

9-1-2005

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Recommended Citation

Hoffpauir, B., & Gleason, E. (2005). Modulation of synaptic function in retinal amacrine cells. *Integrative and Comparative Biology*, 45 (4), 658-664. <https://doi.org/10.1093/icb/45.4.658>

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Modulation of Synaptic Function in Retinal Amacrine Cells¹

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SYNOPSIS. Amacrine cells are interneurons that have diverse functions in retinal signal processing. In order to study signaling and modulation in retinal amacrine cells, we employ a simplified culture system containing identifiable GABAergic amacrine cells. Immunocytochemistry experiments indicate that GABAergic amacrine cells express metabotropic glutamate receptor 5 (mGluR5), a group I mGluR usually linked to the IP3 signaling pathway. Ca²⁺ imaging experiments using an mGluR5-specific agonist indicate that these receptors are functional and when activated, can stimulate temporally diverse Ca²⁺ elevations. To begin to establish the role of these receptors in modulating amacrine cell function, we have used electrophysiological methods to ask whether ion channels are the targets of mGluR5-dependent modulation. Here we discuss our results indicating that activation of mGluR5 leads to enhancement of currents through GABA_A receptors. This enhancement is dependent upon elevations in cytosolic Ca²⁺ and activation of protein kinase C (PKC). To explore the consequences of Ca²⁺ elevations in another context, we have used nitric oxide (NO) donors to mimic the effects of activating the Ca²⁺-dependent synthetic enzyme for NO, neuronal nitric oxide synthase. We find that exposure to NO donors also enhances the amplitude of currents through GABA_A receptors. Together, these results indicate that glutamate from presynaptic bipolar cells has the potential to work through multiple mechanisms to regulate the function of amacrine-to-amacrine cell GABAergic synapses.

Information processing in the visual system begins with the arrival of photons and the initiation of a retinal signal that is ultimately exported to other visual centers in the brain. Between the input side of the retina, the photoreceptors, and the output side of the retina, the ganglion cells, there are multiple classes of interneurons. The activities of these interneurons shape the retinal signal in diverse, and in many cases, unidentified ways. Amacrine cells form a multifunctional group of retinal interneurons that make synapses in the second synaptic layer of the retina, the inner plexiform layer (IPL). In the IPL, amacrine cells receive excitatory glutamatergic input from bipolar cells and primarily inhibitory input from other amacrine cells. Amacrine cells make synapses back onto bipolar cells, onto other amacrine cells and onto ganglion cells. Although our understanding of the function of amacrine cell synaptic circuitry is incomplete, it is clear that the synaptic interactions in which these cells participate are complex (Dowling and Boycott, 1965; Dubin, 1970; Marc and Liu, 2000).

An additional layer of complexity is likely to be engendered by the wealth of modulatory substances found in the retina, especially in the inner retina. These substances include peptides (Morgan, 1983; Vaney *et al.*, 1989) and nitric oxide (Blute *et al.*, 1997; Neal *et al.*, 1998; Fischer and Stell, 1999; Ríos *et al.*, 2000; Crousillac *et al.*, 2003) as well as classical neurotransmitters (Vaney *et al.*, 1989). Although the evidence for the existence of modulatory substances is compelling,

we know very little about their function. In order to begin to understand how the signaling properties of amacrine cells are modulated, we employ a simplified system containing identified amacrine cells derived from the embryonic chicken retina (Gleason *et al.*, 1992). The amacrine cells in culture acquire the ion channels expressed by amacrine cells in the adult chicken retina (Huba and Hofmann, 1991; Huba *et al.*, 1992) and form functional GABAergic synapses with one another. Importantly, the amacrine-to-amacrine GABAergic cell synapses are very common in vertebrate retinæ and have been estimated to constitute about one-third of amacrine cell synapses in the goldfish retina (Marc and Liu, 2000). These synapses have also been demonstrated physiologically in the intact retina (Watanabe *et al.*, 2000; Frech *et al.*, 2001).

Because it is well-established that amacrine cells receive glutamatergic input from bipolar cells, we tested the hypothesis that glutamate can act as a modulator of amacrine cell function. Amacrine cells are known to express both ionotropic (Qin and Pourcho, 1999a, b, 2001; Fletcher *et al.*, 2000; Vandenbranden *et al.*, 2000; Carvalho *et al.*, 2002; Firth *et al.*, 2003; Veruki *et al.*, 2003) and metabotropic glutamate receptors (mGluRs, see below). We explored two facets of glutamate action. First we asked whether mGluRs might mediate modulatory effects of glutamate. Second we addressed the possibility that Ca²⁺ elevations engendered by activation of the full complement of expressed glutamate receptors can alter the function of GABAergic amacrine cells.

THE EXPRESSION OF mGLUR5

Metabotropic glutamate receptors are a family (mGluRs1–8) of G protein-coupled receptors. In the mammalian retina, there is immunocytochemical (Koulen *et al.*, 1997; Cai and Pourcho, 1999) and *in*

¹ From the Symposium *Recent Developments in Neurobiology* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 5–9 January 2004, at New Orleans, Louisiana.

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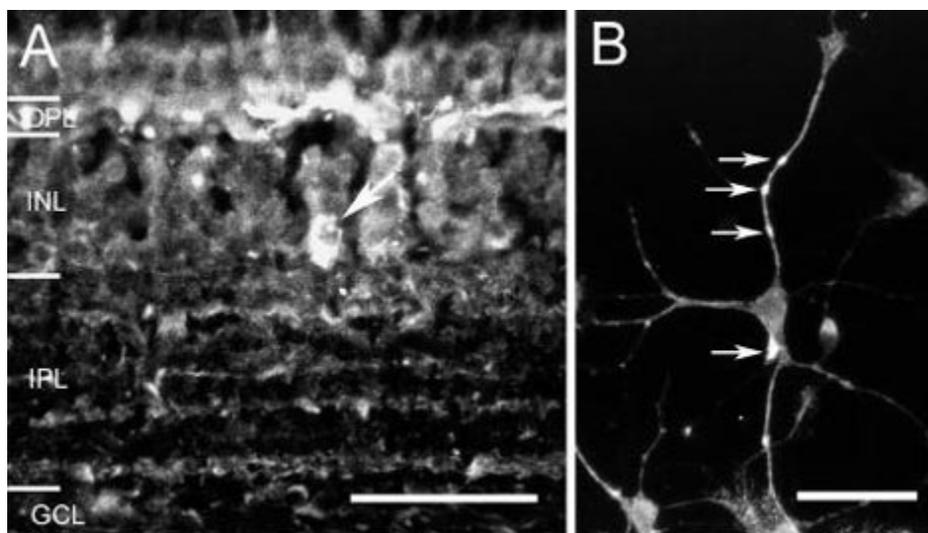


FIG. 1. Expression of mGluR5 in chicken retina and by cultured GABAergic amacrine cells. A. Intense labeling in the outer plexiform layer (OPL) indicates that photoreceptor terminals, bipolar cell processes, horizontal cell processes, or some combination of the three, express mGluR5. Labeling was seen throughout the inner nuclear layer (INL), with a few brightly labeled somata can be found at the inner border of the INL (arrow), a location suggesting that these are amacrine cells. The labeling within the inner plexiform layer (IPL) is found in four distinct strata. Labeling was also seen in cells in the ganglion cell layer (GCL) as well as in ganglion cell axons. Scale bar is 50 μm . B. An amacrine cell is shown bearing a process that contains three hotspots for mGluR5 expression. This amacrine cell is also being contacted by another amacrine cell whose cell body is out of the field of view. At the point of contact, the presynaptic process (bottom arrow) is especially heavily labeled. Scale bar is 50 μm .

situ (Hartveit *et al.*, 1995) evidence that amacrine cells can express mGluRs 1, 5, 4, and 7. We examined the expression of several of these receptors in the chicken retina, but here we will focus on our experiments regarding the expression and function of mGluR5. Polyclonal antibodies raised against rat mGluR5 were tested on frozen sections of adult chicken retina. The labeling patterns indicated that mGluR5 is expressed in all layers of the chicken retina including the inner one-third of the inner nuclear layer where amacrine cell bodies are found (Fig. 1A, arrow) and the IPL where amacrine cells extend their processes. These same antibodies were used on cultured amacrine cells, and we found that all of the amacrine cells in culture expressed mGluR5 (Fig. 1B).

MGLUR5 IS LINKED TO THE IP3 PATHWAY IN GABAERGIC AMACRINE CELLS

Metabotropic glutamate receptor 5 is usually coupled to activation of the IP3 signaling pathway and release of Ca^{2+} from internal stores. To examine the signaling properties of mGluR5 expressed in amacrine cells, we performed Ca^{2+} imaging experiments. For these experiments, cultured cells were loaded with fluo-3 and fluorescence intensity was monitored over time. Activating mGluR5 with the specific agonist, (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), produced cytosolic Ca^{2+} elevations that were temporally diverse. The temporal features of the mGluR5-dependent Ca^{2+} elevations fell into four classes. For a group of forty-two amacrine cells initially tested, 17% of amacrine cells tested produced a Ca^{2+} elevation that had

an initial transient spike, followed by a prolonged shoulder (Fig. 2A). Twenty nine percent of cells responded with a transient spike only (Fig. 2B). A smaller group of amacrine cells had an initial transient spike followed by Ca^{2+} oscillations (9%, Fig. 2C) and 45% of the cells gave prolonged responses that declined slowly and could outlast the 30 minute recording period (Fig. 2D). Some insight into the source of this variability was revealed by experiments performed in nominally Ca^{2+} -free external solution. Under these conditions, responses from all of the cells tested ($n = 59$) fell into the second group in that they exhibited transient Ca^{2+} elevations with no subsequent shoulder or prolonged phase (Fig. 2E). The initial transient Ca^{2+} elevations could be inhibited when cells in Ca^{2+} free solutions were exposed to a phospholipase C inhibitor, U73122 (Fig. 2F). These observations led us to conclude that the initial phase of the mGluR5-dependent Ca^{2+} elevation is due to IP3-mediated Ca^{2+} release from intracellular stores, while the second and most temporally variable phase can be attributed to entry of Ca^{2+} across the plasma membrane. Results from both physiological and immunocytochemical experiments indicate that a TRPC channel is a good candidate for this influx pathway (Sosa *et al.*, 2002; Crousillac *et al.*, 2003).

GABA_A RECEPTORS ARE TARGETS OF MGLUR5-DEPENDENT MODULATION

Once we characterized the expression of mGluR5 and established its linkage to a signaling pathway, our focus turned to identifying targets of modulation.

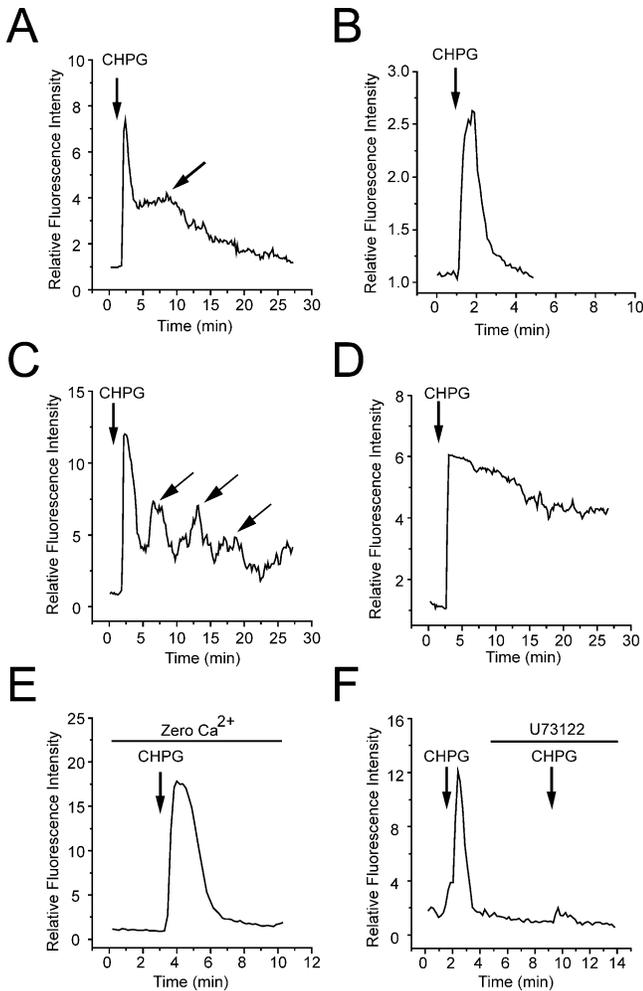


FIG. 2. Activation of mGluR5 stimulates temporally diverse PLC-dependent calcium elevations. CHPG (50 μ M) was added to the bath for 20 sec at the time indicated by the vertical arrow. A. A representative amacrine cell that showed an initial spike in cytosolic Ca^{2+} followed by a prolonged shoulder (arrow). B. The response of a different amacrine cells to the same stimulation shows the spike but no shoulder (note different time scale for this panel). C. These data are representative of the amacrine cells that responded with a spike of Ca^{2+} elevation followed by a period of Ca^{2+} oscillations. D. Data are shown for an amacrine cell where a prolonged Ca^{2+} elevation is elicited. E. Nominally Ca^{2+} -free external solution replaced normal external about 1 min prior to stimulation. In the absence of external Ca^{2+} , 100% of amacrine cells tested ($n = 59$) produced transient Ca^{2+} elevation when exposed to 50 μ M CHPG (20 sec). Data from a representative cell shows this typically transient response. F. Data from a representative cell showing that treatment with U73122 (10 μ M) almost completely abolishes the second response to CHPG. Ca^{2+} -free external was applied approximately one min prior to the first CHPG application.

Analysis of the synapses formed between these GABAergic amacrine cells in culture has established that postsynaptic receptors are GABA_A receptors (Gleason *et al.*, 1993). Given the importance of GABA_A receptors to the properties of amacrine cell synaptic signaling, we asked whether activation of mGluR5 had any modulatory effect on the function of these receptors. Whole-cell voltage clamp recordings were made from

individual amacrine cells. GABA-gated currents were elicited before, during and after activation of mGluR5 with CHPG (Fig. 3A and B). About 66% of the cells responded to CHPG with an enhancement of the GABA-gated current. For these cells, activation of mGluR5 led to a $\sim 10\%$ enhancement of the GABA-gated current amplitude. No changes occurred in the Cl^- equilibrium potential indicating that the enhancement was not due to a change in the Cl^- distribution. Buffering cytosolic Ca^{2+} with BAPTA blocked the current enhancement indicating that the modulation was Ca^{2+} -dependent (Hoffpaur and Gleason, 2002a).

Because signaling via the IP₃ pathway usually leads to activation of PKC, we tested the hypothesis that the mGluR5-dependent modulation of GABA_A was also PKC-dependent. We found that direct activation of PKC also produced enhancement of the GABA-gated current making this enzyme a good candidate for participation in the pathway that modulates the GABA_A receptor (Hoffpaur and Gleason, 2002a). To confirm that the mGluR5-dependent enhancement required the activity of PKC, we asked whether pre-activation of PKC would occlude the effects of activation of mGluR5. In cells that were responsive to CHPG, enhancement of the current with the PKC activator (-)-7-octylindolactam prevented subsequent enhancement by CHPG (Fig. 3, C and D). Additional evidence for this link came from experiments with PKC inhibitors (Hoffpaur and Gleason, 2002a). Together these results indicated that the mGluR5-dependent enhancement of GABA-gated currents occurred through a PKC-dependent pathway.

One of the major objectives of our work is to understand how the function of amacrine cell synapses is modulated. As such, we wanted to determine whether synaptic GABA_A receptors, specifically, were targets of mGluR5-dependent signaling. To address this, we recorded synaptic currents from individual amacrine cells receiving autaptic inputs. To activate synaptic transmission, individual amacrine cells were voltage clamped in the whole cell configuration and held just inside the activation range of the voltage-gated Ca^{2+} currents expressed by these cells (Gleason *et al.*, 1994). With this low level of presynaptic stimulation, quantal currents could be resolved (Fig. 4A). A comparison of the amplitude distributions indicated that the mean peak amplitude of the GABA induced quantal currents was enhanced by about 18% (Fig. 4 B and C). This result indicates that GABA_A receptors at synapses are modulated by activation of mGluR5. Furthermore, it is intriguing that the level of enhancement observed at synapses is nearly twice that found for whole cell currents. This observation raises the possibility that the signaling machinery is somehow targeted to synaptic GABA_A receptors.

THE ROLE OF NITRIC OXIDE AS A MODULATOR IN THE INNER RETINA

Glutamate has the broad potential to regulate amacrine cell function by virtue of its ability to engender

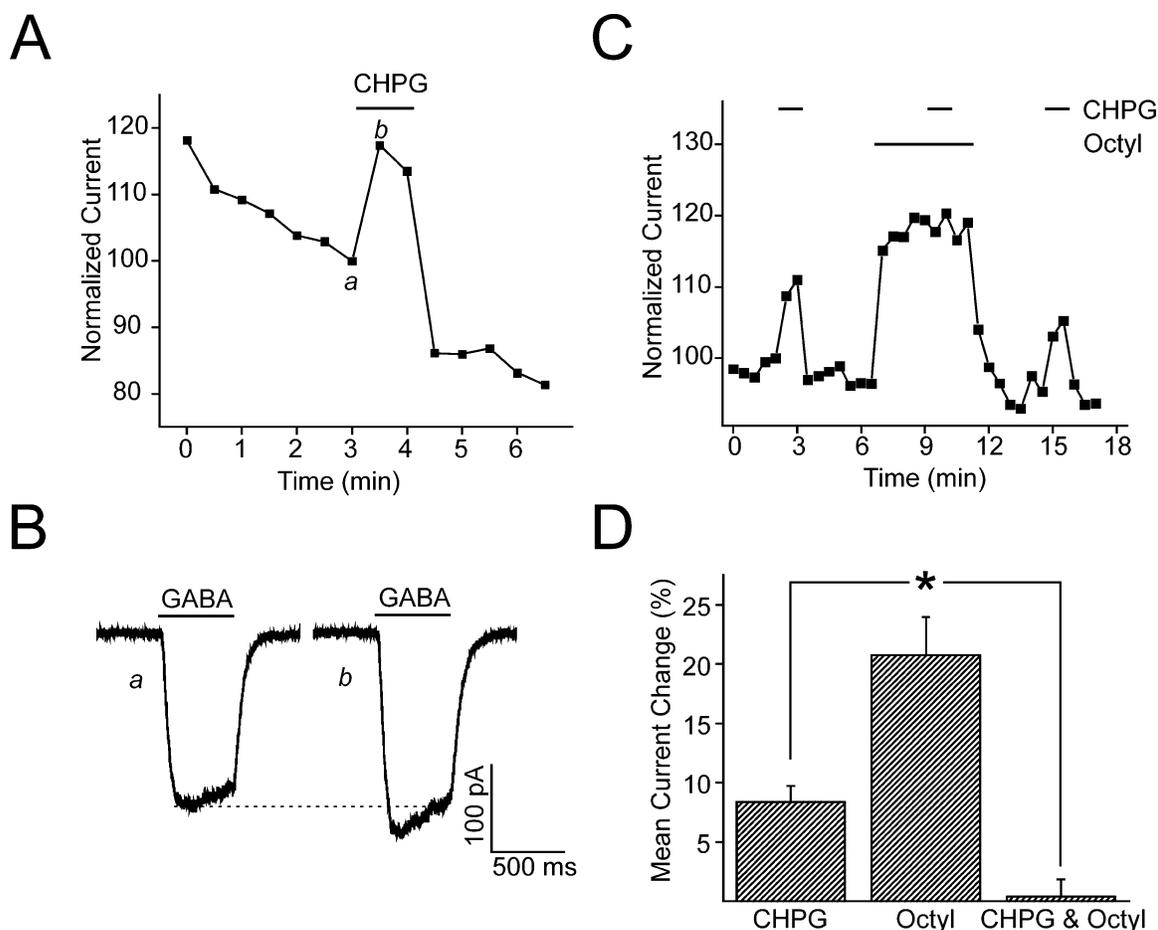


FIG. 3. Activated mGluR5 enhances GABA-gated currents through a PKC-dependent pathway. A. Normalized peak amplitudes of GABA-gated currents plotted over time. GABA-gated currents are enhanced in the presence of the mGluR5-specific agonist, CHPG (300 μ M). B. Whole cell voltage clamp recordings of GABA-gated currents denoted in A, before (a) and during (b) application of CHPG. C. Application of either CHPG (300 μ M) or the PKC activator, octylindolactam (250 nM) enhances GABA-gated currents. The mGluR5-dependent enhancement is occluded when CHPG is applied in the presence of octylindolactam. D. The mGluR5-dependent enhancement is significantly reduced when CHPG is applied after pre-activation of PKC with octylindolactam ($n = 7$; $*P = 0.001$).

Ca²⁺ elevations. These elevations can stem from activation of mGluR5 and the IP3 pathway as well as by activation of ionotropic glutamate receptors. One Ca²⁺-sensitive enzyme that could be activated in this way is neuronal nitric oxide synthase (nNOS). Neuronal NOS is a Ca²⁺/calmodulin-sensitive enzyme that, when activated, converts arginine to citrulline and nitric oxide (NO). NO can then diffuse out of the source cell but its spread is limited because NO has a very brief 1/2 life (3–6 sec). The canonical signaling pathway involves NO-dependent activation of soluble guanylate cyclase, generation of cGMP and stimulation of protein kinase activity.

Expression of nNOS by amacrine cells in the chicken retina was demonstrated by Fisher and Stell (1999) and we have established that cultured GABAergic amacrine cells express this enzyme (Hoffpauir and Gleason, 2002b). The generation of NO and of NO-dependent signaling has been examined in the intact turtle retina (Blute *et al.*, 1998, 1999, 2000; Cao and Eldred,

2001). DAF-2 imaging experiments revealed that activation of N-methyl-D-aspartate (NMDA) receptors generates NO signals (Blute *et al.*, 2000) and stimulates the production of cGMP in amacrine cells. Given these interesting findings, we asked whether the GABA_A receptor might be a target of NO-dependent signaling in GABAergic amacrine cells.

In these experiments, we bypassed activation of nNOS by exposing the cells to nitric oxide donors. We found that 1-Hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC 12) enhanced the GABA-gated current in all cells examined (Fig. 5, $n = 8$). This same effect was achieved with other NO donors (2'-[Hydroxynitrosohydrazino]bisethanamine [NOC-18, 300 μ M], S-Nitroso-N-acetyl-D,L-penicillamine [SNAP, 250 μ M]). In these experiments, we observed no shift in the chloride distribution indicating that the effect was on the GABA_A receptors themselves. Preliminary experiments using an inhibitor of soluble guanylate cyclases indicate that NO is not

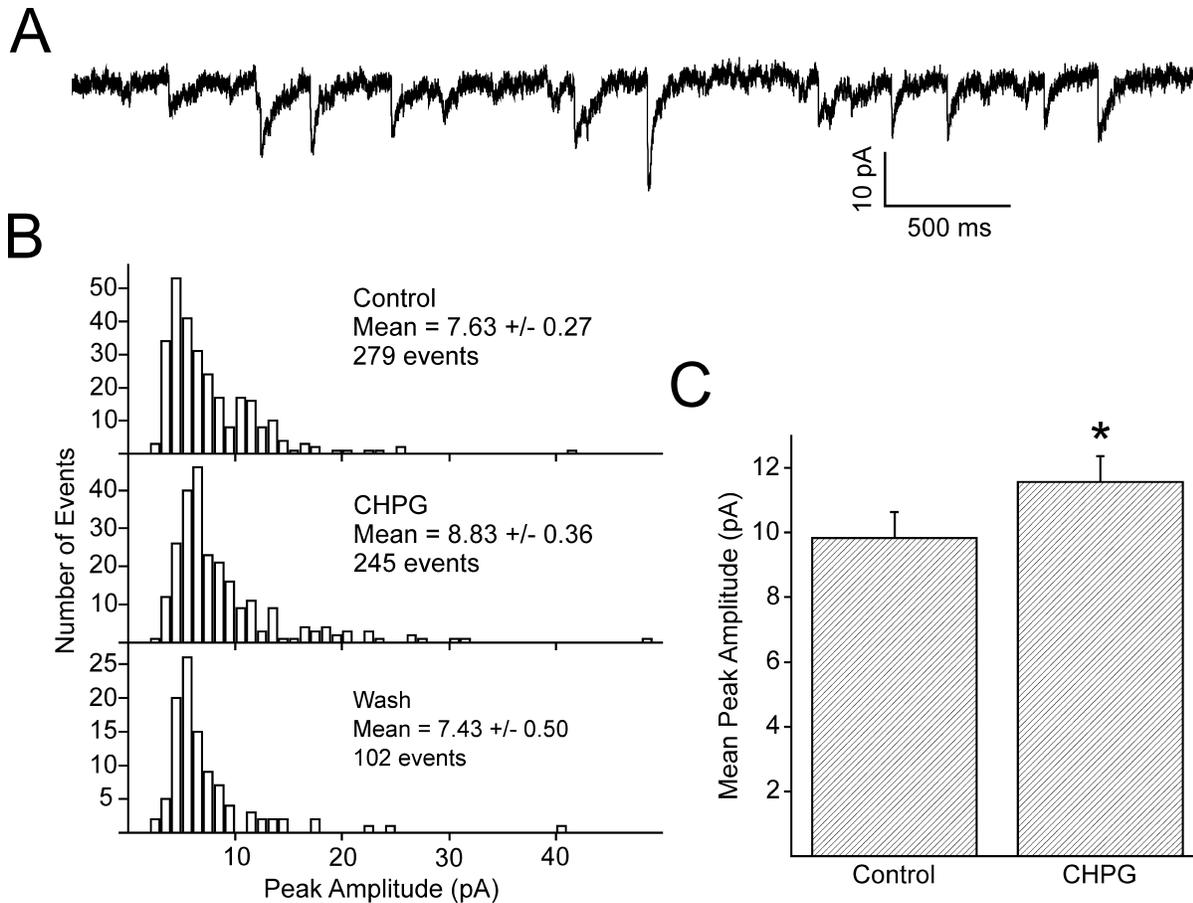


FIG. 4. Activation of mGluR5 modulates synaptic GABA_A receptors. A. GABAergic quantal events recorded from a representative amacrine cell. B. Amplitude distributions of quantal events recorded over 90 seconds before, during, and after CHPG application show that activated mGluR5 reversibly enhances the quantal event peak amplitude. C. On average, CHPG enhances the mean peak amplitude of quantal events by 18% ($n = 6$; $*P = 0.01$).

working via the classical pathway (B.K.H., unpublished data). Interestingly, it has been demonstrated in cultured amacrine cells from the rat that NO inhibits GABA-gated currents in a partially guanylate cyclase-dependent manner (Wexler *et al.*, 1998). Thus, the effects of NO may be species specific and may be influenced by the subunit expression pattern of the GABA_A receptors.

THE EXPANDED ROLE OF GLUTAMATE IN THE INNER RETINA

Our findings indicate that activation of glutamate receptors can lead to enhancement of currents mediated by GABA_A receptors. Activation of mGluR5 leads to a Ca²⁺- and PKC-dependent enhancement of the current that appears to be amplified for GABA_A receptors localized to the synapse. We also demonstrate that nitric oxide can act on GABA_A receptors to enhance their function. Together, the results of these studies suggest that in the retina, glutamate released from bipolar cells not only depolarizes postsynaptic amacrine cells, but activates signaling pathways that can regulate their function (Fig. 6).

Interestingly, our results indicate that signaling through different pathways can have convergent effects on amacrine cell function. It is not surprising that GABA_A receptors are subject to multiple modes of regulation because GABAergic synapses are so prevalent in the IPL. In an immunocytochemical study of the goldfish, Marc and Liu (2000) concluded that at least 92% of the conventional (amacrine cell) synapses in the IPL are GABAergic. We can hypothesize that increased levels of bipolar cell activity and thus increased amounts of glutamate release will lead to an enhancement in inhibitory interactions between amacrine cells (Fig. 6).

An important perspective involves the growing evidence that much of amacrine cell signaling occurs locally. Although amacrine cells can make action potentials (Murakami and Shimoda, 1977; Barnes and Werblin, 1986; Eliasof *et al.*, 1987), action potentials are not required for synaptic transmission. Instead, graded depolarizations are sufficient to stimulate neurotransmitter release (Gleason *et al.*, 1993; Bieda and Copenhagen, 1999). Furthermore, spatially discrete Ca²⁺ signals have been observed in amacrine cell processes

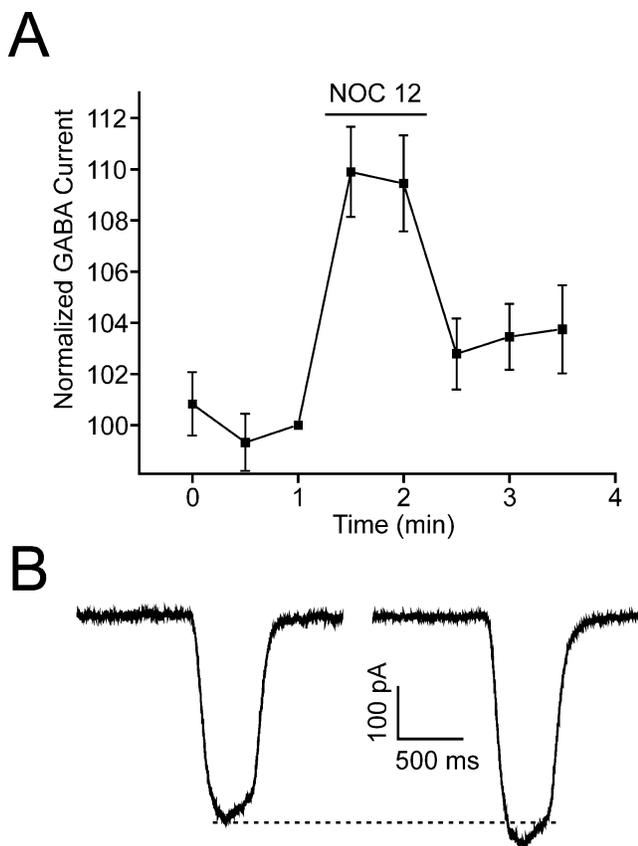


FIG. 5. Nitric oxide enhances GABA-gated currents. A. The mean peak amplitudes of GABA-gated currents are plotted over time (n = 8). Application of the nitric oxide donor, NOC 12 (250 μ M) reversibly enhances the GABA-gated currents. B. Whole cell voltage clamp recordings of GABA-gated currents recorded before (left) and during (right) application of NOC 12.

both in culture (Hurtado *et al.*, 2002) and in the intact retina (Denk and Detwiler, 1999) suggesting that pre-synaptic activity can be highly localized. Thus, the effects of glutamate released from a bipolar cell terminal are probably restricted to nearby amacrine cell processes.

To fully appreciate the consequences of enhancing amacrine-to-amacrine GABAergic synapses, we have to consider the function of these synapses in retinal signal processing. Unfortunately, even though these synapses are very common, we know little about their function. The best-studied amacrine cell synapses are those made onto ganglion cells and the feedback synapses made onto bipolar cells (Masland, 1996). Thus, a divide exists between what we are learning about the modulation of GABAergic amacrine cell synapses and the function of these synapses in the retina. What we learn in culture, however, will help us to direct questions that can be addressed in the near future with studies of amacrine cell signaling in the intact retina.

ACKNOWLEDGMENTS

This work was supported by a National Institutes of Health grant to E. Gleason (EY12204) and a Sigma Xi Grant in Aid of Research to B. K. Hoffpauir.

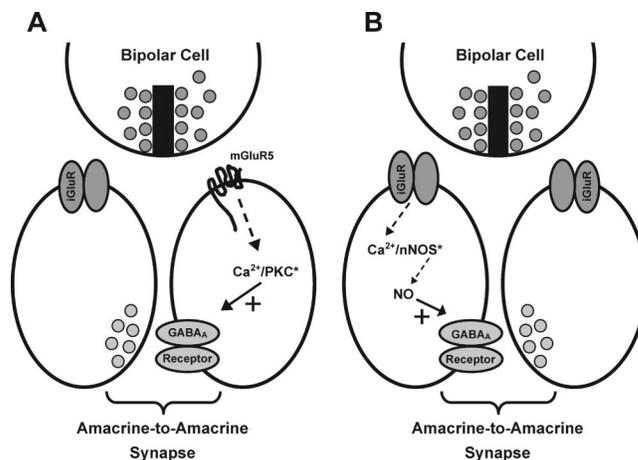


FIG. 6. Role of glutamate-dependent signaling in the inner retina. This diagram represents a subset of the potential interactions between bipolar cells and amacrine cells and between amacrine cells. The bipolar-to-amacrine cell synapse is shown with the presynaptic bipolar cell terminal containing a ribbon synapse. Multiple amacrine cell processes can be found in the region, some of which are post-synaptic to the bipolar cells. A. Glutamate released from the bipolar cell can activate mGluRs expressed on amacrine cell processes. Activation of this signaling pathway leads to a Ca^{2+} - and PKC-dependent enhancement of the currents through GABA_A receptors. B. Glutamate released from the bipolar cell can also activate ionotropic glutamate receptors. Influx of Ca^{2+} can activate nNOS and stimulate the production of NO. NO also has the effect of enhancing the currents through GABA_A receptors. The effect of both of these pathways is to strengthen the inhibitory synapses between amacrine cells. Ionotropic and metabotropic receptors are on separate amacrine cell processes for illustrative purposes only. The precise distribution of glutamate receptor subtypes on amacrine cell processes is not known.

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