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Effects of Nitric Oxide on Inhibitory Receptors of Rod Bipolar Cells of Rat Retina

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The effects of nitric oxide (NO) on inhibitory neurotransmitter receptors and some types of inhibitory receptors in dissociated rod bipolar cell (RBC) were investigated. In the whole cell voltage-clamping mode, the gamma-aminobutyric acid (GABA) activated current showed both sustained and transient components. GABA activated transient current was fully blocked by bicuculine, a $GABA_A$ receptor antagonist. The *cis*-4-aminocrotonic acid (CACA), a $GABA_C$ receptor agonist, evoked the sustained current that was not blocked by bicuculline (BIC). Glycine activated the transient current. These results indicate that the RBCs possess $GABA_A$, $GABA_C$, and glycine inhibitory receptors. Sodium nitroprusside (SNP), a NO analogue, reduced the currents activated by $GABA_A$ receptor only, however, did not reduce the currents activated by either $GABA_C$ or glycine receptors. This study signifies further that only NO depresses the fast inhibitory response activated by $GABA_A$ receptor in RBC. We, therefore, postulate that NO might depress the light-on/off transient inhibitory responses in RBCs in the rat retina.

Key Words: Nitric oxide, Sodium nitroprusside, GABA receptor, Glycine receptor, Whole-cell Patch-Clamp, Rod bipolar cells, Retina

INTRODUCTION

In the rod pathway of mammalian retina, rods are connected to a single type of rod bipolar cell (RBC) whose membrane potential is depolarized by light illumination (Wässle et al, 1991). In the inner plexiform layer (IPL), the axon terminal of RBC makes a ribbon synapse with two types of amacrine cell processes. One of the postsynaptic processes is usually from glycinergic amacrine cells and the other from GABAergic reciprocal or non-reciprocal amacrine cells (Chun & Wässle, 1989; Strettoi et al, 1992; Chun et al, 1993; Wässle et al, 1995; Kim et al, 1998). Some electrophysiological evidences have shown that GABA-induced currents in RBC are mediated by $GABA_A$ or $GABA_C$ receptors in rats (Hartveit, 1996; Euler & Wässle, 1998) and mice (Vaquero & Villa, 1999). Immunocytochemical analysis revealed that $GABA_A$ and $GABA_C$ receptors are located at the axon terminal of RBCs in mammalian retina (Fletcher et al, 1998). In addition, glycine receptor was also found in the axon terminal and somatic/dendritic regions of rat retinal RBCs (Wässle et al, 1998; Cui et al, 2003). These inhibitor receptors contribute to spatial and temporal visual processing by modulating the Ca^{2+} responses and neurotransmitter release at the axon-terminal of bipolar cells (Karschin & Wässle, 1990; Gillette & Dacheux, 1995; Pan & Lipton, 1995; Cui et al, 2003).

Nitric oxide (NO), a free radical gas with a half-life of a few seconds, has various physiological roles in the central nervous system (Moncada et al, 1991; Schuman & Madison, 1994; Ohkuma & Katsura, 2001). NO is generated by oxidation of arginine through the reaction of nitric oxide synthase (Palmer et al, 1988). As a gas, NO freely crosses the cell membranes, and acts as a retrograde messenger at pre-synaptic and their neighboring cells. In the retina, NO is also shown to depress the activity of $GABA_A$ receptors in amacrine cells (Wexler et al, 1998). In this study, we investigated the effects of NO on inhibitory neurotransmitter receptors of RBCs in the rat retina, and found that NO modulates the activation of $GABA_A$ receptor, but not $GABA_C$ or glycine receptors located in RBCs.

METHODS

Preparation of bipolar cells

Bipolar cells were dissociated from Sprague-Dawley rat retina (approximately 150 g of body weight) according to the general method of dissociation, as previously described (Karschin & Wässle, 1990). In brief, rats were anesthetized with ethyl eter and decapitated. After excising the cornea, iris, and vitreous from the enucleated eye, the retina was bared and lifted from the epithelial layer. The retina was placed in an L-cysteine activated, oxygenated enzyme solution (papain 15 units/ml) in a 0.5 ml centrifuge microtube for 10 min. It was washed several times with normal rat Ringer solution containing (in mM): 135 NaCl, 5 KCl,

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ABBREVIATIONS: NO, nitric oxide; RBC, rod bipolar cell; SNP, sodium nitroprusside; I4AA, imidazole-4-acetic acid; BIC, bicuculline; PTX, picrotoxin; CACA, cis-4-aminocrotonic acid.

1 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES (pH 7.4). Subsequently, the microtube was shaken gently for mechanical dissociation of the cells. The dissociated cells were placed on a lectin-coated coverslip and stored in a storage chamber filled with Ringer solution in a refrigerator at 4°C until needed. Cells were used within 8 hours after dissociation.

Immunocytochemistry for PKC immunoreaction

The dissociated cells on the coverslip were immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 0.5 hours and was washed several times with PB. Then, the cells were incubated in 10% normal donkey serum in 0.01M phosphate buffered saline (PBS, pH 7.4) for 1 hour at room temperature in order to block endogenous peroxidase activity. Next, the cells were incubated overnight at 4°C in PBS containing mouse anti-PKC antibody (1 : 500), followed by incubation in PBS containing biotinylated goat anti-mouse IgG (Jackson Immunoresearch Lab, Inc., West Grove, PA, USA) for 2 hours. Finally, a DAB reaction was performed [0.05% 3-3'-diaminobenzidine in 0.05 M Tris-HCL buffer (pH 7.4) with 0.01% H_2O_2 for 10 min], and the cells were rinsed with PBS and coverslipped.

Electrophysiological measurements

Whole-cell patch recordings were performed with a patch-clamp amplifier (Axopatch 1D; Axon Instrument, Foster, CA) using pCLAMP acquisition software. Data were analyzed by Origin software (Microcal Software, Inc., Northampton, MA, USA). Patch electrodes were fabricated from borosilicate glass capillaries (B100-50-10; Sutter Instruments, Novato, CA, USA) by using a horizontal puller (P-87; Sutter Instruments). The electrodes were filled with a pipette solution containing (in mM): 140 CsCl, 1 $CaCl_2$, 5 EGTA and 10 HEPES (pH 7.4 adjusted by adding CsOH). Recordings were made by using patch electrodes with resistance of around 5 MΩ in the normal bath solution, and voltage clamped at −50 mV in the whole-cell configuration. During electrical recording, the dissociated cells placed inside the recording chamber were continuously superfused with an oxygenated normal Ringer solution. GABA and other substances were dissolved in normal Ringer solution and quickly applied to the cell through a drug pipette near the cells (via a gravity-fed Y-tube system). Sodium nitroprusside (SNP) was dissolved in a Ringer solution before use. We purchased all chemicals from Sigma (St. Louis, MO, USA).

RESULTS

Cell identification

The dissociated bipolar cells that show positive PKC immunoreaction could easily be identified based on their morphological characteristics (Fig. 1A; Karschin & Wässle, 1990; Yeh et al, 1990; Wässle et al, 1991).

GABA- and glycine- activated current

Whole-cell currents in the RBCs that keep only the soma and axon-terminal together were measured using voltage clamp recording. The inward whole-cell currents are elicited at the holding potential of −50 mV by adding puffs of 100 μM GABA and 1 mM glycine from a drug pipette near the RBCs. The Cl^- concentrations inside and outside the cells were symmetrical (Fig. 1B and C). While the GABA-activated responses displayed both transient and sustained components, the glycine-activated current showed mainly the transient part, suggesting that RBCs possess both GABA and glycine receptors. These data also agree with other previous electrophysiological recordings in dissociated RBCs of mammalian retinas (Karschin & Wssle, 1990; Yeh et al, 1990; Gillette & Dacheux, 1995; McGillem et al, 2000).

The GABA-activated current can be separated into a sustained component by BIC, a $GABA_A$ receptor antagonist, and a transient component by imidazole-4-acetic acid (I4AA), a $GABA_C$ receptor antagonist (Fig. 2). The transient component of the current activated by 100 μM GABA was almost blocked by 200 μM BIC. Muscimol (50 μM), a $GABA_A$ receptor agonist, evoked only the transient current which was fully blocked by 200 μM BIC (Fig. 2A). The sustained current activated by BIC resistant GABA was blocked by 200 μM I4AA (Fig. 2B). Co-application of GABA and I4AA

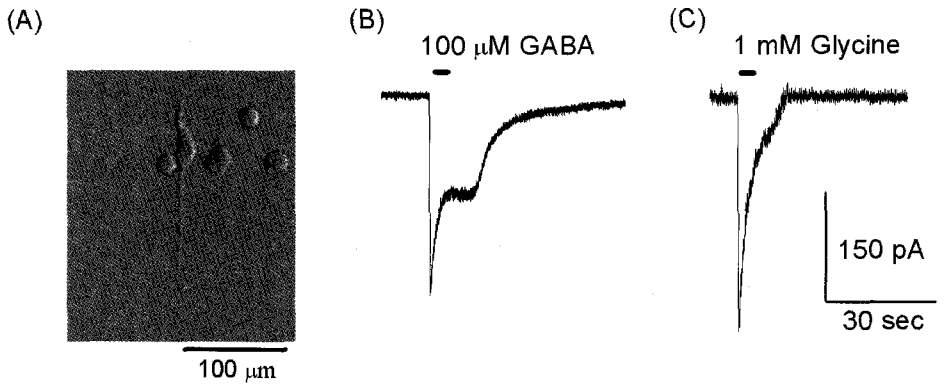

Fig. 1. RBCs dissociated from the rat retina. (A) The bipolar cells show positive PKC immunoreaction characteristic of RBC. (B) & (C) The inward whole-cell currents were elicited by 100 μM GABA and 1 mM glycine at the holding potential of −50 mV, while the Cl^- concentrations were almost symmetrical inside and outside the cell.

elicited the fast transient current. Higher concentration of I4AA was required to induce the current by itself. On the other hand, 100 μM *cis*-4-aminocrotonic acid (CACA), the $GABA_C$ receptor agonist, evoked only the sustained current that was not blocked by 200 μM BIC (Fig. 2C). This CACA activated current was almost blocked by co-application of 200 μM I4AA and 1 mM picrotoxin (PTX). When co-applied with 100 μM CACA and 1 mM PTX, the response was biphasic, which showed the use-dependent binding site PTX at the native $GABA_C$ receptor (Dong & Werblin, 1996). These results show that the GABA-activated currents in RBCs are sum of currents triggered by both $GABA_A$ and $GABA_C$ receptor activation. It is known that rat bipolar cells possess both $GABA_A$ and $GABA_C$ receptors (Feigenspan et al, 1993; Feigenspan & Borman, 1994b; Pan & Lipton, 1995; Hartveit, 1996; Euler & Wässle, 1998; Nelson et al, 1999). Approximately 70% of the GABA current in the RBCs have been found to be mediated by $GABA_C$ receptors (Euler & Wässle, 1998). The glycine responses were completely blocked in the presence of 100 μM strychnine, a glycine receptor antagonist (data not shown).

Modulation of GABA-activated currents by nitric oxide donor

We tested the effects of NO by regulating GABA- and glycine-activated currents with NO analogue SNP that activates soluble guanylate cyclase (Bohme et al, 1984; Schmidt et al, 1993). Thus, while the SNP solution was superfused into the recording chamber, puffs of drugs were applied to the cell for 2~3 sec at intervals of 1~2 minutes, depending on the types of drugs. When exposed to 1 mM SNP, only the fast component of GABA responses was reduced by 57.3±3.1% (n=4), and the responses were recovered by exposing back to normal Ringer solution (Fig. 3A). The most dramatic reduction response appeared within 2 minutes after the exposure to SNP. We selected the first GABA pulse response as the control and the smallest response as the comparison. If the concentration was higher than 1 mM SNP or application lasted for more than 2 minutes, it was difficult to observe recovery of the depressed component in GABA responses. The remaining sustained GABA response did not change with longer application (over 10 minutes). This phenomenon suggests that SNP affects only $GABA_A$ receptor activation. Co-application of GABA and I4AA accelerated the $GABA_A$ response by blocking $GABA_C$ receptors (Fig. 2B). SNP also decreased the fast inward current (evoked by co-application of 100 μM GABA and 200 μM I4AA) at every 120 seconds (Fig. 3B). SNP depressed the muscimol-induced currents by 84.5± 0.9% (n=7) from the control (Fig. 3C).

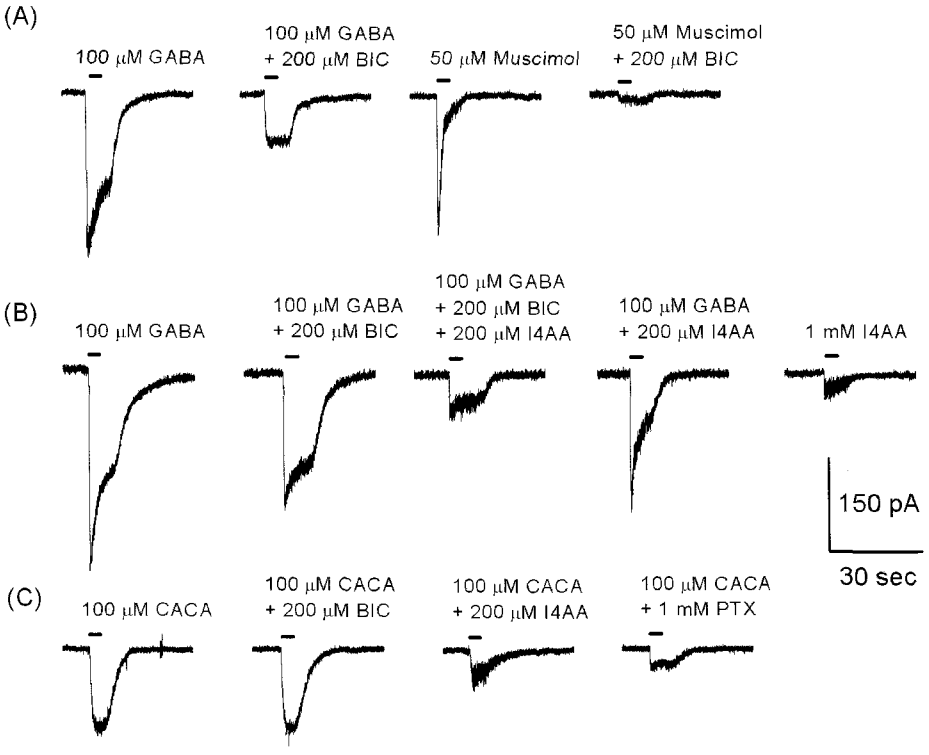

Fig. 2. RBC responses to GABA. (A) The fast component activated by 100 μM GABA was blocked by co-application of 200 μM bicuculline (BIC). 50 μM muscimol induced only the fast current blocked by 200 μM BIC. (B) BIC-resistant current was blocked by co-application of 200 μM imidazole-4-acetic acid (I4AA). Transient current was evoked by co-application of GABA and I4AA. A small current was evoked by 1 mM I4AA alone. (C) 100 μM *cis*-4-aminocrotonic acid (CACA) evoked only the sustained current that was not blocked by co-application of 200 μM BIC. 100 μM CACA activated current was almost blocked by co-application of 200 μM I4AA or 1 mM PTX.

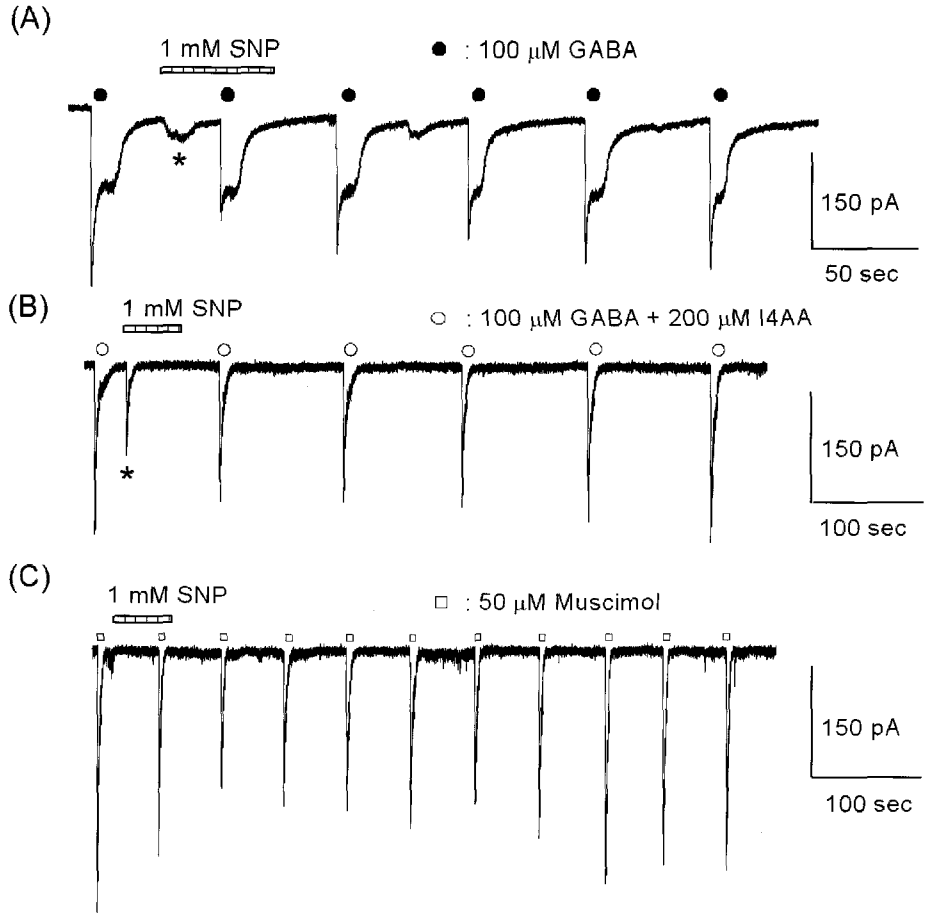

Fig. 3. Sodium nitroprusside (SNP) reduced $GABA_A$ current on RBCs. (A) SNP (1 mM) reduced only the fast component of the GABA responses. (B) SNP reduced the transient current that was evoked by co-application of 100 μM GABA and 200 μM I4AA. (C) SNP reduced muscimol (50 μM) activated current. Symbol (*) indicates an experimental artifact.

No effect of SNP on $GABA_C$ and glycine receptors

Fig. 4 shows that SNP had no effect on the $GABA_C$- and the glycine-activated currents. The sustained current induced by 100 μM CACA was not reduced in the presence of 1 mM SNP (Fig. 4A). SNP did not affect also the glycine-activated transient current (Fig. 4B).

DISCUSSION

GABA- and glycine- activated currents

BIC sensitivity has been used as a useful indicator to distinguish $GABA_A$ from $GABA_C$ receptors (Johnston, 1996; Zhang et al, 2001). In the present research, BIC was found to successfully antagonize the transient part of GABA-activated current in dissociated rat retinal RBCs. In these cells, 50 μM muscimol evoked only the transient current that was fully antagonized by 200 μM BIC. I4AA has earlier been shown to be an antagonist on the $GABA_C$ receptors (Qian & Dowling, 1994; Pan & Lipton, 1995; Picaud et al, 1998; Chang et al, 2000). In our study, I4AA sufficiently antagonized the BIC-resistant and sustained currents, and then evoked the transient current when co-applied with GABA. The glycine receptor was also activated in the RBCs. These results imply that the RBCs possess $GABA_A$, $GABA_C$ and glycine receptors. However, we did not attempt to confirm whether these receptors are localized at dendrite or axon-terminal.

Modulation of GABA-activated currents by nitric oxide donor

Our present data showed the suppression of $GABA_A$ receptors by NO donor in rat retinal RBCs. The mechanism underlying the inhibition of $GABA_A$ current by NO remains unclear: NO may inhibit $GABA_A$ currents via direct activation of $GABA_A$ receptor, whereas NO may inhibit $GABA_A$ currents also by decreasing PKA phosphorylation. Through a molecular cloning with Xenopus oocytes, Fukami et al. (1998) reported that NO acts directly at the GABA receptor, and the γ_{2s} subunit was involved in NO's action. The cGMP analogue 8-Br-cGMP failed to induce NO-like effect. In contrast, however, cGMP increased PKG-mediated phosphorylation while concurrently decreasing PKA phosphor-

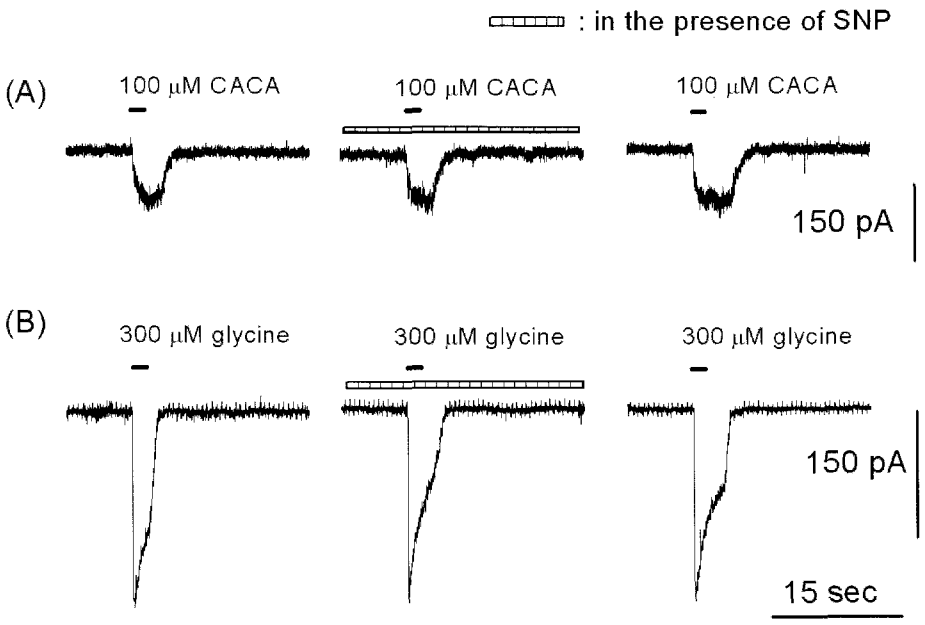

Fig. 4. Effects of SNP on responses induced by 100 μM CACA and 300 μM glycine. (A) The sustained current induced by 100 μM CACA was not reduced in the presence of 1 mM SNP. (B) SNP (1 mM) did not affect the glycine-activated fast currents.

ylation, when cGMP-dependent protein kinase was stimulated in cultured retinal amacrine cells (Wexler et al, 1998). These events lead to the state where NO depressed the $GABA_A$ receptor-gated currents. Several studies have shown that adenylate cyclase activators enhance $GABA_A$ currents in mammalian retina (Veruki & Yeh, 1992; Feigenspan & Bormann, 1994a). However, to the best of our knowledge, this is the first study to carry out the modulation of $GABA_A$ receptor-activated current by NO donor in the dissociated rat retinal RBCs, using the whole cell configuration.

RBCs have synapses in amacrine cell processes (AI and AII types) at the axon-terminal. Only AI amacrine cells provide a reciprocal synapse onto the RBCs axon-terminal (Kolb & Famiglietti, 1974). The majority of these amacrine cell processes that make conventional synapses onto RBCs in rat and cat retinas are mostly GABAergic cells with little glycinergic cells (Pourcho & Goebel, 1985; Freed et al, 1987; Kim et al, 1998). The effect of NO inhibition on $GABA_A$ receptor occurs only when the RBCs receive GABA neurotransmitter from amacrine cells. Therefore, information on the timing of GABA activation is crucial. In this study, NO was able to modulate the fast inhibition of GABA activities, and it could be one of the factors that inhibit glutamate release pathway at RBCs axon-terminal.

In mammalian retinas, it is well established that interneurons form two major pathways: the rod bipolar pathway (through an AII amacrine cell) and the cone bipolar pathway. Both pathways ultimately converge upon the same ganglion cells. Suppression of $GABA_A$ receptor activity by NO in RBC may construct more sustained light response due to the presence of less GABA-activated fast inhibitory signals. This increased sustained response offers signals to cone bipolar cell's axon terminal through AII amacrine cells, and the signals reach ganglion cells. Depending on localizations of GABAergic cell synapses and $GABA_A$ receptors in RBCs, the light-on or -off transient responses are altered. It is considered to be most likely due to the effect of $GABA_A$ receptor's desensitization. During light-adapted condition (under prolonged light exposure), HC responses become faster, and the responses to short light steps become longer (Frumkes & Wu, 1990). Consequently, the bipolar cell responses are more transient under light-adapted conditions. If the signal inputs on dendrites of GABAergic horizontal cells in RBCs are dominant, NO might depress the light-on transient inhibitory responses on RBCs. If the inputs from GABAergic amacrine cells are dominant, NO might depress the light-off transient inhibitory responses on RBCs. Additional studies of NO effects on cone bipolar cells might unravel greater alteration in ganglion cell responses because much higher $GABA_A$ receptor existence ratios have been found in several types of cone bipolar cells than in RBCs.

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