

Reversible modulation of GABA_A receptor-mediated currents by light is dependent on the redox state of the receptor

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Abstract

Light has recently been shown to be a physical modulator of GABA_A receptor activity. Here, we further characterize the effects of light on a native cortical and retinal population of GABA_A receptors, and identify a possible mechanism for light induced potentiation using recombinant receptors. GABA-induced currents in cortical neurons were observed to be rapidly and reversibly potentiated following exposure to a brief flash of light (0.5–2 s; >280 nm) directed via an optical fibre (50 µm i.d.). GABA_A receptor-mediated responses in retinal ganglion cells were also enhanced by light, while glycine-induced currents in these cells were unaffected by the same stimulus. We also determined that physiological levels of light, that is, those that would normally reach the retina, also enhanced GABA-induced currents. Finally, we observed that chemical reduction of recombinant $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$ GABA_A receptors by dithiothreitol substantially attenuated the effects of light. These results suggest that GABA_A receptors can be reversibly modified by a brief pulse of light via an allosteric mechanism that is intimately linked to redox modulation.

Introduction

The majority of fast inhibitory neurotransmission in the mammalian forebrain is mediated by activation of the GABA_A receptor, a Cl⁻ selective ion channel. A functional GABA_A receptor has been proposed to be a pentameric protein, comprised of subunits from five known families α_{1-6} , β_{1-3} , γ_{1-3} , δ_1 , ϵ_1 (Baumann *et al.*, 2001; Klausberger *et al.*, 2001). Differential expression of these subunits has been shown to cause substantial changes in the pharmacology and physiology of assembled GABA_A receptors (Hevers & Lüddens, 1998). Physiological responses mediated by GABA_A receptors are modulated by a number of different compounds including zinc, steroids, and benzodiazepines (Seighart, 1995). Furthermore, these receptors are also sensitive to redox modulation; chemical reduction of the receptor potentiates currents, while chemical oxidation depresses currents *in vitro* (Pan *et al.*, 1995; Amato *et al.*, 1999; Pan *et al.*, 2000).

In addition to chemical modulators, heat, stretch as well as light, have been shown to modify the activity of ligand-gated channels (Chung & Kuyucak, 1995; Ascher *et al.*, 1988; Casado & Ascher, 1998; Leszkiewicz *et al.*, 2000; Chang *et al.*, 2001). Previously, our laboratory demonstrated that *N*-methyl-D-aspartate (NMDA) receptors, but not non-NMDA receptors, are functionally and reversibly modified by brief pulses of light (Leszkiewicz *et al.*, 2000). We have also shown that inclusion of a redox-insensitive NR1 subunit can abolish the effects of light on currents mediated by recombinant NMDA receptors (Leszkiewicz & Aizenman, 2002). The results presented here demonstrate that light pulses directed towards native and recombinant GABA_A receptors reversibly enhance currents mediated by these channels. We also show that this effect is dependent on the redox state of the receptor. We propose that light and redox

modulation of ion channels may be intimately linked together, altering receptors via common allosteric mechanisms.

Methods

Neuronal cell culture

Cortical neurons were dissociated from E16 Sprague–Dawley rats as described previously (Hartnett *et al.*, 1997). Pregnant rats were killed by CO₂ inhalation immediately prior to removal of embryos. Embryonic cortical cells were dissociated by incubation with trypsin and plated onto 35 mm tissue culture dishes containing five poly-L-lysine coated glass coverslips. Growth media contained 80% Dulbecco's modified Eagle's medium with 10% heat-inactivated, iron-supplemented bovine calf serum, 10% Ham's F-12 media, as well as 25 mM Hepes, 24 U/mL penicillin, 24 U/mL streptomycin and 2 mM L-glutamine. Two weeks after plating, non-neuronal cell growth was arrested with a 72-h treatment with 2 mM cytosine arabinoside, after which the growth media contained only 2% serum and no F-12. Cells were used for electrophysiology experiments in the fourth week after dissociation. Isolated postnatal mouse retinae were treated with papain, dissociated with gentle trituration, and cultured as previously described (Aizenman *et al.*, 1988; Barres *et al.*, 1988). Electrophysiological recordings from putative, morphologically identified Y-like or α -like retinal ganglion cells were performed 6–8 h after plating (Fukada, 1977; Perry, 1979; Leszkiewicz *et al.*, 2000).

Chinese hamster ovary cell culture

Chinese hamster ovary (CHO) cells were grown and transfected as previously described (Boeckman & Aizenman, 1996; Brimecombe *et al.*, 1997). Briefly, CHO cells were grown in Ham's F-12 media containing 10% fetal bovine serum and 2 mM L-glutamine and passaged 2–3 times a week for a total of no more than 30 times. CHO cells were seeded at 2.8×10^5 cells per well into 6-well plates 24(h) prior to

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transfection. The cDNAs for GABA_A receptor subunits have been previously ligated into mammalian expression vectors (a gift from D. Lynch, University of Pennsylvania). Cells were transfected in serum-free medium with 6 μ L LipofectAMINE reagent (Invitrogen, Carlsbad, CA, USA) and a total of 1.4 μ g of DNA per well (0.05 μ g DNA GFP, 0.675 μ g DNA each GABA subunit). Four hours after transfection, cells were refed with media containing 10% serum. Recordings were performed 24–48 h after transfection.

Electrophysiology

Electrophysiological recordings were performed at room temperature using the whole cell configuration of the patch clamp technique (Tang & Aizenman, 1993). Cells were bathed in a solution containing (in mM): 150 NaCl, 1.0 CaCl₂, 2.8 KCl, 10 Hepes, 0.25 tetrodotoxin (in the case of neurons) and pH was adjusted to 7.2 with NaOH. Electrodes were pulled to a resistance of 1.5–3 M Ω when filled with internal solution containing (in mM): 140 CsF (or 140 CsCl), 10 EGTA/CsOH, 1 CaCl₂ and 10 Hepes (pH adjusted to 7.2 with CsOH). Signals were amplified using an Axopatch 1D patch-clamp amplifier (Axon Instruments, Union City, CA, USA), filtered at 1 kHz and digitized at 2 kHz. The reference electrode was an Ag/AgCl wire bridged with 2 M KCl/1% agarose. Gamma-aminobutyric acid (GABA), glycine, *N*-methyl-D-aspartate (NMDA; with 10 μ M glycine), dithiothreitol (DTT), and 5,5-dithio-*bis*-(2-nitro-benzoic acid) (DTNB) were dissolved in solution and applied via a fast perfusion system (Warner Instruments, Hamden, CT, USA).

Light stimulus

Light was directed onto cells as previously described (Kandler *et al.*, 1999; Leszkiewicz *et al.*, 2000). Briefly, light from a 100W mercury lamp (Oriel Corporation, Stratford, CT, USA) was focused through an electronic shutter (Vincent Associates, Rochester, NY, USA) and guided onto the preparation utilizing a 50 μ m fused-silica fibre. A 280-nm long pass filter (Oriel) was placed in the path of the light beam to minimize UV damage to the cell. Both 1% and 3% neutral density filters (Oriel) were placed in the light path to attenuate the light source to physiological levels as noted in the text (Leszkiewicz *et al.*, 2000). The fibre produced a 75–100 μ m diameter light spot covering the soma and proximal dendrites of a cultured cortical neuron.

Results

NMDA and GABA-induced currents were recorded using the whole-cell configuration of the patch-clamp technique both before and after exposing the neuronal soma and proximal dendrites to a brief pulse of light (>280 nm, 1–2 s). As previously seen for NMDA currents, GABA mediated responses also became substantially enhanced following the light stimulus (Fig. 1A). We next examined whether the light induced potentiation of GABA-induced responses could also be observed during the agonist exposure. Under these conditions, GABA currents were rapidly potentiated by the light pulse and were observed to slowly decay after the light stimulus, although still remaining enhanced when compared to baseline (Fig. 1B).

Previously, we had shown that light enhanced NMDA-induced currents in retinal ganglion cells (Leszkiewicz *et al.*, 2000). While NMDA receptors have limited expression in the retina, GABA_A receptors are expressed ubiquitously throughout this organ (Nag & Wadhwa, 1997). As such, whole-cell GABA-induced and, for comparison, glycine-induced currents were measured in an acute retinal ganglion cell preparation both before and after a brief pulse of light. GABA-induced currents in these cells were modified in a similar manner to the currents mediated by cortical GABA_A receptors (Fig. 2A). In contrast, glycine receptor-mediated responses were not potentiated by light, and in some cases, currents mediated by this inhibitory receptor were actually depressed following light exposure (Fig. 2A). Receptor light sensitivity was quantified by measuring the ratio of maximal current amplitudes before and after a brief light pulse (I^*/I ; Fig. 2B; Leszkiewicz *et al.*, 2000).

The intensity of the light stimulus utilized in the previous experiments is close to 100 times that of direct sunlight (Leszkiewicz *et al.*, 2000). However, NMDA receptor mediated responses are enhanced even after these levels of light are attenuated by 99%, by placing a neutral density filter within the light path (Leszkiewicz *et al.*, 2000). The retina probably receives these levels of light during a bright, clear day (Leszkiewicz *et al.*, 2000). Therefore, we determined whether these ‘physiological’ levels of light could also alter responses mediated by GABA_A receptors. After establishing a stable baseline amplitude for GABA-induced currents, attenuated levels of light (1% or 0.03% that of control) were continuously applied to the soma and proximal dendrites, while whole-cell GABA-induced currents were measured

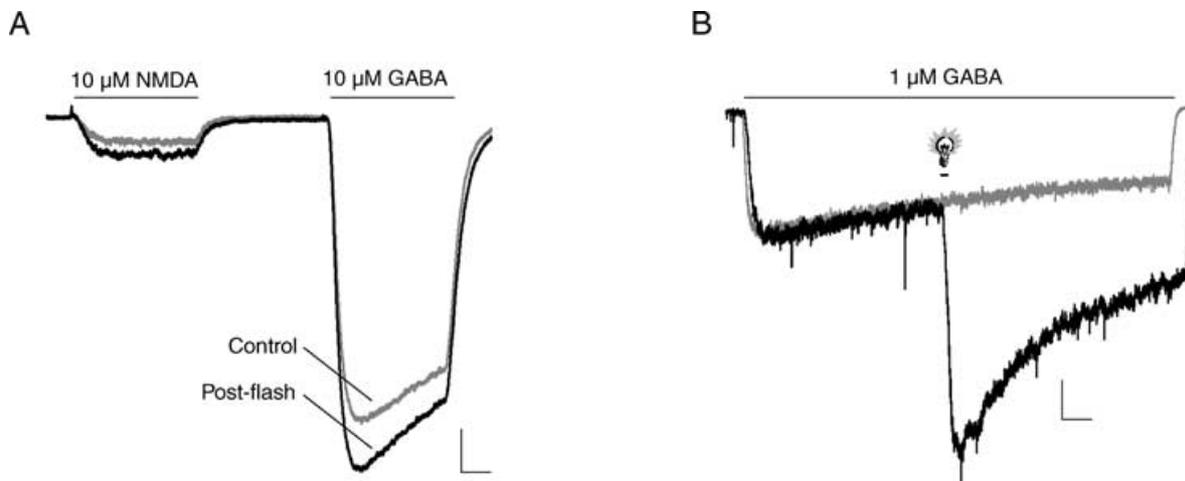


FIG. 1. (A) Whole cell NMDA (10 μ M) and GABA (10 μ M)-induced currents in cortical neurons before (grey trace) and shortly after (10 s; black trace) a brief light pulse (2 s, >280 nm). Membrane potential, -60 mV; scale bars, 500 pA and 500 ms. (B) A brief pulse of light (500 ms, >280 nm) was focused upon the soma and proximal dendrites during a whole cell GABA (1 μ M) mediated response (black trace). A control response from the same cell is also shown (grey trace). Membrane potential, -60 mV; scale bars, 200 pA and 2 s.

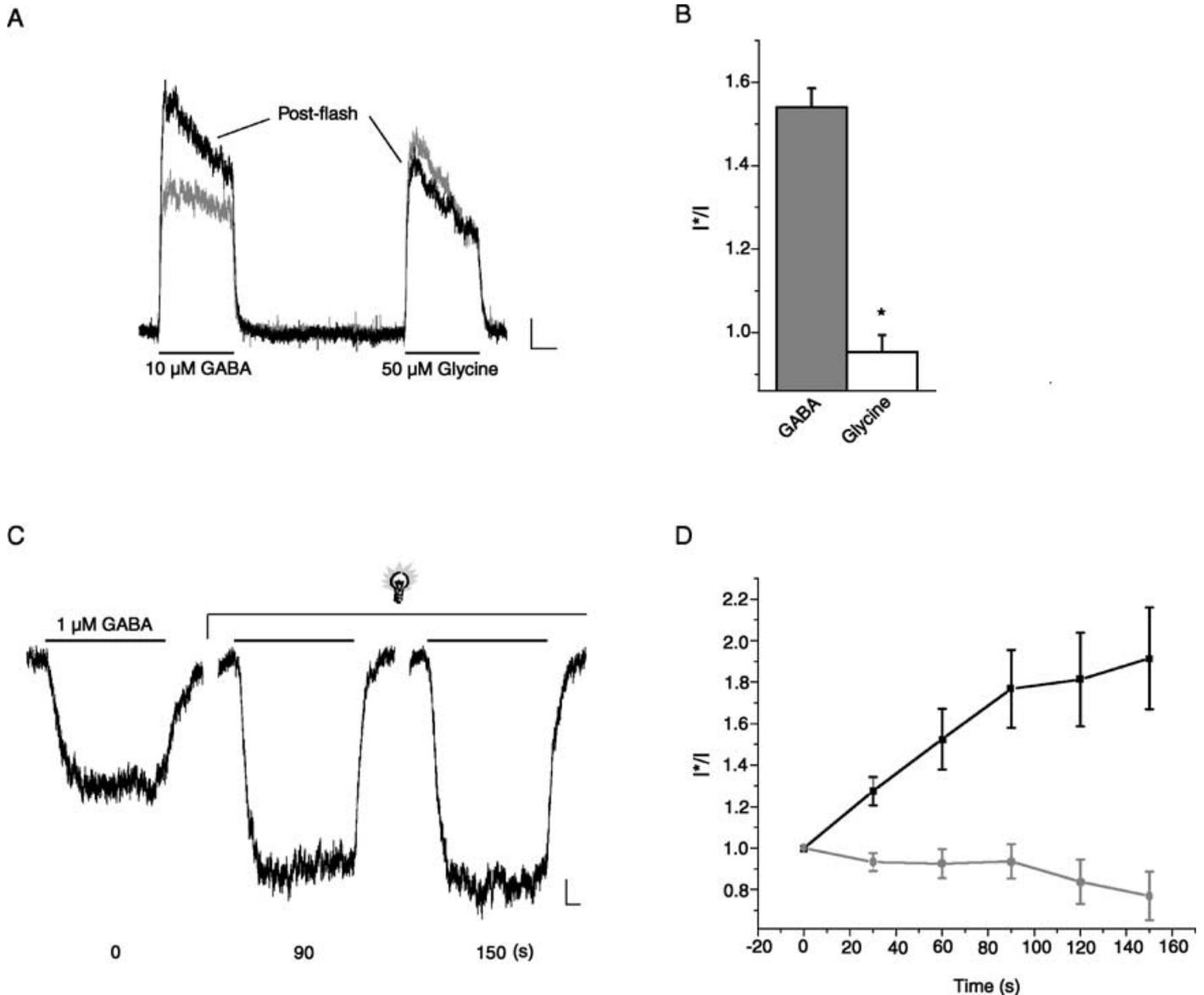


FIG. 2. (A) Whole cell GABA (10 μ M) and glycine (50 μ M) induced currents in retinal ganglion cells before (grey trace) and shortly after (5 s; black trace) a brief light pulse (2 s, >280 nm). Similar effects were seen in five cells. Membrane potential, -30 mV; scale bars, 25 pA and 1 s. (B) Peak response amplitudes for responses such as those seen in A were measured and a postflash to preflash ratio (I^*/I) was calculated ($*P < 0.05$, significantly different from 1, one sample two-tailed t -test; $n = 5$). (C) Whole-cell GABA (1 μ M) mediated responses in cortical neurons both before and during a continuous attenuated light stimulus (1% of control; light bulb). Similar effects were seen in six cells. Membrane potential, -30 mV; scale bars, 50 pA and 500 ms. (D) Peak response amplitudes were measured and an I^*/I ratio was calculated both before (0 s) and during (30–150 s) a continuous pulse of 1% transmitted light (black line) and 0.03% transmitted light (grey line), summary of $n = 10$ cells.

every 30 s. We observed that GABA currents were indeed enhanced by this light stimulus, with the responses becoming maximally potentiated 90 s after the start of exposure to light attenuated to 1% of the control (Fig. 2C). In contrast, light attenuated to 0.03% of the control had no apparent effect on the GABA-induced responses (Fig. 2D). These results suggest that light may play a role as a modulator of ion channel function in the retina.

While other investigators have determined that the light-induced potentiation of recombinant GABA_A receptors may be irreversible following a long UV pulse of light (60 s; Chang *et al.*, 2001), we measured an observable decay of the light enhancement of currents following a much briefer pulse (1–2 s). GABA-induced currents were measured before and for five minutes after a brief light stimulus (Fig. 3A). After this time point a subsequent light pulse was used to

determine whether the receptors remained light sensitive following the decay (Fig. 3A). We found that the light induced potentiation of GABA_A receptors reversed back to baseline after 3–5 min (Fig. 3B). Also, we observed that GABA-mediated responses could be enhanced once again by the second light stimulus (Fig. 3A). These data demonstrate that the light induced change that we have studied in both NMDA and GABA_A receptors is mediated by reversible modification to the receptor.

Chang *et al.* (2001) reported that the irreversible potentiation of GABA induced currents by light was manifested by a change in the EC₅₀ for the agonist. We thus investigated whether the reversible effects of light also affected the GABA concentration–response profile. Neurons were exposed to various concentrations of GABA (0.3 μ M–300 μ M), both before and after a brief pulse of light (Fig. 4A;

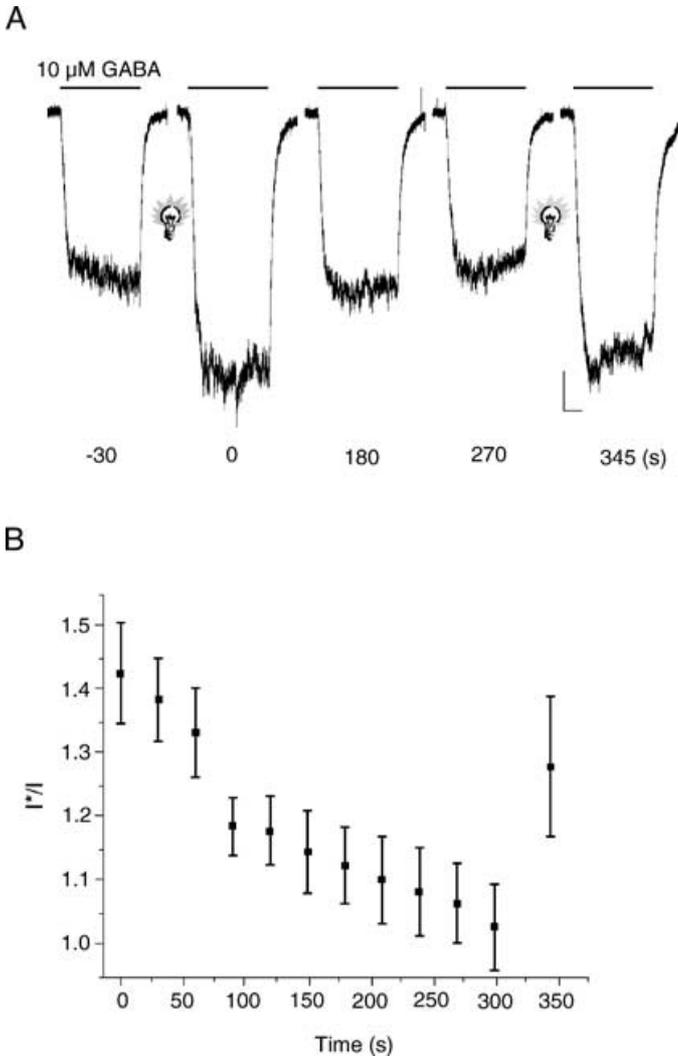


FIG. 3. (A) Whole cell GABA ($1\ \mu\text{M}$) induced currents in cortical neurons before ($-30\ \text{s}$) and after ($0\text{--}270\ \text{s}$) a brief light pulse ($2\ \text{s}$, $>280\ \text{nm}$). A subsequent light pulse repotentiated currents ($345\ \text{s}$). Similar effects were seen in seven cells. Membrane potential, $-60\ \text{mV}$; scale bars, $100\ \text{pA}$ and $1\ \text{s}$. (B) Peak response amplitudes were measured for responses such as those seen in A and an I^*/I ratio was calculated for all time points during each trial, summary of $n = 7$ cells.

$2\ \text{s}$; $>280\ \text{nm}$). Both preflash and postflash responses from each cell were normalized to the preflash response to $3\ \mu\text{M}$ GABA (Fig. 4B). We noted no change in the GABA EC_{50} following light stimulus, similar to what was seen during our NMDA receptor studies (Leszkiewicz *et al.*, 2000). Therefore, the reversible light enhancement of receptor currents does not likely involve alterations in the GABA binding site and are probably manifested by the structural alteration of a different site on the receptor.

GABA_A receptors have been shown to be redox sensitive (Pan *et al.*, 1995; Amato *et al.*, 1999; Pan *et al.*, 2000), and we have reported that an intact and oxidized functional redox site is necessary for the light induced potentiation of NMDA induced currents (Leszkiewicz & Aizenman, 2002). Therefore, we evaluated whether the effects of light would also be altered by the oxidation or reduction of recombinant $\alpha_1\beta_2$ GABA_A receptors transfected in CHO cells. We chose to use CHO cells for these experiments, as the light stimulus we utilize does not illuminate the entire population of receptors in our cultured

neurons (due to their large and elaborate dendritic processes; Leszkiewicz *et al.*, 2002). Trials consisted of first testing the light sensitivity of receptors that had been previously oxidized by exposure to $500\ \text{nM}$ DTNB for $2\ \text{min}$ ($-\text{DTT}$; Fig. 4C). Receptors were chemically reduced by exposing cells to $5\ \text{mM}$ DTT ($+\text{DTT}$), until currents were stably enhanced (approximately $4\text{--}7\ \text{min}$ after incubation with the reducing agent). GABA induced currents were then measured both before and after a brief pulse of light. Finally, a 2-min incubation with DTNB was performed to re-oxidize the receptors (Fig. 4C). Our results, which are summarized in Fig. 4D, demonstrate that the chemical reduction of the GABA_A receptor nearly completely abolished light induced potentiation.

Previous investigators have observed that the addition of the γ_{2S} subunit to the $\alpha_1\beta_2$ receptor significantly decreases the redox sensitivity of the channel (Amato *et al.*, 1999; Pan *et al.*, 2000). However, a recent study by Wilkins & Smart (2002) demonstrated that the lack of an apparent effect of DTT on γ_{2S} -containing receptors was due to a decrease in the degree of Zn^{2+} sensitivity in this receptor configuration. Hence, some of the effects of DTT on recombinant receptors were due to the ability of this reducing agent to bind zinc. Nonetheless, a residual, large redox sensitivity was shown to be still present in both $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$ receptors (Wilkins & Smart, 2002). We believe, however, that the light effects we observe are not due to an alteration in the Zn^{2+} -binding properties of recombinant GABA_A receptors as we observed no difference between the light-induced potentiation of $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$ receptor mediated responses (I^*/I : $\alpha_1\beta_2$, 1.29 ± 0.03 ; $\alpha_1\beta_2\gamma_{2S}$, 1.27 ± 0.05 ; $n = 4$ and 6 , respectively; Fig. 4C).

Discussion

The results from this study demonstrate that a brief pulse of light potentiates GABA-induced currents in a reversible manner. In addition, we have shown that chemically reducing the GABA_A receptor with DTT abolishes the light sensitivity of the channel. Furthermore, physiological levels of light enhanced GABA_A-receptor mediated responses, and GABA-induced currents measured in retinal ganglion cells are also potentiated following a brief pulse of light. Decay of the light-induced effects demonstrates that light, at least under our experimental conditions, does not permanently alter the function of the receptor. Finally, we do not believe that the effects of light are due to heat or generation of reactive oxygen species (ROS). First, our light source does not produce local changes in temperature (as measured with a microthermocoupler probe; Leszkiewicz *et al.*, 2000), and second, ROS would be expected to oxidize the receptors and thereby depress the responses, rather than potentiate them (Pan *et al.*, 1995; Amato *et al.*, 1999; Pan *et al.*, 2000).

As we have proposed before, modulation of ion channel function by both redox agents and light seem to be structurally linked within the receptor (Leszkiewicz & Aizenman, 2002). Specifically, we have shown that both GABA and NMDA receptor mediated responses are enhanced following exposure to light stimuli, and that reducing agents occlude the effects of light on these channels (Fig. 4D; Leszkiewicz and Aizenman, 2002). Mutation of two cysteines on the NR1 subunit, responsible for redox modulation of the majority of NMDA receptor configurations (Sullivan *et al.*, 1994; Brimacombe *et al.*, 1999), nearly abolishes the effects of light on this channel (Leszkiewicz & Aizenman, 2002). Expression of the γ_{2S} subunit along with the $\alpha_1\beta_2$ subunits decreases, but does not abolish, the redox sensitivity of the ion channel (Amato *et al.*, 1999; Pan *et al.*, 2000). A recent paper by Wilkins & Smart (2002) demonstrated that low concentrations of DTT ($2\ \text{mM}$) potentiate GABA-induced currents mediated via $\alpha_1\beta_2$ receptors by binding to, and decreasing Zn^{2+} -mediated block of the

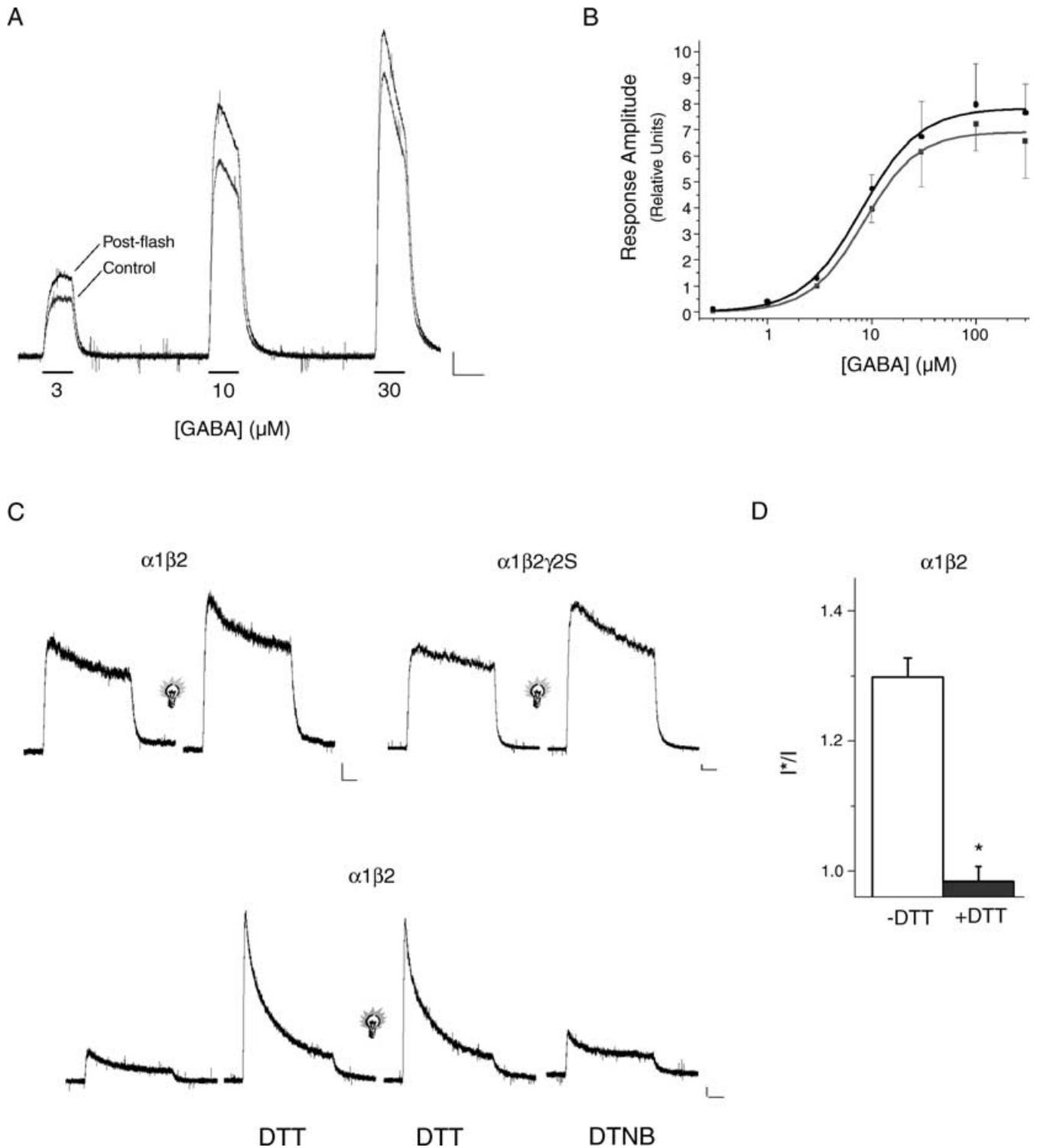


FIG. 4. (A) Representative whole cell GABA (3–30 μM) induced currents in cortical neurons before (grey trace) and shortly after (5 s; black trace) a brief light pulse (2 s, >280 nm). Membrane potential, -30 mV ; scale bars, 250 pA and 1 s. (B) Current amplitudes were normalized to the 3 μM GABA-induced responses for each current trace. Preflash and postflash data were plotted and fit with a sigmoidal dose–response curve (EC_{50}^{pre} , 7.94 ± 0.60 ; EC_{50}^{post} , $8.4 \pm 0.76\ \mu\text{M}$). (C). Top, GABA-elicited responses (1 μM) from recombinant $\alpha_1\beta_2$ receptors (left) and $\alpha_1\beta_2\gamma_2\text{s}$ (right) expressed in CHO cells both before and after a brief flash of light (light bulb; 2 s, >280 nm). Scale bars, 50 pA and 500 ms. Bottom, GABA-mediated responses from recombinant $\alpha_1\beta_2$ receptors before and after 5 min of 5 mM DTT incubation. Chemically reduced receptors were then exposed to brief flash (2 s, >280 nm), followed by chemical oxidation with 500 mM DTNB (2 min). Scale bars, 50 pA and 500 ms. (D) I^*/I ratios demonstrating the absence of light induced potentiation following chemical reduction ($*P < 0.05$, significantly different from 1, one sample two-tailed t -test, $n = 10$ cells).

ion channel. At high concentrations (10 mM), DTT still potentiates currents via a redox-based process (Wilkins & Smart, 2002). Although $\alpha_1\beta_2\gamma_{2S}$ receptor-mediated responses can be potentiated by high DTT concentrations, low concentrations of this reducing agent are unable to affect these receptors' responses, suggesting that this subunit configuration is much less sensitive to Zn^{2+} blockade. We have found that the light sensitivity of the $\alpha_1\beta_2\gamma_{2S}$ receptor combination was no different than that of the $\alpha_1\beta_2$ receptor, similar to what was reported by Chang *et al.* (2001). This result suggests that redox properties are important for the effects of light on ion channel function. As such, the presence of the γ_{2S} subunit does not interfere with the reversible or irreversible effects of light on recombinant GABA_A receptors.

Redox agents potentiate GABA-induced currents without modifying the EC₅₀ of the receptor for its agonist (Amato *et al.*, 1999; Pan *et al.*, 2000). Similarly we have observed that the reversible effects of light on GABA_A receptor function do not alter the apparent affinities of these receptors for their respective agonists. Our results differ from those of Chang *et al.* (2001), who showed that intense UV light exerts its effects on the channel by altering the EC₅₀ of the GABA_A receptor for its agonist. Interestingly however, these authors observed that $\alpha_1\beta_2\gamma_{2S}$ receptor-mediated responses were potentiated by light without a change in the EC₅₀. Therefore, there are likely to be multiple effects of light on GABA_A receptors.

Additional evidence from studies involving other ion channels, strengthens our hypothesis that light and redox modulation of ion channels are structurally linked. For example, it has been shown that cyclic nucleotide gated channel currents are depressed following both light and exposure to reducing agents (Broillet & Firestein, 1996; Middendorf *et al.*, 2000). We have shown in this study that glycine receptor-mediated responses, which have been demonstrated to be somewhat depressed following exposure to a reducing agent (Pan *et al.*, 1995), may also be decreased following a brief pulse of light (Fig. 2C). Additionally, we have previously demonstrated that redox insensitive non-NMDA receptors are also insensitive to light (Leszkiewicz *et al.*, 2000). These observations indicate that an intact functional redox site is necessary for light-induced receptor alterations, and that changes in the redox state of the receptor may also affect the light sensitivity of the ion channel.

Light-mediated disruption of disulphide bonds mediated via tryptophan residues has been previously suggested (Vladimirov, 1970), and this phenomenon has been experimentally demonstrated in two proteins, cutinase and α -lactalbumin (Prompers *et al.*, 1999; Vanhooen *et al.*, 2002). The disruption of the disulphide bonds appears to be induced as the indole nucleus of the tryptophan residue absorbs the light energy and forms a thioether bond with one of the two cysteine residues. However, this process has been deemed irreversible (Vanhooen *et al.*, 2002), and therefore we do not believe it provides a mechanistic explanation for the enhancement of GABA_A receptor currents under our experimental conditions.

While we have been able to demonstrate that the light induced potentiation of GABA and NMDA currents is reversible, other studies have reported that there is an absence of reversibility of GABA_A receptors and cyclic nucleotide gated channels following light exposure. In our studies we have used intensities 2×10^9 photons/s/ μm^2 ($0.09 \mu\text{W}/\mu\text{m}^2$) and wavelengths of light greater than 280 nm (Leszkiewicz *et al.*, 2000). Middendorf *et al.* (2000) altered cyclic nucleotide gated channels with light of similar intensities 1.96×10^8 photons/s/ μm^2 that included the entire UV light spectrum. Chang *et al.* (2001), used a much higher intensity of UV light ($8 \mu\text{W}/\mu\text{m}^2$, 312 nm) and for greater exposure durations (60 s). As such, the differences in reversible vs. irreversible alterations of ion channel function by light may be entirely due to the stimulus exposure conditions.

Wade *et al.* (1988) evaluated the physiological effects of light on the central structures of the CNS by measuring the amount of light that would reach the brain in the intact skull of an adult rat. Although the fur, skin and bone can attenuate much of the light, these researchers calculated the intensity of light within the skull to be 5% of the original stimulus (Wade *et al.*, 1988). Thus it seems plausible, but somewhat unlikely, that light may exert some effects on the modulation of ion channels in the cerebral cortex or other central structures. Nonetheless, the results presented in our study demonstrate a potential physiological role for the effects of light on ion channels expressed in retina. Here, we have shown that GABA-mediated responses are potentiated in response to what may constitute 'physiological' levels of light. We have similarly reported that NMDA receptors are sensitive to this attenuated light source, which results in levels of light that can reach the retina (Leszkiewicz *et al.*, 2000). In addition, both GABA-induced and NMDA-induced currents in retinal ganglion cells are potentiated following a brief pulse of light (Fig. 2A; Leszkiewicz *et al.*, 2000). Hence, light may eventually be demonstrated to play an important role in the modulation of synaptic circuits within the retina.

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Abbreviations

CHO, Chinese hamster ovary; DTNB, 5,5'-dithio-bis-(2-nitro-benzoic acid); DTT, dithiothreitol; GABA, gamma-aminobutyric acid; NMDA, *N*-methyl-D-aspartate.

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