a pulled glass pipette attached (tip < 10 μm) through a hole which was punctured 0.5 mm posterior to the limbus by a 30-gauge needle. All procedures were performed under dim red illumination (λ>620 nm) to avoid light-adapting the rods. The pre-injection and post-injection recordings were typically performed in the same session on the

same eye. After injection the ERG was monitored until the drug effects on the waveform were stable (approximately 45 min). The volume of agent injected was 1 – 1.2 μl for mouse and 2 μl for BN rat. Intravitreal concentrations of the pharmacologic agents were estimated by assuming the vitreal volume to be roughly 20 μl for mouse and 40 μl for rat. Multiple concentrations were tested for most agents.

Figure 2, A and B, illustrates the effect of vitreal concentration of TPMPA, (Tocris Biosciences, Ellisville, MO), and 2-AEMP prepared as described Xie et al. 2011, on the b-wave amplitude in response to a flash of 1.3 log sc td s. Vitreal concentrations for TPMPA ranged from 0.50 – 1000 μM and for 2-AEMP, 0.1 – 20 mM. The figure shows that increasing the concentration of TPMPA or 2- AEMP progressively reduced the b-wave amplitude. The lowest tested dose of TPMPA or 2-AEMP did not affect the b-wave amplitude. However, an extremely high dose (20 mM) reduced the amplitude by 80%. 2-AEMP doses of 0.5 to 1 mM (vitreal conc.) and TPMPA doses of 5 to 50 μM yielded effects on the ERG similar to those seen in the $GABA_CR^{-/-}$ mouse, when compared to B6, as illustrated in the representative example on the right side of the figure. The pH values for 1 mM TPMPA and 20 mM 2-AEMP were close to 7.4; thus, the effects of extreme doses were not due to nonphysiological pH of injection. Figure 2D shows the relationship between intravitreal dose and normalized b-wave amplitude evoked by flash of 1.3 log sc td s for TPMPA and 2-AEMP. The b-wave peak amplitude after injection was normalized to that before the injection of TPMPA (4 – 5 mice per dose) or of 2-AEMP (3 – 4 mice per dose), and the b-wave amplitude reduction was determined by subtracting this normalized value from unity. The Hill equation (equation 1), was fit to the relations between antagonist dose and b-wave amplitude reduction.

$$R/R_{max}=(C^n)/(C_{50}{}^n+C^n) \quad (1)$$

In this equation, R is the b-wave amplitude reduction, R_{max} is the maximal amplitude reduction, C is the concentration of test agent, and C_{50} is the level of C that produces a response amplitude of one half R_{max} and n is the Hill coefficient. The fitting of eq. 1 (and of eq. 2 in the Results) to the data was carried out using the Marquardt–Levenberg algorithm in SigmaPlot 10 (Systat Software Inc, USA). The dose-response functions for TPMPA and 2-AEMP were well described by the Hill equation in response to flash of −1.1 and 1.3 log sc td s, as shown by the parameters of the fits in Table 1. The curves that were generated based on the parameters for 1.3 log sc td s are also shown in Figure 2D. In response to flash of 1.3 log sc td s, the TPMPA fit yielded C_{50} = 50 uM and n = 0.77. For 2-AEMP, the fit yielded C_{50} = 1.42 mM and n = 1.68. Sensitivity to TPMPA was 28-fold greater than for 2-AEMP. TPMPA exhibited negative cooperative binding (n < 1) whereas 2-AEMP showed positive cooperative binding (n >1). For the experiments described below, we selected 1 mM as the fixed dose of 2-AEMP and 50 uM as that for TPMPA, as the effects of these doses were similar to those changes observed in $GABA_CR^{-/-}$ mice (see Figure 2C and later comparisons in this paper). In addition, both of these selected doses were close to the C_{50} of the respective agent's dose-response function.

The following agents and vitreal concentrations were also used in mice: 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid hydrobromide (SR95531, 50 μM) to block $GABA_A$ receptors, 3-aminopropyl (diethoxymethyl) phosphinic acid (CGP35348, Tocris Biosciences, 2 mM) to block $GABA_B$ receptors, baclofen (Tocris Biosciences,100 μM) to activate $GABA_B$ receptors, 3- aminopropyl-(methyl)phosphinic acid (3-APMPA, Sigma Aldrich,250 μM, 100 μM) to block $GABA_C$ receptors, and tetrodotoxin (TTX, Sigma-Aldrich, St. Louis, MO, 3 μM) to block sodium-dependent spiking activity. TPMPA (50 uM), SR95531 (100 μM) and CGP35348 0.5 mM) were used for intravitreal injections in rats.

Data analysis

DA-ERG a-wave amplitudes were measured at a fixed time on the a-wave leading edge (6 ms) after the brief flash, and also at the trough (i.e., peak) of the a-wave. After oscillatory potentials (OPs > 50 Hz) were removed by filtering, the amplitude of the b-wave was taken as the excursion between the a-wave trough and b-wave peak, when an a-wave was present. In the absence of the a-wave, b- wave amplitude was taken as the excursion from pre-flash baseline to b-wave peak. DA-ERG b-wave amplitudes were also determined both in the presence or absence of the a-wave at a fixed time near the peak of the b-wave, 110 ms after flash onset for DA-ERG (Saszik et al. 2002) and 50 ms for LA- ERG. OPs (50 – 300 Hz) were extracted starting from the trough of a-wave for strong flashes. OP amplitudes were quantified by calculating the root-mean-square (RMS) of the extracted OPs in the signal window between 0 and 100 ms for LA-ERG and DA-ERG for strong flash energies (−2.3 to 1.6 log sc td s) and between 50 and 150 ms for DA-ERG at weak flash energies (−6.0 to −2.3 log sc td s). The record between 700 and 800 ms was selected as the unstimulated window for quantification of the background noise. Repeated-measures ANOVA was performed to determine whether there was a significant difference in the stimulus response functions for control versus treated conditions. In the DA-ERG, the testing ranges for the a-wave and b-wave were from −3 to 1.6 log sc td s. OPs were tested from −3 to 1.6 log sc td s. The pSTR and nSTR were tested from −6 to −3 log sc td s (Saszik et al. 2002). In the LA-ERG, a-wave, b-wave, OPs and PhNR were all tested from −2.3 to 1.6 log sc td s. A paired *t*-test was used to determine significant difference in amplitude at a single flash strength.

Results

Effects of GABA antagonists on isolated rod bipolar cells

Previous work has demonstrated the specificity of the relatively new $GABA_CR$ antagonist, 2- AEMP, tested in cell expression systems (Xie et al. 2011). Here effects of 2-AEMP on $GABA_CR$ were tested on rod bipolar cells isolated from the rat retina. The effect of 2-AEMP on GABA-elicited currents in isolated rod bipolar cells is illustrated in Figure 3. The recordings in Figure 3A were from a single bipolar cell in response to, sequentially, 10 μM GABA, 10 μM GABA plus 100 μM 2-AEMP, and 10 μM GABA. Co-application of the 2-AEMP with 10 μM GABA produced a pronounced inhibition of the GABA-elicited response, and this inhibition was fully reversible. 2-AEMP inhibited the GABA- elicited response when the $GABA_A$ antagonist bicuculline was included with the GABA for all recordings (Figure 3B). 2-AEMP had no effect ($p>0.05$) on the current elicited by $GABA_A$

agonist THIP (Figure 3C). Figure 3D shows that 2-AEMP significantly inhibited the GABA current ($p<0.01$), and the GABA current after $GABA_AR$ was blocked by bicuculline. However, 2-AEMP did not significantly alter the GABA-elicited current after $GABA_CR$ was blocked by TPMPA, or the current elicited by $GABA_AR$ agonist, THIP ($P>0.05$). Therefore, most or all of 2-AEMP's antagonist activity reflects its antagonism at the GABA binding site of $GABA_CR$.

Effect of inactivation of $GABA_CR$ on the DA-ERG

Inactivation of $GABA_CR$ reduced b-wave maximum amplitude—ERGs were recorded in mice, so that the effect of genetic inactivation of $GABA_CR$ could be compared with effects of pharmacological blockade of $GABA_CR$. Figure 4A shows representative DA-ERGs recorded from B6 and $GABA_CR^{-/-}$ mice in response to brief flashes of increasing stimulus strength. The typical control ERGs shown as grey traces had a prominent positive b-wave that grew in amplitude with increasing stimulus strength. The b-wave peaked at 110 ms for flashes less than −1 log sc td-s that mainly stimulate the rod pathway (Robson et al. 2004). In response to weaker flashes (<−4 log sc td s), the positive response was dominated by a small, more sensitive positive wave called the pSTR, which peaked at 110 ms as well (Saszik et al. 2002). The pSTR and nSTR responses are described more fully below. In response to stronger flashes that lead to activity in the cone pathways as well (Abd-El-Barr et al. 2009), the negative a-wave was present and the b-wave peak moved to earlier times. ERGs recorded from the $GABA_CR^{-/-}$ mouse had a-waves similar to those of B6, shown as black traces. Both the amplitude and latency of the a-wave in the $GABA_CR^{-/-}$ mouse were similar to those of the B6 mouse. However, maximum amplitude of the b-wave in the $GABA_CR^{-/-}$ mouse was smaller than that in the B6 mouse, as originally reported by Herrmann et al. (Herrmann et al. 2011). Specifically, in the $GABA_CR^{-/-}$ mouse, b-wave amplitude rose with increasing flash strength to a value representing about 50% of the maximum exhibited in B6 mouse, and then stopped increasing in amplitude. Thus, the initial portion of the b-wave in the $GABA_CR^{-/-}$ mouse was unaltered. The plots shown at the right of the responses in Figure 4 show the isolated OPs (> 50 Hz) in response to flashes of 1.2 and −2.3 log sc td s, respectively. OP amplitudes were enhanced in the $GABA_CR^{-/-}$ mouse, as reported previously (McCall et al. 2002).

Based on the dose-response relations for TPMPA and 2-AEMP shown in Figure 2, 50 μM for TPMPA and the much higher dose of 1 mM for 2-AEMP, were selected for experiments that compared b-waves after injection of these antagonists with b-waves in the $GABA_CR^{-/-}$ mouse. Figure 4B, C, and D show superimposed ERGs for the B6 control recording and for the ERG recorded from the same eye after the intravitreal injection of TPMPA, 3-APMPA or 2-AEMP. All three $GABA_CR$ antagonists reduced b-wave maximum amplitude by truncating the response, as observed for the $GABA_CR^{-/-}$ mouse (Figure 4A) and again neither the a-wave nor the early portion of the leading edge of b-wave were changed by loss of $GABA_CR$ function. Consistent with the absence of $GABA_CR$, intravitreal application of TPMPA in the $GABA_CR^{-/-}$ mouse did not affect the DA-ERG (Figure 4E). The insets to Figure 4B-D illustrate the OPs in response to a flash of 1.2 log sc td s. Amplitudes of isolated OPs (>50 Hz) were enhanced by all three antagonists. Effects of 3-APMPA, are

shown for a concentration of 250 μM. Application of 100 μM 3-APMPA, had less effect on the b-wave amplitude (not shown).

Figure 5 shows stimulus response functions for averaged results obtained from 4 – 5 animals per group for dark adapted ERGs in B6 mice, in $GABA_CR^{-/-}$ mice, and B6 mice after injection of TPMPA or 2-AEMP. (For 3-APMPA, averages were not shown because only 2 animals were tested at the dose illustrated in Figure 4.) Table 2A shows results of repeated measures ANOVA analysis for comparison of the stimulus-response function. The b-wave stimulus-response relation in $GABA_CR^{-/-}$ mice differed significantly ($F=563$, $p<0.001$) from that in B6 mice. The attenuation commenced at −2.6 log sc td s (*t*-test, $p<0.05$), became more pronounced as b-wave saturation was approached (−1 log sc td s), and then stabilized. As shown in Table 2B, the maximal reduction was about 50%, on average, when b-wave amplitude was measured at 110 ms after flash onset, and 45% when measured from a-wave trough to b-wave peak. When measured at 50 ms after the flash, much earlier on the rising edge of the b-wave, the b-wave amplitude was not reduced until the flash energy reached −0.2 log sc td s. Past that point, the b-wave amplitude of $GABA_CR^{-/-}$ mice saturated, whereas amplitude for B6 increased up to the strongest flash used, 1.6 log sc td s (Figure 5A), which likely elicited a mixed rod-cone response. (Abd-El-Barr et al. 2009).

Similar to the effects of genetic inactivation of $GABA_CR$, the stimulus response relations for the b- wave amplitude after intravitreal injection of TPMPA ($F=30$, $p=0.01$) or 2-AEMP ($F=13$, $p<0.05$) were significantly different from those before injection. The attenuation commenced around −2.6 and −2.9 log sc td s respectively, increased up to b-wave saturation (−0.9 log sc td s) and then stabilized with maximal 45% reduction for TPMPA and 30% reduction for 2-AEMP when b-wave was measured at 110 ms after flash. When measured from peak to trough, the maximal reduction was 42% for TPMPA and 28% for 2-AEMP, compared to pre-injection. After saturation, the reduction in b-wave amplitude determined at 50 ms continued to increase up to the strongest flashes used (data not illustrated).

The a-wave amplitudes were not affected by genetic inactivation of $GABA_CR^{-/-}$ ($p>0.05$), or injection of either TPMPA ($p>0.05$) or 2-AEMP ($p>0.05$) measured at 6 ms after the flash or at the a- wave trough. $GABA_CR^{-/-}$ mice exhibited larger OPs (RMS amplitude) than B6 ($F=8$, $p<0.05$) over the flash range of −3 to −1 log sc td s only. These data are plotted using log RMS in Figure 5G so that the larger amplitudes in the $GABA_CR^{-/-}$ mice to stimuli below −1 log sc td s are visible. Over the stronger flash range of −1 to 1.6 log sc td s, OP amplitudes were also significantly enhanced after the injection of TPMPA ($F=32$, $p<0.05$) (40% increase on average) or of 2-AEMP ($F=16$, $p<0.05$) (30% enhancement on average). The RMS of the noise was unaltered in $GABA_CR^{-/-}$ mice ($p>0.05$) and after injection of TPMPA ($p>0.05$) or 2-AEMP ($p>0.05$). RMS noise level was below 6 in all groups, as shown in Figure 5G, H, and I.

Effect of inactivation of $GABA_CR$ on the STR—Grey traces in Figure 6 show typical mouse brief flash DA-ERGs elicited by weak flashes from −6 to −3 log s td s. A slow negative potential, the nSTR, was present in response to a stimulus of −5.1 log sc td s peaking at 200 ms after the flash. With slightly stronger stimuli, a positive wave, the pSTR, preceded the nSTR, peaking at 110 ms. Genetic elimination of $GABA_CR$ or intravitreal

injection of TPMPA, 3-APMPA or 2-AEMP did not consistently alter the pSTR amplitude or latency. pSTR amplitudes measured at 110 ms after the stimulus flash in *GABA$_C$R$^{-/-}$* mice, illustrated in Figure 6E, did not differ ($p>0.05$) from those of the B6 for flashes of −6 to −4 log sc td s. However, nSTR amplitude was affected differently by inactivation of GABA$_C$R. In Figure 6A, the nSTR recorded from *GABA$_C$R$^{-/-}$* mouse was slightly lower in amplitude than for B6 at a flash strength of −5.1 log sc td s, and then reached the same amplitude as that of the B6 at the flash strength of −4.2 log sc td s. Figure 6E shows that nSTR amplitude in *GABA$_C$R$^{-/-}$* mice (n=6) was lower ($F=544$, $p<0.01$) over the range of −6 to −4.5 log sc td s, compared to B6 mice, and eventually reached the same maximum nSTR amplitude as the B6 mice but at a flash strength (−4.2 log sc td s) higher than that for B6 mice (−4.8 log sc td s). Therefore, the entire stimulus-response function shifted to a slightly higher stimulus strength in the *GABA$_C$R$^{-/-}$* mice. To quantify this finding, the nSTR stimulus-response relation from the smallest response to saturation in each *GABA$_C$R$^{-/-}$* and B6 mouse was analyzed using the generalized Naka-Rushton function (i.e. the Naka-Rushton function (Fulton and Rushton 1978) in which *n* is allowed to vary). This function (eq. 2) is algebraically similar to the Hill equation used above, but its terms have been altered to reflect the stimulus-response relationship in ERG recordings:

$$V=(V_{max}\,I^n)/(I^n+I_0{}^n) \quad (2)$$

In eq. 2, *V* (volts) is the response amplitude, V_{max} is the maximum amplitude, *I* (intensity) is the stimulus strength, I_0 is the level of *I* that produces a response amplitude of one half V_{max}, and *n* denotes the slope of the function where *I* is equal to I_0. The nSTR stimulus-response relations of both *GABA$_C$R$^{-/-}$* ($r^2=0.98$) and B6 ($r^2=0.98$) mice were well described by the function. A measure of sensitivity, I_0, was significantly different ($p<0.05$) between *GABA$_C$R$^{-/-}$* (−5.0 log sc td s) and B6 (−5.5 log sc td s) mice. The saturated amplitude (V_{max}) of the nSTR was not significantly ($p>0.05$) different between *GABA$_C$R$^{-/-}$* (35.6 μV) and B6 (36.0 μV) mice. The exponent, *n*, was fairly close to 2.5 in both cases.

In contrast to the findings in the *GABA$_C$R$^{-/-}$*, the nSTR after TPMPA (50 μM) treatment became slightly larger on average ($F=12.3$, $p=0.04$) over the flash range of −6 to −3 log sc td s where the nSTR was present. 3-APMPA (100 μM), increased nSTR amplitude even more ($F=11.1$, $p=0.04$). In contrast, pSTRs were not enhanced. For the enhanced nSTR, neither TPMPA nor 3-APMPA consistently changed the timing of the response, and the nSTR saturated at the same flash strength as in the pre-injection recordings, around −4.5 log sc td s. Application of 2-AEMP did not significantly alter the nSTR amplitude ($p>0.05$); the tendency to be larger was slight.

Given TPMPA's GABA$_B$R agonist properties, we investigated whether GABA$_B$ receptors were involved in producing the reduced amplitude of the b-wave after TPMPA, by adding CGP, a GABA$_B$R antagonist, when TPMPA was injected. Figure 7A shows the responses before and after injection of the GABA$_B$R antagonist CGP. CGP alone did not alter any components of the ERG. In Figure 7B and 7 F, co-application of CGP with TPMPA slightly reduced the nSTR amplitude ($F=9.7$, $p=0.008$), but did not change b-wave amplitude, compared to the response exhibited after TPMPA injection alone. These results indicate that

the main effect of TPMPA on $GABA_BR$ activity is the enhancement of the nSTR. The reduction of b-wave amplitude over much of the stimulus response function beyond the pSTR range seen in Figure 5 was due only to effects on $GABA_CR$.

The effect of blockade of $GABA_A$ receptors on the DA-ERG also was investigated. Figure 8A shows the responses before and after intravitreal injection of $GABA_AR$ antagonist SR95531 in one B6 mouse. After SR95531, the b-wave reached the same maximal amplitude as the control, but the b- wave peak was narrower. The b-wave stimulus-response relation measured after SR95531 injection at 110 ms after the flash was not significantly different from that measured before the injection ($p>0.05$), as shown in Figure 8C. After blockade of $GABA_ARs$, the maximum pSTR was reduced, a negative STR-like potential was enlarged, and slow oscillations occurred in all 5 tested animals. Intravitreal injection of TTX, a voltage-gated Na^+ channel blocker, partly suppressed the enhanced inner retina responses, suggesting the dependence of these enhanced responses on the spiking activity of inner retinal neurons (Figure 8B). The OPs were all removed after blockade of $GABA_AR$, except for a positive OP1-like wavelet that appeared in the trough of a-wave (extracted OPs for 1.2 log sc td s flash). The amplitude of the a- wave leading edge measured at 6 ms was not affected by $GABA_AR$ blockade ($p>0.05$). However, when measured at the trough, the a-wave amplitude was enhanced ($F=150$, $p<0.01$) by SR95531 in response to flashes of −1.7 to −0.7 log sc td s. For stronger flashes, this enhancement did not occur. The enhancement of the a-wave for midrange flash strengths may be related to a negative-going postreceptoral contribution to the a-wave from the second rod pathway (Robson and Frishman 1996), that may normally be modulated by $GABA_AR$.

Effect of inactivation of $GABA_CR$ on the LA-ERG

As shown in Figure 9 (grey traces), a typical mouse LA-ERG in response to brief stimulus has a prominent b-wave, a negative a-wave which is visible for strong flashes and OPs superimposed on the b-wave. In the $GABA_CR^{-/-}$ mouse, the b-wave did not reach the maximal amplitude observed for the B6 mouse, but, as observed in the DA-ERG, the leading edge of b-wave of the $GABA_CR^{-/-}$ mouse was similar to that of the B6 mouse, and the main effect was truncation of responses to strong stimuli. Figure 9D shows the average b-wave amplitude, measured at 50 ms, after OPs (>50 Hz) were removed. Results of a repeated-measures ANOVA test of stimulus-response functions are summarized in Table 3A, and percent changes in b-waves and OPs, in Table 3B). The b-wave peak amplitude over the range of stimuli tested in $GABA_CR^{-/-}$ mice was significantly different ($F=31.7$, $p<0.001$) from that in B6 mice. The b-wave amplitude attenuation increased up to b-wave saturation at 2.3 log sc td s with about 30% reduction maximally. The a-waves were unchanged in amplitude ($F=1.03$, $p>0.05$). The overall amplitude of the OPs was larger in $GABA_CR^{-/-}$ mouse, but the number of wavelets was constant. The stimulus-response relation for OP (>50 Hz) amplitude, in terms of RMS in $GABA_CR^{-/-}$ mice differed significantly ($F=9.1$, $p=0.01$) from that in B6 mice, and represented about a 60% increase. The noise RMS was not altered. Flashes durations of 200 ms produced an even greater enhancement of the OPs (data not shown).

Figures 9B and 9C show responses obtained before and after TPMPA (50 μM) or 2-AEMP (1 mM) injection. Similar to the ERG of $GABA_CR^{-/-}$ mice, TPMPA or 2-AEMP attenuated the maximal amplitude of b-wave and left the leading edge of b-wave and a-wave unchanged. The relations in Figure 9E, F showed that b-wave maximal amplitude was significantly reduced after TPMPA (n=8) or 2-AEMP (n=5) injection. The amplitude reduction grew as flash strength increased, up to b-wave saturation at 2.3 log sc td s, with 60% reduction maximally for TPMPA and 40% for 2-AEMP. OPs amplitudes were significantly enhanced after TPMPA (35% maximal increase) and after 2-AEMP (20% increase), compared to the control. The RMS of the noise in the unstimulated window was not altered by TPMPA or 2-AEMP injection, similar to the finding under DA conditions (data not shown). TPMPA appears to have enhanced a negative-going wave after the b-wave, called the photopic negative response (PhNR) which, like the nSTR in mice, originates from inner retina (Chrysostomou and Crowston 2013). This enhancement was not seen in the ERGs of $GABA_CR^{-/-}$ mice, or after application of 2-AEMP. As observed for the nSTR, the enhancement may have been due to the $GABA_B$ agonist properties of TPMPA.

Effect of $GABA_B$ receptor activation on the DA- and LA-ERG

To investigate whether the $GABA_BR$ agonist properties of TPMPA and 3-APMPA (Tian and Slaughter 1994) affect the mouse ERG, effects on DA- and LA-ERGs of baclofen, a selective $GABA_B$ agonist were examined. For the DA-ERG, the effect of 100 μM baclofen in one B6 mouse is shown in Figure 10. After injection of baclofen, the nSTR increased in amplitude. The effect of baclofen on the amplitude of the nSTR was similar to, but much larger than, the effects of TPMPA and 3-AMPA shown in Figure 6. The stimulus-response relations are shown in Figure 10C, D and E. Baclofen significantly increased the nSTR amplitude ($F=26.7$, $p<0.01$) over −6 to −2 log sc td s, and caused it to saturate at stronger flash strengths (−3 log sc td s), compared to the pre-injection control. As shown in Figure 10B, the enhanced nSTR could be removed almost entirely by TTX, indicating a role for spiking activity of inner retinal neurons in its generation. TTX injection also nearly eliminated the most sensitive nSTR in B6 mice (data not shown). The a-wave was unchanged by baclofen application, and the b-wave amplitude was unaffected, except for responses to weak flashes (< −1 log sc td s) where it was decreased. Here, the decrease in b-wave amplitude likely reflected algebraic summation of the b- wave with the enhanced nSTR of opposing polarity. The OPs (>50 Hz) were significantly ($F=18$, $p<0.01$) enhanced by baclofen.

Figure 11A shows the effect on the LA-ERG of a B6 mouse of injection of 100 μM baclofen. Application of baclofen did not affect the timing of the a-wave, or b-wave, although the b-wave appeared to be slightly reduced in amplitude. In contrast, baclofen caused a substantial PhNR to emerge after the b-wave. Injection of TTX blocked baclofen's effect, and the induced PhNR was eliminated. Figure 11C, D, E shows the stimulus-response functions for the PhNR measured at 100 ms after the flash, for the b-wave measured at 50 ms, and for the a-wave measured at the trough. Baclofen injection significantly ($F=8.6$, $p<0.05$) increased the amplitude of PhNR over the stimulus range. This enhancement became substantial at 1 log sc td s, and reached full effect at 1.7 log sc td s, with the maximal amplitude of −80 μV. These results indicate that baclofen enhanced the spiking

activity of inner retinal neurons in the LA-ERG, which increased PhNR amplitude, similar to the enhancement of the spike-driven nSTR in the DA-ERG.

The b-wave amplitude was reduced after baclofen injection, although effects were variable, and this change was not significant when measured at 50 ms ($F=3.5$, $p>0.05$) and just barely significant when measured from trough to peak ($F=1.5$, $p<0.05$). The a-wave amplitude was unaltered ($F=0.32$, $p>0.05$).

Rat DA-ERG: effect of $GABA_CR$ and $GABA_AR$ antagonists

Figure 12A (grey traces) shows typical BN rat DA-ERG responses to brief flashes. As shown above for B6 mice, the pSTR and nSTR were the most sensitive responses, and as flash strength increased, the b-wave grew in amplitude; a-wave preceded the b-wave for strong flashes. After TPMPA injection, the a-wave and initial rise of the leading edge of the b-wave were superimposed with the pre-injection responses, but, as in the mouse, peak response amplitudes were truncated, and the maximum response was about 50% of that before injection. TPMPA did not alter the pSTR and nSTR, but slightly increased the OPs amplitude. Figure 12B shows stimulus response functions for averaged results obtained from 3 animals before and after TPMPA injection.

Figure 12C compares the rat DA-ERGs before and after injection of $GABA_AR$ antagonist SR95531. As in the mouse, SR95531 produced a large STR-like potential. It was composed of a negative wave peaking at 200 ms and a positive wave peaking at 400 ms after the flash. The pSTR was not visible, perhaps due to the large nSTR. SR95531 enhanced the peak amplitude of the b-wave in this subject, as well as in one other rat in which it was tested. The a-wave was unaltered in its leading edge, but slightly larger at the trough, consistent with a post-photoreceptor contribution to the a-wave trough under normal conditions.

These results show that effects of $GABA_AR$ and $GABA_CR$ antagonists on the DA-ERG in rat were similar to those in mouse, except that b-wave amplitude was enhanced by the $GABA_AR$ antagonist in rat ERG, but not on average in mouse ERG. This may be because more of the GABA current is mediated by $GABA_A$ vs $GABA_CR$ in rod and ON cone bipolar cells in rat retina than in mouse retina (Euler and Wässle 1998; (Eggers, McCall et al. 2007).

Discussion

This study addresses the contribution of $GABA_C$ receptors to visual signal processing in the retina, a topic of long-standing and continuing wide interest in retinal neuroscience (Feigenspan et al. 1993; Lukasiewicz et al. 2004). As a foundation for the present experiments, we confirmed that inactivation of *$GABA_CR$ in vivo* by genetic elimination of *$GABA_CR$* or by $GABA_CR$ receptor antagonist TPMPA in mouse (and rat in DA-ERG) at selected concentrations leads to reduction of the b-wave maximum amplitude and enhancement of OPs in DA- and LA-ERGs, as has been described previously in mouse and rat (Herrmann et al. 2011; Kapousta-Bruneau 2000; McCall et al. 2002). With use of the *$GABA_CR^{-/-}$* mouse, and with TPMPA as a comparison $GABA_C$ antagonist, we have analyzed the effects, on components of the ERG, of the recently described $GABA_CR$ antagonist 2- AEMP. Previous work in oocytes and in neuroblastoma cells transfected with

$GABA_CR$ demonstrated the selectivity of 2-AEMP for $GABA_CR$ *in vitro* (Chowdhury et al. 2007; Xie et al. 2011). In the present study, patch-clamp recording of rat retinal bipolar cells indicated a primary competitive antagonistic action for 2-AEMP at $GABA_CR$ in those cells. The effects of 2-AEMP on ERG b-wave amplitude were found to be similar to the alterations from normal observed in $GABA_CR^{-/-}$ mice in the present study and in Hermann et al (Herrmann et al. 2011). The effects were also similar to effects of other $GAAB_CR$ antagonists, TPMPA and 3-APMPA, on the b-wave. However effects of 2-AEMP on nSTR of inner retina were different, and closer to those that occurred due to genetic inactivation of $GABA_CR$. Co-administration of the $GABA_B$ antagonist CGP with TPMPA eliminated the latter's effect on the nSTR. Therefore, it is likely that the effects of TPMPA and 3-APMPA on the nSTR are caused by $GABA_BR$ agonist activity rather than a $GABA_CR$ effect.

2-AEMP, a selective $GABA_CR$ antagonist

The present study provides evidence *in vivo* in mice that 2-AEMP, a phosphonic acid analog of GABA, has a similar effect as TPMPA and 3APMPA and genetic deletion of $GABA_CR$, on both DA- and LA-ERG. Specifically, antagonism of $GABA_CR$ or deletion of the receptor caused reduction of b-wave amplitude and enhancement of OPs. Structurally, 2-AEMP is quite similar to 3-APMPA (Figure. 1), differing only by the substitution of an oxygen atom for the methylene group (CH2) adjacent to phosphorus in 3-APMPA. Despite this small difference in structure, there appears to be a great difference in the relative effects at $GABA_BR$. 3-APMPA is a potent $GABA_B$ agonist that increased the magnitude of the nSTR by 200% at 100 μM concentration. In contrast, 2-AEMP had no detectable effect on the nSTR at a concentration (1 mM) that produced comparable reductions in the b-wave amplitude. TPMPA, which is a weaker $GABA_B$ agonist than 3-APMPA (Ragozzino et al. 1996), had an intermediate effect on the nSTR. Thus, the cyclic tetrahydropyridyl side chain of TPMPA may be needed to attenuate the $GABA_BR$ agonism of 3-APMPA, whereas the 2-aminoethoxy side chain of 2- AEMP, which is isosteric to the side chain of 3-APMPA, appears to convey intrinsically weak agonism at $GABA_BR$. Because to date only a few 2-aminoethyl phosphonates have been tested for interaction with GABA receptors (Cates et al. 1984; Chowdhury et al. 2007; Xie et al. 2011), and none have been specifically tested at $GABA_BR$, conclusive testing of the structure-activity properties just noted will require further investigations. Overall, the present and previous evidence for 2-AEMP's selectivity for $GABA_CR$, together with its ease of synthesis, emphasize the desirability of its further study in the field of $GABA_CR$ pharmacology.

TPMPA was more potent at $GABA_CR$ than 2-AEMP, based on the dose-response functions for effects of TPMPA and 2-AEMP on the b-wave amplitude in DA-ERG (Figure. 2). The relative potency of TPMPA vs 2-AEMP observed in present study was more similar to that in ρ1 $GABA_CR$ expressing *X. lavevis* oocytes (Chowdhury et al. 2007) than that in human ρ1 $GABA_CR$ expressing neuroblastoma cells (Xie et al. 2011). This may have resulted from different microenvironments in whole retina vs *in vitro*, for example, different concentrations of GABA present in the testing system than *in vivo*, and also to different testing approaches (bipolar cell light responses reflected in ERG vs. GABA currents in other preparations).

Compared to genetic elimination of $GABA_CR$ in mice, the $GABA_CR$ antagonists, TPMPA and 2- AEMP caused a much greater reduction of the b-wave amplitude when extremely high doses were applied. This may have resulted from the amplified effects on other GABA receptors by extremely high doses, for example, perhaps by activating $GABA_ARs$. At doses near the C(50), both TPMPA and 2- AEMP yielded effects on the b-wave similar to those observed in the $GABA_CR^{-/-}$ mouse.

Inactivation of $GABA_C$ receptors attenuates maximum b-wave amplitudes

Selective blockade of either $GABA_AR$ and or $GABA_BR$ alone did not reduce b-wave amplitudes in mouse ERGs in the present study, which is in agreement with previous reports in rat (Kapousta-Bruneau 2000), mouse, (Herrmann et al. 2011; Smith et al. 2013) and frog (Kupenova et al. 2008), and with the report that no immune reactivity of $GABA_BR$ was found in the bipolar cells in mouse retina (Koulen et al. 1998). Retinal $GABA_A$ receptors are less sensitive and are more rapidly desensitized than $GABA_CR$ (Cates et al. 1984; Eggers and Lukasiewicz 2011; Lukasiewicz and Shields 1998). Activation of retinal $GABA_AR$ is likely to have an inhibitory effect on bipolar cell responses, and these receptors likely do not mediate the tonic effects on membrane potential that have been ascribed to $GABA_CR$ in the retina. This may explain why blockade of $GABA_AR$ in rats in the present study increased, rather than decreased, b-wave amplitude.

Although blockade of $GABA_BRs$ did not alter b-wave amplitude, agonism of $GABA_BRs$ by baclofen did lead to some reductions in b-wave amplitudes. However, these were probably a subtractive effect: the positive-going b-wave was pulled down by the enlarged negative going nSTR and PhNR waves from the inner retina (see below).

The effect of GABA receptors on signals from third order neurons

Although b-wave amplitude was similarly reduced in $GABA_CR^{-/-}$ mice, or by injection of TPMPA, 3- AMPA or 2-AEMP in B6 mice, the amplitude of nSTR was affected differently across the treatments. In $GABA_CR^{-/-}$ mice, the nSTR was less sensitive than in B6 by about a factor of 2.5, but maximal amplitude was similar. In contrast 2-AEMP had essentially no effect on the nSTR, whereas both TPMPA and 3-AMPA enlarged the nSTR. Both TPMPA and 3-AMPA are known to have $GABA_B$ agonist properties. A higher dose of 3-APMPA (250 μm) had a greater effect on nSTR, increasing nSTR maximum amplitude by 245%, compared with 100 μm 3-APMPA dose which increased nSTR by 200% (data not shown). Baclofen, a specific $GABA_B$ agonist, produced an even larger enhancement (~290%) of the nSTR amplitude at the doses that were used, and also enhanced the PhNR of the LA-ERG. These findings provide evidence that the increased nSTR and PhNR amplitudes were due to stimulation of $GABA_B$ receptors. In this regard, the more recently developed 2-AEMP was shown to be more selective for $GABA_CR^{-/-}$ than TPMPA or 3-AMPA in the present study.

Antagonism of $GABA_AR$ also increased inner retinal responses, but not with the same time course as the enhancement caused by agonism of $GABA_BR$. $GABA_A$ receptors are widely expressed in amacrine and ganglion cells, as well as in bipolar cells, and they provide feedback and feed forward inhibition (Chavez et al. 2010; Eggers and Lukasiewicz 2006a; Eggers et al. 2007). When $GABA_A$ receptors are fully activated by exogenous GABA, the

shunting inhibition can be strong enough to completely overwhelm excitatory currents, resulting in loss of all light-evoked responses in third order neurons (Bolz et al. 1985; Frumkes and Nelson 1995). This is the likely main cause of the loss, post intravitreal GABA injection in mice, of both nSTR and pSTR in the DA-ERG, as well part of the cause of loss of OPs (Saszik et al. 2002). Conversely, the nSTR was significantly enhanced after blockade of $GABA_A$Rs in the present study. Inactivation of $GABA_C$R, found mainly in bipolar cells, also leads to an enhancement of some inner retinal responses, manifest as an increase in the amplitude of the OPs, presumably due to removal of inhibition. The enhancement might be caused by the same mechanism as that enhancing spontaneous and light-evoked spiking in On retinal ganglion cell (Lukasiewicz et al. 2004) and in A17 amacrine cells (Eggers and Lukasiewicz 2006b) in $GABA_CR^{-/-}$ mice.

The nSTR was not greatly affected in the $GABA_CR-/-$ mice except for being slightly less sensitive than in B6 mice. The slight nature of the effect may have resulted from the interaction of two opposite effects of the $GABA_C$R inactivation: 1) loss of inhibitory effects of $GABA_C$R on bipolar cells, leading to enhanced bipolar cell signaling, and 2) loss of the tonic augmentory effects of GABACR on bipolar cell signaling, proposed by Herrmann et al. (2011). The pSTR did not appear to be altered at all in $GABA_CR^{-/-}$ mice or post injection of $GABA_C$R antagonists. The pSTR may be more related to the Off than On ganglion cell responses. (Saszik et al. 2002) (Abd-El-Barr et al. 2009). The expression of $GABA_C$R in Off bipolar cells is limited, and responses of optic nerve fibers of Off ganglion cells in extracellular recordings in $GABA_CR^{-/-}$ mice indicated that Off pathway signals are hardly affected by loss of $GABA_C$R (Lukasiewicz et al. 2004).

$GABA_B$ receptors are expressed in amacrine cells and retinal ganglion cells. These metabotropic GABA receptors also participate in inner retinal feedback and feed forward circuits. The present study has shown that activating $GABA_B$ receptors enhances negative-going ERG waves, nSTR and PhNR, that originate from inner retina, and are spike-dependent. Why does baclofen enhance the inner retinal neuronal responses, instead of suppressing them? One possibility is that the $GABA_B$R agonist inhibits the inhibition from amacrine cells, and thereby disinhibits bipolar cells, leading to increased glutamate release by bipolar cells to postsynaptic third order retinal neurons (Song and Slaughter 2010). This could occur via $GABA_B$Rs located either presynaptically to modulate calcium channels or postsynaptically to modulate potassium channels (Slaughter and Bai 1989; Slugg et al. 2003). For instance, in the presynaptic terminals of GABAergic amacrine cell, $GABA_B$R activation inhibits the n- type (Shen and Slaughter 1999) voltage-gated calcium channels, thereby reducing GABA release. Postsynaptically in some GABAergic amacrine cells, binding of GABA to $GABA_B$R leads to the potassium outflow which hyperpolarizes the cell and reduces the GABA release. Another possibility is that the enhancement of nSTR by baclofen results from tonic activation of $GABA_B$R in the inner retina. This is consistent with previous studies showing that activation of $GABA_B$R by baclofen, hyperpolarized the membrane and augmented On and Off light evoked excitatory postsynaptic potentials (EPSPs) and spiking of third order neurons in tiger salamander retinal slice (Tian and Slaughter 1994) probably by potentiating L-type voltage-gated calcium channels in ganglion cells (Shen and Slaughter 1999). Baclofen was also reported to increase firing in On

ganglion cells in cat retina under some stimulus conditions (Müller et al. 1992). When the $GABA_BR$ antagonist, CGP was applied, the opposite effect was observed: the dark membrane potential was slightly depolarized and the EPSPs amplitudes were slightly reduced (Tian and Slaughter 1994). Finally, it is important to note that the presence of a PhNR in mice is controversial, since the PhNR is not always obvious (Miura et al. 2009). The present results obtained with baclofen (Figure 11), together with our observation that a small PhNR also emerged in the LA-ERG when TPMPA was injected rat (not shown), suggest the existence of circuitry for generating a PhNR in mouse although this circuitry may not be sufficiently active under normal conditions to consistently produce a robust PhNR.

Conclusions

2-AEMP, a relatively new $GABA_CR$ antagonist, has similar effects on the ERG as other known $GABA_CR$ antagonists, and similar also to those produced by genetic inactivation of $GABA_CR$ in mouse. The attenuation of the b-wave caused by $GABA_CR$ inactivation, observed in this and previous studies, suggests a role for those receptors in augmenting the amplitude of the signal from bipolar cells, in addition to their documented inhibitory role in modulating bipolar cell output. Negative-going ERG waves originating in inner retina, the nSTR and PhNR, can be amplified by $GABA_BR$ agonists, and $GABA_AR$ antagonists in the rodent retina, indicating that these waves are normally strongly modulated by inhibitory circuits in the inner retina.

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Highlights

- $GABA_C$ receptor antagonist 2-AEMP blocked GABA-induced current in rod bipolar cells.
- Knockout or blockade of $GABA_C$ receptors reduced maximum ERG b-wave amplitude.
- Blockade of $GABA_{A\ or\ B}$ receptors, did not reduce b-wave amplitude.
- $GABA_B$ agonist properties of TPMPA and 3-APMPA did not alter b-wave amplitude.
- $GABA_B$ agonists increased amplitudes of ERG waves originating from inner retina.

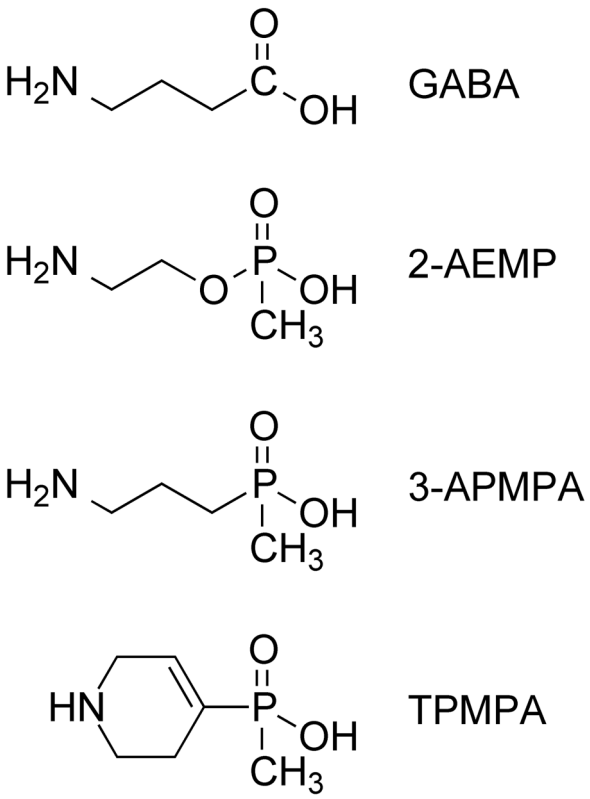

Figure 1.

Chemical formulae for GABA, 2-AEMP, TPMPA and 3-APMPA. The figure shows the structural similarities and differences between the inhibitory neurotransmitter, GABA and the $GABA_CR$ antagonists used in this study that varied in specificity for $GABA_CR$ (see Discussion). GABA, ã-aminobutyric acid; 2-AEMP, 2-aminoethyl methylphosphonate; phosphonic analogues: 3-APMPA (3- aminopropyl-(methyl) phosphinic acid) and TPMPA (1,2,5,6-tetrahydropyridine-4-yl-methylphosphinic acid).

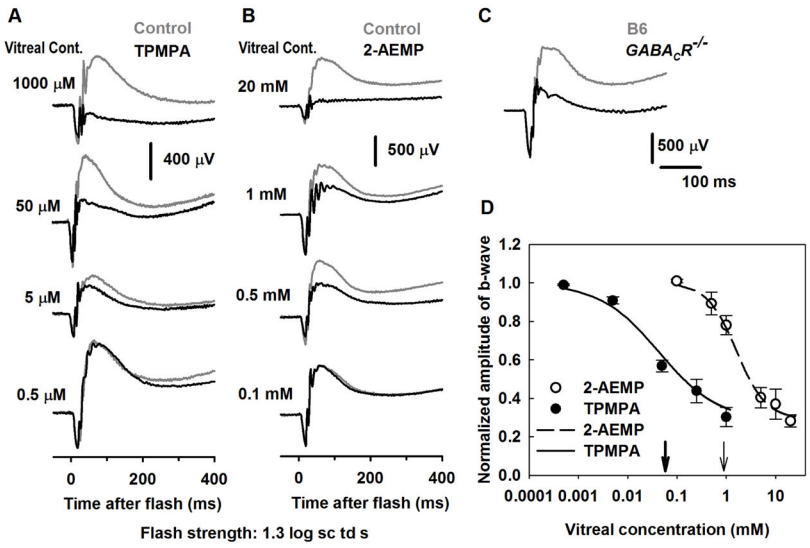

Figure 2.

Dose response function of the effect of GABA$_c$R antagonists, TPMPA and 2-AEMP on b-wave amplitude in DA-ERGs of mice. DA-ERG responses to a flash of 1.3 log sc td s are shown before (grey lines) and after (black lines) intravitreal injection of (**A**) TPMPA or (**B**) 2-AEMP over a range of increasing vitreal concentrations from bottom to top and for (**C**) *GABA$_C$R$^{-/-}$*. (**D**) Normalized b- wave amplitudes were plotted against the vitreal concentration for TPMPA (filled circles) and 2-AEMP (open circles) in response to flash of 1.3 log sc td. The solid and dashed lines show the best fitting Hill equations. b-wave amplitudes measured from trough to peak after injection were normalized to amplitudes before injection. Error bars represent standard error of the mean.

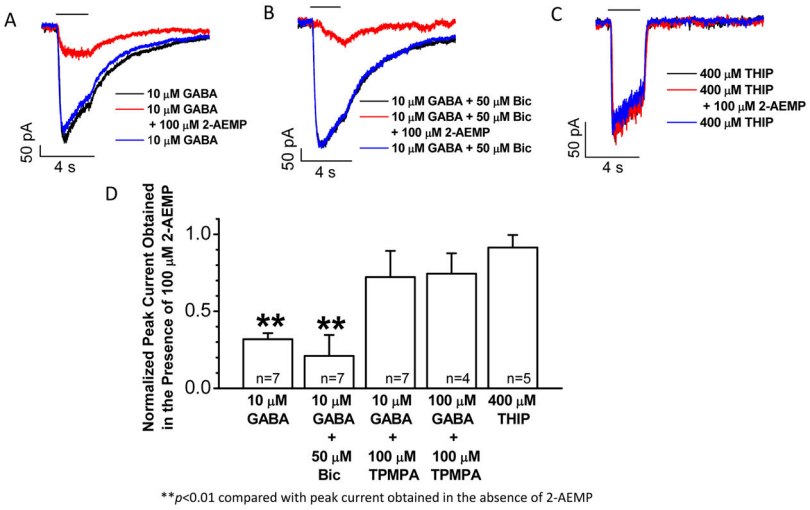

Figure 3.

The effect of 2-AEMP on isolated rat retinal bipolar cells. (**A**) Test of 100 μM 2-AEMP on the response to 10 μM GABA. Responses recorded from a single cell upon treatment with 10 μM GABA alone, co-applied 10 μM GABA plus 100 μM 2-AEMP. Black trace was obtained before, and blue trace after, the response to the GABA plus 2-AEMP mixture (red trace). (**B**) Test of 100 μM 2-AEMP on the response to 10 μM GABA plus 50 μM bicuculline. Responses recorded from a second cell upon treatment, sequentially, with 10 μM GABA plus 50 μM bicuculline (black trace), 100 μM 2-AEMP plus 10 μM GABA plus 50 μM bicuculline (red trace), and 10 μM GABA plus 50 μM bicuculline (blue trace). (**C**) Test of 100 μM 2-AEMP on the response to 100 μM THIP. Responses recorded sequentially with 100 μM THIP alone (black trace), 1 μM GABA plus 100 μM THIP (red trace), and 100 μM THIP (blue trace). (**D**) Peak current in the presence of additional 100 μm 2-AEMP was normalized to that in the absence of 2-AEMP, obtained to 10 μM GABA alone, 10 μM GABA plus 50 μM bicuculline, 10 μM GABA plus 100 μM TPMPA, 100 μM GABA plus 100 μM TPMPA and 100 μM THIP. Error bars represent standard deviation.

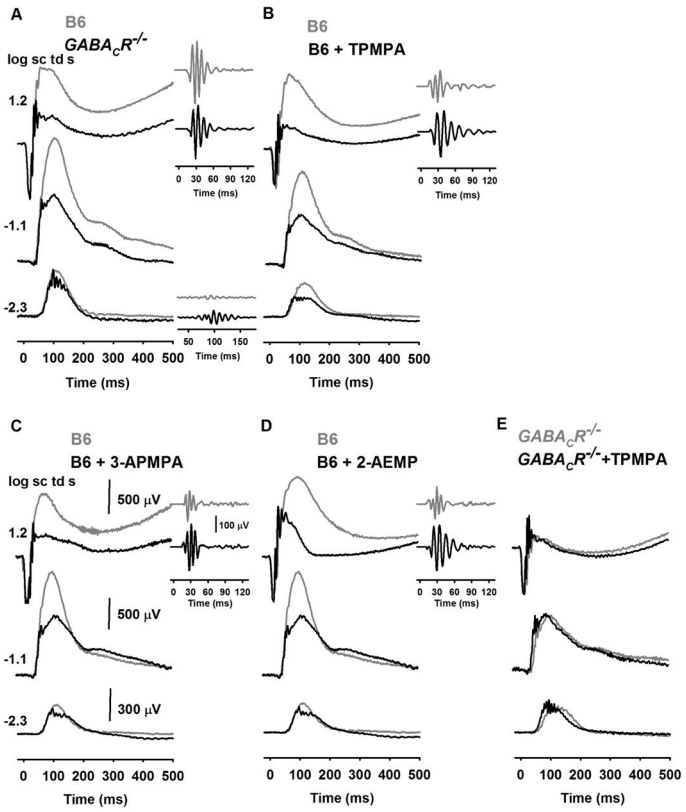

Figure 4.

The effect of genetic elimination of $GABA_CR$ vs effects of $GABA_CR$ antagonists on the mouse DA-ERG. DA-ERG responses elicited by brief full-field flashes with energy increasing from bottom to top were recorded in (**A**) B6 (grey lines) and *GABA$_C$R$^{-/-}$* mice (black lines), and before (grey lines) and after (black lines) intravitreal application of (**B**) TPMPA, (**C**) 3-APMPA and (**D**) 2-AEMP in the B6 mice, and before (grey lines) and after (black lines) intravitreal application of (**E**) TPMPA in *GABA$_C$R$^{-/-}$* mice. The plots inserted on the right hand side of the responses to 1.2 and −2.3 log sc td s flash show the extracted OPs (50 – 300 Hz).

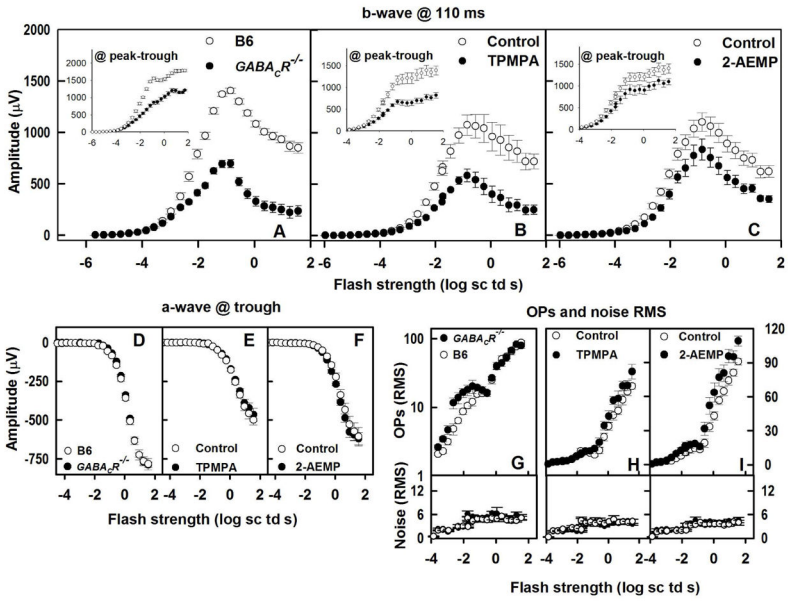

Figure 5.

The effect of $GABA_CR^{-/-}$ (n=6), TPMPA (n=5) and 2-AEMP (n=5) on the amplitude of mouse DA-ERG parameters. Amplitudes of b-wave measured at 110 ms after flash and from a-wave trough to peak (inserts) are plotted against flash strength recorded from (**A**) B6 (open circles) and $GABA_CR^{-/-}$ (black filled circles) mice, and before (open circles) and after (black filled circles) application of (**B**) TPMPA and (**C**) 2-AEMP. Amplitudes of a-wave measured at the trough are plotted against flash energy before (open circles) and after (black filled circles) application of (**E**) TPMPA, (**F**) 2-AEMP and from (**D**) $GABA_CR^{-/-}$ mice. RMS of OPs over the initial 100 ms window are plotted against flash energy in the upper plots before (open circles) and after (black filled circles) application of (**H**) TPMPA, (**I**), 2-AEMP and from (**J**) $GABA_CR^{-/-}$ mice. The lower plots show RMS of noise in the unstimulated window 700–800 ms after the flash. Error bars represent standard error of the mean.

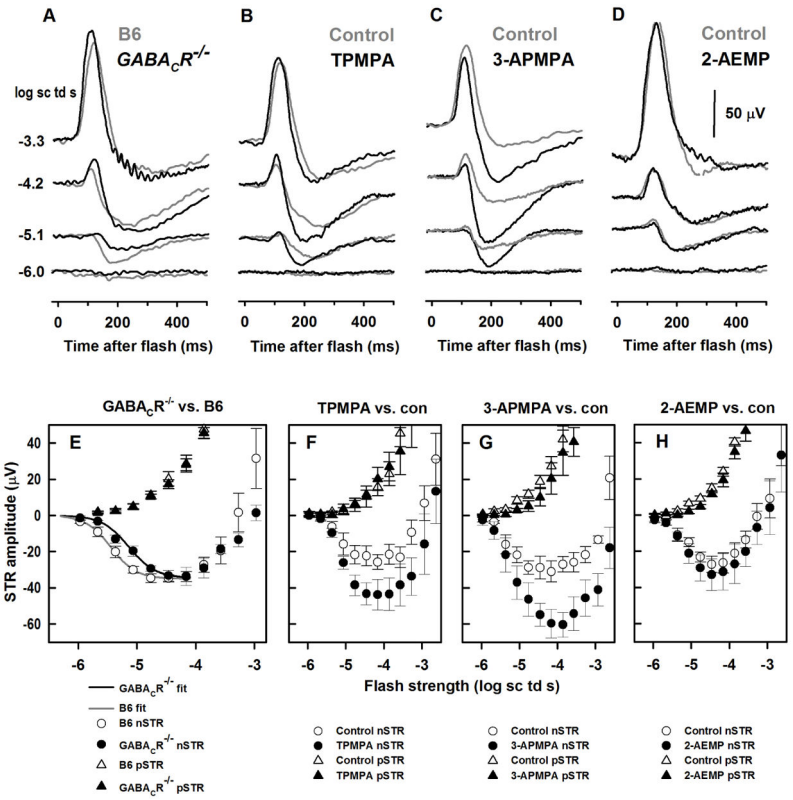

Figure 6.

The effect of GABA$_C$R antagonists and *GABA$_C$R$^{-/-}$* on the STR of mouse DA-ERG. DA-ERG responses elicited by weak flashes (−6 to −3 log sc td s) are shown before (grey lines) and after (black lines) intravitreal application of (**B**) TPMPA, (**C**) 3-APMPA (**D**) 2-AEMP and from (**A**) B6 (grey lines) and *GABA$_C$R$^{-/-}$* (black lines) mice. Amplitudes of pSTR (triangles) measured at 110 ms after flash and nSTR (circles) measured at 200 ms are plotted against flash energy before (open) and after (black) application of (**F**) TPMPA, (**G**) 3APMPA, (**H**) 2-AEMP and from (**E**) *GABA$_C$R$^{-/-}$* mice. The solid lines show the best fitting generalized Naka-Rushton functions. Error bars represent standard error of the mean.

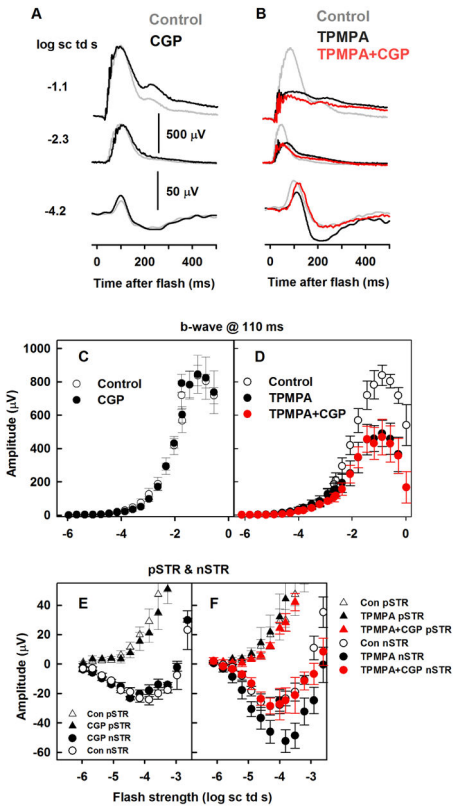

Figure 7.

The effect of $GABA_BR$ on the b-wave amplitude of mouse DA-ERG. (**A**). DA-ERG recordings elicited by brief full-field flashes before (grey lines) and after (black lines) intravitreal injection of CGP. (**B**). DA-ERG recordings before (grey lines) and after (black lines) application of TPMPA and with additional application of CGP after TPMPA (red lines). Stimulus response function for b-wave and STR amplitude before (open circles) and after (black filled circles) the treatment are plotted for (**C, E**) CGP (n=4), for (**D, F**) TPMPA (n=4, black filled circles and triangles) and TPMPA+CGP (n=4, red filled circles and triangles). Error bars represent standard error of the mean.

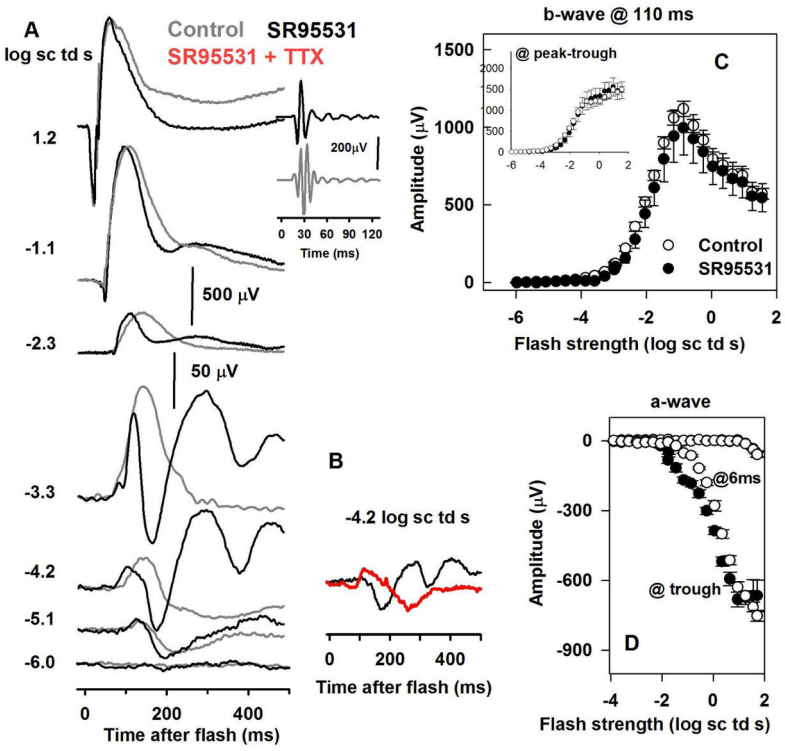

Figure 8.

The effect of $GABA_AR$ antagonist SR95531 on the DA-ERGs in B6 mice. (**A**) DA-ERG recordings before (grey lines) and after (black lines) intravitreal injection of SR95531. (**B**) DA-ERG responses after SR95531 (black line) and with additional application of TTX after SR95531 (red lines). Stimulus response function before (open circles) and after (black filled circles) application of SR95531 (n=5) are plotted for (**C**) b-wave amplitude measured at 110 ms after the flash and also from a-wave trough to peak (inserted), for (**D**) a-wave amplitude measured from trough to baseline. Error bars represent standard error of the mean.

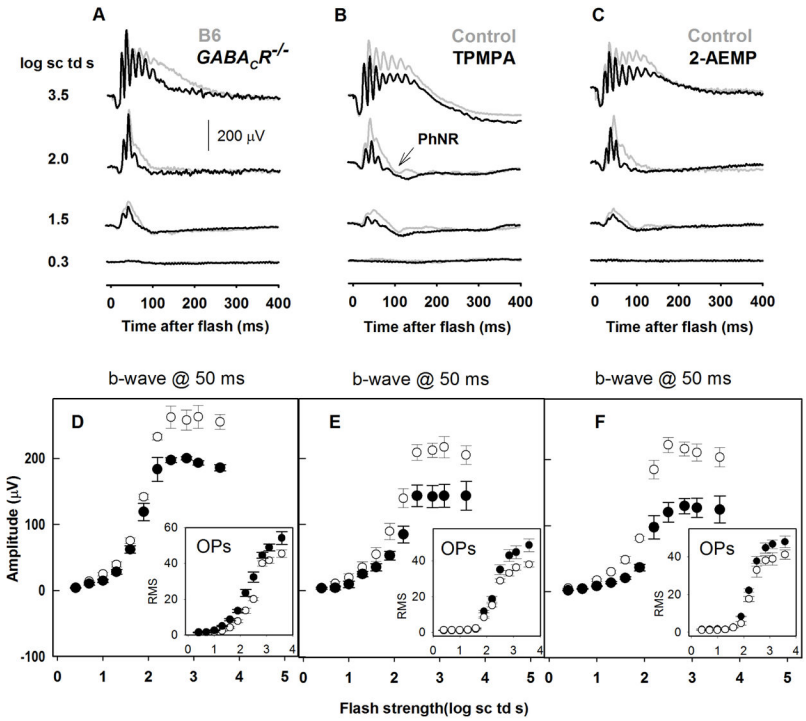

Figure 9.

The effect of inactivation of $GABA_CR$ on the mouse LA-ERG. LA-ERG responses elicited by brief full-field flashes with a rod-saturated background before (grey lines) and after (black lines) intravitreal injection of (**B**) TPMPA and (**C**) 2-AEMP and from (**A**) $GABA_CR^{-/-}$ mice. Stimulus response function of b-wave, and OPs RMS are plotted against stimulus strength before (open circles) and after (filled circles) injection of (**E**) TPMPA and (**F**) 2-AEMP and for (**D**) B6 (open circles) and $GABA_CR^{-/-}$ (filled circles) mice. B6. Error bars represent standard error of the mean.

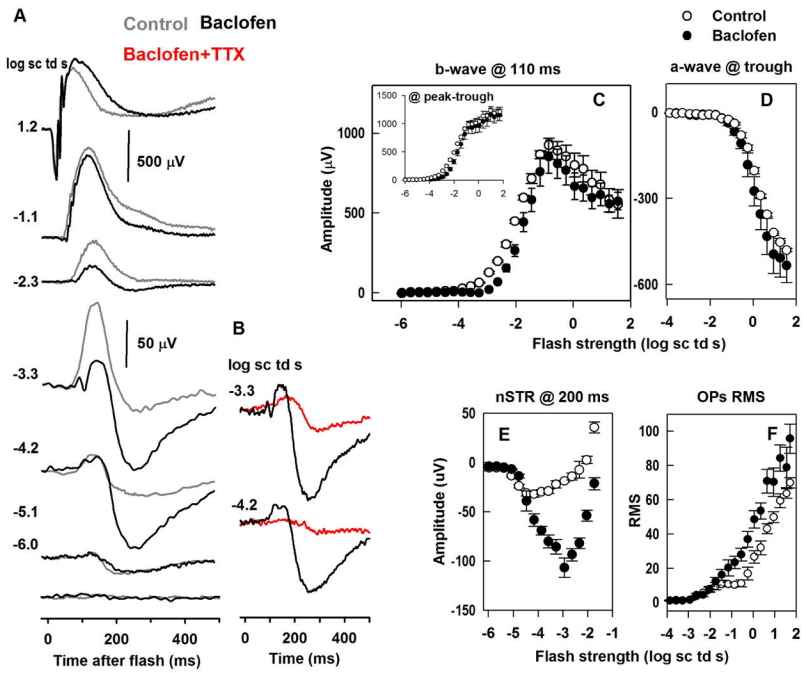

Figure 10.

The effect of $GABA_BR$ agonist baclofen on the DA-ERGs in B6 mice. The figures shows (**A**) DA-ERG recordings before (grey lines) and after (black lines) intravitreal injection of baclofen and (**B**) with additional application of TTX after baclofen (red lines). Stimulus response function before (open circles) and after (black filled circles) application of baclofen (n=4) are plotted for (C) b-wave amplitude measured at 110 ms after the flash and also from a-wave trough to peak (inserted), for (D) a-wave amplitude measured from trough to baseline and for (E) nSTR amplitude measured at 200 ms, and for (F) OPs RMS and noise RMS. Error bars represent standard error of the mean.

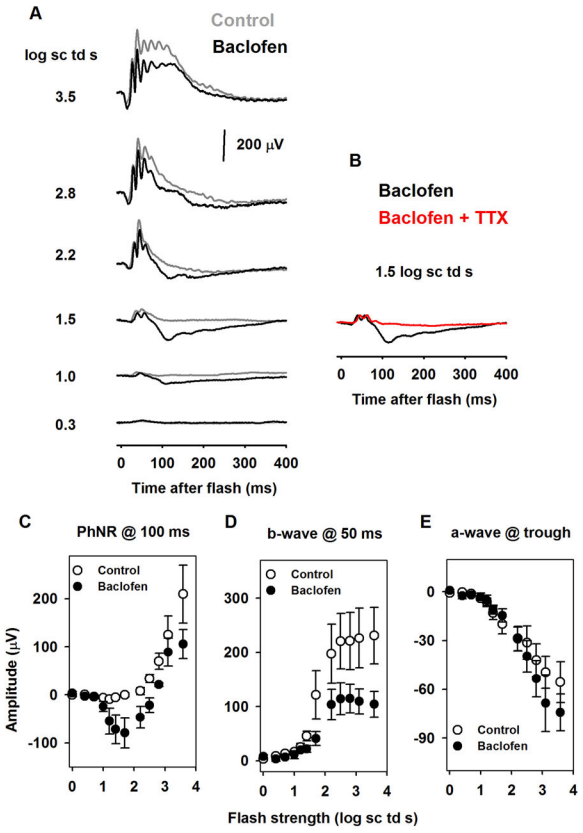

Figure 11.

The effect of GABABR agonist baclofen on the LA-ERG. (A) LA-ERG recordings before (grey lines) and after (black lines) intravitreal injection of baclofen. (B) Responses of additional application of TTX (black line) after baclofen injection (grey line). Stimulus response functions before (open circles) and after (black filled circles) application of baclofen (n=4) are plotted for (C) the PhNR amplitude measured at 100 ms after the flash, for (D) b-wave amplitude measured at 50 ms and for (E) a-wave amplitude measured at the trough. Error bars represent standard error of the mean.

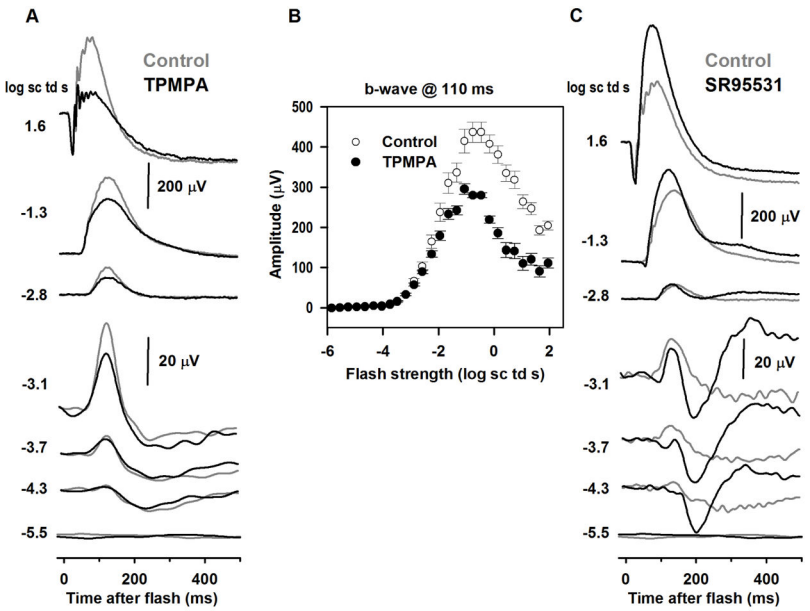

Figure 12.

The effect of TPMPA on the DA-ERG in Brown Norway rats. (A) DA-ERG recordings before (grey lines) and after (black lines) intravitreal injection of TPMPA. (B) The b-wave amplitude measured at 110 ms after the flash, plotted against flash energy, before (open circles) and after (black filled circles) application of TPMPA (n=3). Error bars represent standard error of the mean. (C) Responses before (grey lines) and after (black lines) injection of SR95531.

Table 1

Parameters of Hill equation fit to the dose-response function

	Flash 1.3 log sc td s		Flash −1.1 log sc td s	
	TPMPA (n=4–5 per dose)	**2-AEMP (n=3–4 per dose)**	**TPMPA (n=4–5 per dose)**	**2-AEMP (n=3–4 per dose)**
r^2	0.90	0.91	0.88	0.96
F value	56.4	73.9	53.0	180
p value	<0.0001	<0.0001	<0.0001	<0.0001
r_{max}	0.79 (0.11)	0.70 (0.07)	0.82 (0.14)	0.79 (0.05)
n	0.72 (0.23)	1.46 (0.41)	0.77 (0.30)	1.68 (0.37)
C_{50}	0.04 (0.03)	1.62 (0.47)	0.05 (0.03)	1.42 (0.27)

The b-wave was measured from trough to peak for a flash of 1.3 log sc td s and at 110 ms for a flash of −1.1 log sc td s. Data are presented as mean and standard error.

Table 2

Table 2A. Comparisons of stimulus-response functions for a- and b-waves, and OPs of DA-ERGs

	$GABA_CR^{-/-}$ vs. B6 n=6		TPMPA vs. control n=5		2-AEMP vs. control n=5	
	F value	**p value**	**F value**	**p value**	**F value**	**p value**
b-wave amp (μV) @ 110 ms	563.0	<0.001	30.1	0.01	12.8	<0.05
b-wave amp (μV) @ peak-trough	194.0	<0.001	43.0	<0.01	8.4	<0.05
a-wave amp (μV) @ trough	0.28	>0.05	0.51	>0.05	1.30	>0.05
OPs amp (RMS)	8.1	<0.05	31.6	<0.05	16.2	<0.05

Table 2B. Percent change (mean +/− SD) in b-wave and OP amplitudes

	$GABA_CR^{-/-}$ vs. B6 n=6	TPMPA vs. control n=5	2-AEMP vs. control n=5
b-wave amp @110 ms reduction	50±17%	45±10%	31±5%
b-wave amp @peak-trough reduction	45±7%	42±6%	28±4%
OPs amp (RMS) enhancement	80±36%	41±18%	34±16%

DA-ERG b-wave stimulus response functions were compared over the range of −3 to 1.6 log sc td s. OP amplitudes for *$GABA_CR^{-/-}$* were compared with B6 amplitudes for the range over which they were enhanced, −3 to −1 log sc td s. For TPMPA and 2-AEMP, OPs were compared from −3 to +1.6 log sc td s. The F values and p values in part A were derived from repeated measures ANOVA tests.

Table 3

Table 3A. Comparisons of stimulus-response functions for a-waves, b-waves and OPs of LA-ERGs

	$GABA_CR^{-/-}$ vs. B6		TPMPA vs. control		2-AEMP vs. control	
	F value	**p value**	**F value**	**p value**	**F value**	**p value**
b-wave amp @ 50 ms	31.7	<0.001	33.7	<0.01	44.8	<0.01
b-wave amp @ peak-trough	24.9	<0.01	22.1	<0.01	416.6	<0.01
a-wave amp @ 6 ms	1.03	>0.05	4.8	>0.05	0.96	>0.05
a-wave amp @ trough	2.36	>0.05	5.00	>0.05	0.02	>0.05
OPs amp	9.1	<0.05	10.1	<0.05	10.1	<0.05

Table 3B. Percent change (mean +/− SD) in b-wave and OP amplitudes

	$GABA_CR^{-/-}$ vs. B6	TPMPA vs. control	2-AEMP vs. control
b-wave amp @ 50 ms reduction	23±8%	36±12%	46±12%
OPs amp (RMS) enhancement	51±17%	26±7%	21±9%

LA-ERG b-wave, a-wave and OPs functions for control and treated were compared over the entire range of flash strengths (from 0.3 to 3.5 log sc td s for b-waves, 1.5 −3.5 for OPs.)