A novel modulatory role for nitric oxide in retinal amacrine cells

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A NOVEL MODULATORY ROLE FOR NITRIC OXIDE IN RETINAL AMACRINE CELLS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by

Emily Ann McMains
A.B., Brown University, 2000
December, 2008
Dedication

For my mother and my mentor,

Thyre Yocum McMains

1951-2008
Acknowledgements

I will always be deeply indebted to my graduate advisor, Dr. Evanna Gleason, for her support, encouragement, and enthusiasm throughout my time at LSU. Dr. Gleason was the one who convinced me to pursue my doctorate in biology in the first place, and whatever success I have had in my research is, in large part, due to her calm and consistent guidance. She has taught me how to be a better writer, a clearer thinker, and a more organized scientist. And I look forward to many more years of collaboration and conversation with her.

I also wish to thank the members of my graduate committee for their insights and their assistance during the past five years. I sincerely thank Dr. Kurt Svoboda for his dedication to all of the graduate students that he advises and for his interest in and enthusiasm for my work. I thank Dr. John Lynn for his constant support and his guidance on certain aspects of my imaging projects. And I also wish to thank Dr. John Caprio for thought-provoking discussions and particularly for stepping in as a member of my committee in the last year of my graduate work. Finally, I would like to acknowledge Dr. Jim Belanger for his role on my graduate committee. Though Dr. Belanger is no longer at LSU and was not a member of my final graduate committee, his participation in my studies has been invaluable. I thank him for his insightful questions and his always useful advice.

My time in the Gleason Lab would have been very lonely without the presence of my wonderful labmates: Brian Hoffpauir, Madhumita Sen, Scott Crousillac, and Merve Tekmen. My graduate student colleagues provided stimulating conversations, encouragement during occasional discouraging times, and, most importantly, a sense of
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with them. In particular, I dedicate this dissertation to my mother, Thyre McMains, who was not able to see me graduate, but who never doubted that I would. Her intellect, humour, grace, and strength will guide me and my work for the rest of my life.
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<td>2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DAF-2</td>
<td>4,5-diaminofluorescein</td>
</tr>
<tr>
<td>DAF-FM</td>
<td>4-amino-5-methylamino-2',7'-difluorofluorescein</td>
</tr>
<tr>
<td>DIDS</td>
<td>4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EE</td>
<td>Embryonic equivalent</td>
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<tr>
<td>FCCP</td>
<td>p-trifluoromethoxy carbonyl cyanide phenyl hydrazone</td>
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<td>GABA</td>
<td>Gamma amino butyric acid</td>
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<tr>
<td>GCL</td>
<td>ganglion cell layer</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>INL</td>
<td>Inner nuclear layer</td>
</tr>
<tr>
<td>IPL</td>
<td>Inner plexiform layer</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro-RNA</td>
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<td>NMDA</td>
<td>N-methyl D aspartate</td>
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<td>1-Hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-1-triazene</td>
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<td>NOC-18</td>
<td>2,2'-(Hydroxynitrosohydrazino)bisethanamine</td>
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<td>NOS</td>
<td>Nitric oxide synthase</td>
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<td>ODQ</td>
<td>1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one</td>
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<td>ONL</td>
<td>Outer nuclear layer</td>
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<td>OPL</td>
<td>Outer plexiform layer</td>
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<td>Abbreviation</td>
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<td>PCD</td>
<td>Programmed cell death</td>
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<td>Primary micro-RNA</td>
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<td>RNA-induced silencing complex</td>
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<td>sGC</td>
<td>Soluble guanylate cyclase</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SNAP</td>
<td>S-nitroso-N-acetylpenicillamine</td>
</tr>
<tr>
<td>SNARF1-AM</td>
<td>Carboxy-semaphorhodfluor acetoxymethyl ester</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetra-ethyl ammonium</td>
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Abstract

Nitric oxide is a gaseous signaling molecule that is produced by subsets of each cell type in the vertebrate retina. Though there is evidence that nitric oxide (NO) can affect multiple cellular processes in the retina, much remains unknown, especially with respect to its function in the inner retina. We have used a simplified system of cultured amacrine cells (interneurons that signal in the inner retina) to explore the role of nitric oxide in amacrine cell signaling. We find that physiological concentrations (100’s of nM – low µM) of nitric oxide (NO) transiently invert the sign of voltage responses mediated by GABA or glycine receptors by shifting the equilibrium potential for chloride ($E_{Cl^-}$) to more positive values. The direction of the shift in $E_{Cl^-}$ is consistent with a transient elevation of intracellular chloride. The physiological consequence of this shift is that NO can switch inhibitory synapses into excitatory synapses. Manipulations of extracellular chloride demonstrate that the shift in $E_{Cl^-}$ is not caused by the transport of chloride across the plasma membrane into the cytosol. Instead, NO mediates a release of chloride from an internal compartment. Analysis of cellular pH using the pH indicator dye, SNARF-1AM, reveals that NO also induces a transient acidification of the cytosol that displays a similar time course to the cytosolic chloride elevation. Using measurements of chloride reversal potential ($E_{Cl^-}$) to monitor changes in intracellular chloride levels, we found that alkalinization of the cytosol with NH$_4$Cl resulted in a negative shift in $E_{Cl^-}$, consistent with a decrease in internal chloride. Acidification of the cytosol with amiloride induced a positive shift in $E_{Cl^-}$, consistent with a low cytosolic pH-driven increase in internal chloride. Furthermore, NO-induced positive shifts in the $E_{Cl^-}$ were reduced in a basic cellular environment. Finally, when we strongly buffered
cytosolic pH with 125 mM HEPES in the recording pipet, we found that the ability of NO to alter cytosolic chloride levels was reduced. These results indicate that NO-induced changes in cellular pH are both sufficient and necessary to alter chloride distribution across internal membranes in neurons. The discovery that this redistribution can change the sign of central synapses has potentially broad implications for our understanding of the role of this signaling molecule in the CNS.
Chapter 1

Introduction
The vertebrate retina is unique in that it is both a sensory organ and a part of the central nervous system (CNS). It therefore contains both sensory receptive elements as well as elements that integrate sensory signals and transmit them to other areas of the brain for further processing. Because of this dual sensory and integrative role, the retina is a more accessible model for many aspects of brain function. My dissertation will focus on a particular retinal interneuron. Like other CNS interneurons, amacrine cells are numerous and diverse, but all of them serve in some capacity to integrate incoming information. Much progress has been made in understanding primary synaptic pathways in the retina, but much less is understood about how these pathways are modulated by other synaptic inputs and signaling molecules. Synaptic modulation can be accomplished via alterations of the strength, frequency, and/or quality of presynaptic output or postsynaptic responses. Modulators include, but are not limited to, presynaptic ion channels, postsynaptic second messengers, and even paracrine and endocrine factors. Nitric oxide (NO) is a physiological, gas phase signaling molecule that has been demonstrated to modulate many different CNS processes. My work not only provides evidence for a new form of NO-dependent modulation in the retina, but also raises further questions about the role of NO in CNS signaling.

**Retinal Amacrine Cells**

In vertebrates, light travels through all layers of the retina before reaching the photoreceptors where it is converted into a neural signal that is communicated to bipolar cells, and then from bipolar cells to ganglion cells. From here, the retinal signal is sent to visual centers in other parts of the brain. This is the primary vertical signaling
pathway in the retina. The visual signal, however, is also shaped by lateral interactions mediated by horizontal cells (in the outer retina) and amacrine cells (in the more complex inner retina), (Cook and McReynolds, 1998). Amacrine cells form synaptic connections with bipolar cells, ganglion cells, and other amacrine cells. In the highly laminar structure of the vertebrate retina, amacrine cell bodies are found in the inner nuclear layer (INL) and the ganglion cell layer (GCL) and their synaptic connections are located in the inner plexiform layer (IPL), (Dowling and Boycott, 1965; Dubin, 1970).

Two known functions of amacrine cells are to mediate directional selectivity and changes in sensitivity to light intensity (Euler et al 2002, Roska et al 1998). Directional selectivity is the ability of retinal ganglion cells to be preferentially stimulated by light moving in a particular direction (Barlow et al 1964). Directional information is encoded as calcium transients in the dendrites of starburst amacrine cells and directly transmitted to ganglion cells (Euler et al 2002). Two different types of amacrine cells mediate changes in the sensitivity of ganglion cells to light intensity (Roska et al 1998). Narrow-field GABAergic amacrine cells inhibit transient ON and ON/OFF ganglion cells. This inhibition occurs after a delay of ~150ms after a light stimulus, which is brought about by glycinergic inhibition of narrow dendritic-field amacrine cells by wide dendritic-field amacrine cells (Roska et al 1998). These two very different functions depend on the morphological and biochemical diversity of retinal amacrine cells and highlight the potential functional diversity of these interneurons.

In the mammalian retina, amacrine cells constitute over a third of the cells in the inner nuclear layer (Strettoi and Masland, 1995) and participate in most synapses in the inner plexiform layer (Dubin, 1970). In nonmammalian retinas, amacrine cells are
present in even greater numbers, (Zhang et al 2004). In addition to their numerical richness, they display diverse patterns of synaptic connections (Marc and Liu, 2000). In our lab, we focus on GABAergic amacrine cells. Even though there are subsets of amacrine cells with other neurotransmitter phenotypes, more than 90% of amacrine cells in a cyprinid fish retina are GABAergic (Marc and Liu, 2000). Furthermore, a majority of CNS interneurons primarily release GABA. Therefore, understanding GABAergic amacrine cell signaling is not only important for understanding visual signaling in the retina, but also useful more generally for shedding light on interneuronal signaling in the CNS.

**The Cell Culture System**

The number and complexity of synapses in the inner retina makes physiological analyses at the level of a single cell or single synapse extremely difficult. Hence, we use a simplified system consisting of dispersed chick retinal neurons. This culture system allows us to observe signaling events in isolated cells or in pairs of amacrine cells, using both electrophysiological and digital imaging recording methods. Previous work has shown that after about 6 days in culture, amacrine cells express the same voltage-gated and ligand-gated ion channels expressed in the intact, adult chicken retina (Huba and Hofmann, 1990). Furthermore, in this same time frame, cultured amacrine cells form functional GABAergic synapses with each other (Gleason et al., 1989; 1993).

**Nitric Oxide in the Retina**

NO is produced when nitric oxide synthase (NOS) catalyzes the oxidation of arginine to citrulline and NO. The activation of the neuronal form of nitric oxide
Nitric oxide (NO) has emerged as an important signaling molecule in the vertebrate retina. Subsets of all retinal cell types, including amacrine cells, express nNOS (Shin et al., 1999; Blute and Eldred, 1997; Fischer and Stell, 1999) and produce nitric oxide in response to calcium-calmodulin stimuli (Neal et al., 1998; Blute et al., 2003). We have observed high levels of nNOS expression in both cultured amacrine cells (see Appendix 1) and subsets of cell bodies and processes in the INL and IPL (where amacrine cell processes are found) of the chicken retina (Crousillac et al., 2003). Direct imaging of NO in the turtle retina (Blute et al 2000, Eldred and Blute 2005) demonstrates that NO itself is made by subsets of amacrine cells, where it often is restricted to not only the cell that produced it, but also the region of the cell in which it was produced. NO production was monitored with the NO-sensitive dye, DAF-2. In contrast to diffuse labeling in ganglion cells, DAF-2 labeling in retinal tissue was sharply defined in amacrine cell bodies and processes indicating that NO produced in an...
amacrine cell soma may not diffuse out of the soma. NO production restricted to individual varicosities, which are often indicative of synaptic sites, in the IPL was also observed (Blute et al 2000, Eldred and Blute 2005). This restricted diffusion of NO means that NO is acting as a local modulator rather than a diffuse one.

Though a characterization of the role of NO in the retina is far from complete, NO has been implicated in a number of signaling pathways in this tissue (for reviews see Goldstein et al, 1996; Cudeiro and Rivadulla, 1999). For example, in isolated frog rods, NO attenuates light responses by cGMP-dependent mechanisms. (Schmidt et al, 1992). Nitric oxide reduces electrical coupling between horizontal cells (Xin and Bloomfield, 2000). NO also reduces gap-junction coupling between A2 amacrine cells and cone bipolar cells, suggesting a role for nitric oxide in switching from rod to cone vision (Mills and Massey, 1995). Especially relevant to my project, in the ferret retina, light responses of ganglion cells were greatly dampened in response to application of high (1mM) concentrations of a spontaneous NO donor (SNAP). This effect was demonstrated to be due to an increase in presynaptic (probably amacrine cell) inhibition (Wang et al, 2003). Because we have found that NO increases excitation of inhibitory amacrine cells, (see below), one can envision a scenario in which the NO-dependent reduction in ganglion cell light responses is mediated by an increase in the inhibitory output from NO-producing amacrine cells onto ganglion cells (See model: Chapter 2, Figure 10).

How much NO do retinal neurons produce? Measurements with an NO electrode at the surface of whole-mounted rat retinas revealed an NO concentration of 15uM (Groppe et al, 2003). Other measurements in mammalian retina agree with
retinal NO concentrations in the low micromolar range (Donati et al, 1995; Heiduschka and Thanos, 1998). In the bulk of the experiments reported here, we have exposed isolated amacrine cells to NO concentrations of at most 2µM. Though the amount of nitric oxide produced and “felt” by individual amacrine cells is not known, these concentrations indicate that we are working within the physiological range of the retina.

In Chapter 2, I explore the effects of NO on cultured retinal amacrine cells. Brian Hoffpauir, a former graduate student in our lab, conducted a number of the experiments reported in this chapter. His work on this project, along with my own, was first published as an article in the Journal of Neurophysiology in 2006 of which we were co-first authors. In this chapter, I was directly responsible for the work presented in Figs: 2F-G, 3C-D, 4, and 7-9. In Chapter 3, I investigate the mechanisms by which NO modulates amacrine cell signaling, specifically the role of cellular pH on NO-mediated processes.

References


Heiduschka, P. and Thanos, S. NO production during neuronal cell death can be directly assessed by a chemical reaction in vivo. 1998. Neuroreport 9: 4051-4057.


Chapter 2

Nitric Oxide Transiently Converts Synaptic Inhibition to Excitation in Retinal Amacrine Cells*

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Introduction

Amacrine cells are interneurons in the inner retina that extend laterally and form synaptic connections with bipolar, ganglion, and other amacrine cells. These lateral interactions play a key role in determining the nature of retinal output (for reviews, see Lagnado 1998; Taylor and Vaney 2003), so understanding the function and regulation of amacrine cells is critical to understanding retinal signal processing.

Nitric oxide (NO) has been implicated as a signaling molecule at all levels of the visual system (for review, see Cudeiro and Rivadulla 1999). Nitric oxide synthase (NOS) catalyzes the production of NO and citrulline from arginine. The neuronal form of this enzyme (nNOS) is constitutively expressed and stimulated by elevations of cytosolic Ca\(^{2+}\). In the canonical pathway, NO stimulates soluble guanylate cyclase (sGC) activity. NO is also known to affect target protein function through S-nitrosylation (Davis et al. 2001; Stamler et al. 1997) and by other direct interactions including formation of dinitrosyl iron complexes (Rogers et al. 2003).

Neuronal NOS has been found in subsets of each class of neuron in the vertebrate retina (Blute et al. 1997; Chun et al. 1999; Fischer and Stell 1999; Kim et al. 1999; Rios et al. 2000; Shin et al. 1999), and NO itself has been detected in a similar array of retinal cells (Blute et al. 2000, 2003; Neal et al. 1998). In the chicken retina, a subset of amacrine cells expresses high levels of nNOS, and distinct sublaminae of nNOS labeling occur throughout the inner plexiform layer of the retina (Crousillac et al. 2003; Fischer and Stell 1999; Rios et al. 2000). Furthermore, measurements at the inner surface of the retina indicate that this tissue can generate high (micromolar) levels of NO (Donati et al. 1995; Groppe et al. 2003; Heiduschka and Thanos 1998).
The physiological effects of NO production have been examined for some cell types that signal in the inner retina. In the rabbit retina, NO reduces gap-junction coupling between A2 amacrine cells and cone bipolar cells (Mills and Massey 1995). Wexler and colleagues (1998) have shown that NO depresses GABA$_A$ receptor function in cultured rat amacrine cells. In the ferret retina, recordings of ganglion cell activity reveal a dramatic NO-dependent decrease in light responses (Wang et al. 2003). Although effects of NO have been identified in multiple retinal cell types, the full range of NO function in the inner retina remains unknown.

To further examine how NO influences amacrine cell signaling, whole cell recordings were made from cultured GABAergic amacrine cells and amacrine cell synaptic pairs derived from embryonic chick retinae. We show that NO alters amacrine cell activity by affecting the functional properties of GABA$_A$ receptors and, much more dramatically, by converting inhibitory synapses to excitatory synapses through a GABA receptor-independent mechanism. This mechanism involves an NO-induced redistribution of Cl$^-$. Our results indicate that the redistribution of Cl$^-$ does not stem from Cl$^-$ moving across the plasma membrane but is instead due to release of Cl$^-$ from an internal compartment.

**Methods**

**Cell Culture**

Retinal cultures were prepared from 8-day-old chick embryos and maintained as previously described (Hoffpauir and Gleason 2002). Experiments were performed on isolated amacrine cells or isolated pairs of amacrine cells 6–14 days (EE14 to PE1) after plating. After 6 days in culture, these cells are considered to be mature, based on
several physiological criteria including expression of the appropriate voltage- and ligand-gated ion channels (Huba and Hofmann 1991; Huba et al. 1992) and the formation of functional GABAergic synapses with other GABAergic amacrine cells. The properties of the amacrine-to-amacrine cell GABAergic synapses formed in culture have been previously characterized (Gleason et al. 1993). The uniformity of neurotransmitter phenotype indicates that the amacrine cells in culture represent a subset of amacrine cell phenotypes found in the intact retina.

Hippocampal cultures were made from 18-day-old rat (Fischer 344) embryos. Micro-dissected hippocampal tissue was obtained from Neuromics (Bloomington, MN) and prepared and maintained according to the supplier’s protocols. Recordings from single hippocampal neurons were made after 4–7 days in culture. No physiological assessments of the relative maturity of these cells have been made. According to their developmental timeline alone, they would be considered embryonic (EE22–EE25).

Electrophysiology

Whole cell recordings were made using an Axopatch 1-D amplifier, Digidata 1322A data-acquisition board, and Clampex 9.2 software (Axon Instruments, Union City, CA). A reference Ag/AgCl pellet in 3M KCl was connected to the culture dish via an agar bridge containing 3M KCl. Patch electrodes were pulled from thick-walled borosilicate glass (1.5mm OD, 0.86mm ID; Sutter Instruments, Novato, CA) using a P-97 Flaming/Brown Puller (Sutter Instruments). Tip resistance values were 5–10MΩ for ruptured-patch recordings and 3–5MΩ for perforated-patch recordings as measured in the bath. All recordings were made at room temperature (22–24°C) and were corrected for junction potential error. Voltage ramp data were leak-subtracted. Because
we used both perforated- and ruptured-patch to study the NO-shift in $E_{GABA}$ (see Results), we wanted to determine whether the recording configuration had an effect on the amplitude of the shift. To achieve this, we recorded NO-induced shifts in $E_{GABA}$ on 1 day with one batch of NO solution. For these experiments, we alternated between the two recording configurations and found no significant differences in the amplitude of the shifts ($n = 3$ in each configuration; $p = 0.999$).

**Solutions**

Unless otherwise indicated, all reagents were purchased from Sigma, St. Louis, MO. GABA application was achieved in 10–20ms through computer-controlled perfusion barrel movements. This method was also used to switch between normal and zero external Cl$^-$ for the experiment depicted in Fig. 2.8A. All other solution changes were achieved in ~500ms by opening and closing the valves upstream of a manifold feeding one common barrel. The compositions of external and internal solutions are shown in Tables 2.1 and 2.2, respectively. TTX (300nM) and LaCl$_3$ (25–50μM) were added to external solutions (with the exception of the 0Cl$^-$ external solution) for single-cell voltage clamp (but not current clamp) recordings to block voltage-gated Na$^+$ and Ca$^{2+}$ currents, respectively. For ruptured-patch recordings, the following reagents were added to internal solutions (Cs$^+$-A, unless otherwise indicated): 50U/ml creatine phosphokinase, 3mM adenosine 5’-triphosphate (ATP) dipotassium salt, 1mM ATP-disodium salt, 20mM phosphocreatine (Calbiochem, La Jolla, CA), 2mM guanosine 5’-triphosphate (GTP) sodium salt. For perforated-patch recordings, either amphotericin B (synaptic pairs, Fig. 2.4) or gramicidin (Figs. 2.5 and 2.6) were added to normal internal or Cs$^+$ internal, respectively, to a final concentration of 10μg/ml.
Table 2.1: External Solutions

<table>
<thead>
<tr>
<th></th>
<th>NaCl</th>
<th>KCl</th>
<th>CaOH</th>
<th>CaCl₂</th>
<th>MgCl₂</th>
<th>HEPES Cl⁻</th>
<th>Glucose</th>
<th>NMG-Cl⁻</th>
<th>Na⁻</th>
<th>Iseth</th>
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<tbody>
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<td>Normal</td>
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<td>3.0</td>
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<td></td>
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<td></td>
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<tr>
<td>TEA-A</td>
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<td>20.0</td>
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<td>0 K⁺, 0 Na⁺</td>
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<td></td>
<td>3.0</td>
<td></td>
<td>10.0</td>
<td>5.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 K⁺/0 Na⁺</td>
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<td>5.6</td>
<td>135.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEA-B</td>
<td></td>
<td></td>
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<td></td>
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<td>5.0</td>
<td>5.6</td>
<td>134.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Cl⁻</td>
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<td>0.4</td>
<td>10.0</td>
<td>5.6</td>
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</tr>
<tr>
<td>0 Cl⁻, H</td>
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<td>1.0</td>
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<td>20.0</td>
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<tr>
<td>0 Cl⁻, H</td>
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</tr>
</tbody>
</table>

Contents of external recording solutions are given in millimolar. The pH of external solutions was adjusted to 7.4 using an appropriate base. Glucose concentration was varied +/-10% to alleviate deviations from the normal osmolarity for some of the solutions. NMG, N-methyl-D-glucamine; TEA, tetraethylamonium; iseth, isethionate.

S-Nitroso-N-acetyl-D,L-penicillamine (SNAP), 1-hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC-12), 2,2'-(hydroxynitrosohydrazino) bisethanamine (NOC-18), and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, sodium salt (carboxy-PTIO) were obtained from Dojindo Molecular Technologies (Gaithersburg, MD) and stored at −20°C. The final pH of external solutions containing the nitric oxide donors was readjusted to 7.4 with NaOH (SNAP and NOC12). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was prepared at 10mM in DMSO.

Furosemide and bumetanide were prepared as 300mM stocks in DMSO.

**NO-bubbled Solutions**

Pure NO solutions were prepared by bubbling solutions with argon for 15min followed by 15min of bubbling with pure soda lime-filtered NO. NO solutions were tightly sealed, protected from light, and stored at 4°C. Although the method for preparing NO was standardized, the efficacy of different batches was variable. For most experiments,
Table 2.2: Internal solutions

<table>
<thead>
<tr>
<th></th>
<th>Cs</th>
<th>CsCl</th>
<th>K</th>
<th>KCl</th>
<th>CaCl₂</th>
<th>MgCl₂</th>
<th>HEPES</th>
<th>EGTA</th>
<th>NaCl</th>
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</thead>
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<td>2.0</td>
<td>10.0</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cs⁺-B</td>
<td>135.0</td>
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<td>0.1</td>
<td>2.0</td>
<td>10.0</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>High</td>
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<td>2.0</td>
<td>10.0</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Cl⁻</td>
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<td>5.0</td>
<td>0.1</td>
<td>2.0</td>
<td>10.0</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MES</td>
<td>145.0</td>
<td>5.0</td>
<td>0.1</td>
<td>2.0</td>
<td>10.0</td>
<td>1.1</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cs⁺-H</td>
<td>130.0</td>
<td>15.0</td>
<td>0.1</td>
<td>3.0</td>
<td>10.0</td>
<td>1.1</td>
<td></td>
<td></td>
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</tbody>
</table>

Contents of internal recording solutions are given in millimolar. Methane sulfonate is abbreviated MES. Internal solutions Cs⁺-A and high Cl⁻ were supplemented with the ATP regeneration system components (see Methods). Cs⁺-H was supplemented with 2mM disodium ATP. The pH of the internal solutions was adjusted to 7.4 using an appropriate acid or base.

Injections between 10 and 50µl were able to elicit shifts of 15–35mV. When standard volumes of a batch of NO solution became less efficient, a new batch was obtained.

The NO solutions were delivered into the perfusion system manually with a Hamilton syringe via in-line injections (10–50µl depending on potency, see following text). In-line injection of dye-containing solutions indicated that NO exposure was limited to 1–3s with most of the NO arriving in the first 500 ms. NO was bubbled into either H₂O or TEA external. No differences in the effects of NO in the two solutions were observed. For synaptic recordings, NO was bubbled into a 40mM HEPES solution to minimize low pH effects on Ca²⁺ channels and other synaptic proteins. The concentration of NO in these solutions was measured using an ISO-NO meter (NOMK2 system) with an ISO-NOP electrode (World Precision Instrument, Sarasota, FL) for a batch and volume that was demonstrated to be effective in eliciting cellular responses. The mean NO concentration measured at the perfusion outlet was 2.0 +/- 0.3µM. This value represents the upper
limit of NO coming in contact with a cell. For SNAP solutions, ~100nM NO was detected in external solution containing 250µM SNAP (2h after preparation). With the exception of Fig. 2.1, the data shown were obtained using NO-bubbled solutions.

“Air-exposed” NO solutions were made by exposing NO-bubbled solution to air for ≥10–30min. For both fresh and air-exposed NO solutions, pH values typically ranged between 2 and 3. Injections of the appropriate amount of pH 2.5, NO-free solutions were routinely used as controls and none of the effects reported here were reproduced by low pH alone. Although extreme care has been taken to standardize the preparation, handling and injections of these volatile solutions, some variability remains. As such, only those cells receiving the same volumes, from the same batch of NO-bubbled solutions were used for quantitative comparisons. Data are reported as means +/- SD and statistical significance was determined using the t-test. Data shown in Figs. 2.5, B and D, and 2.6F were analyzed using the paired t-test.

Results

Effects of NO on GABA-gated Currents

To explore the effects of NO, we recorded whole cell GABA\textsubscript{A} receptor currents (Hoffpauir and Gleason 2002) from individual amacrine cells. For these experiments, Cs\textsuperscript{+}-A internal and TEA-A external solutions were used, and recordings were made in the ruptured-patch recording configuration. Under these conditions, we find that NO produces three effects in amacrine cells. First, moderate NO donor concentrations (250 µM SNAP, ~100nM NO, see Methods) caused a small (~15%) enhancement of the GABA-gated current amplitude that was not due to alteration in the reversal potential of
the current ($E_{\text{GABA}}$, Fig. 2.1). Similar effects were observed with NOC 12 (250µM) and NOC 18 (300µM, not shown). The SNAP-dependent enhancement was significantly

![Figure 2.1](image)

**Figure 2.1:** Moderate concentrations of nitric oxide (NO) enhance GABA<sub>A</sub> receptor function. **A,** Peak current amplitudes of inward currents elicited by 500-ms applications of GABA (20µM) are normalized to the current amplitude just prior to application of S-nitroso-N-acetyl-D,L-penicillamine (SNAP). **B,** Whole cell voltage-clamp recordings of GABA-gated currents from a representative amacrine cell recorded before (black trace) and in the presence of (gray trace) SNAP. Scale bars are 100pA and 500ms. **C,** Mean response amplitudes from cells exposed to 2 concentrations of SNAP and in the presence of the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, sodium salt (carboxy-PTIO). Mean current change is calculated as the percent difference of GABA-gated current amplitudes measured immediately before SNAP application compared with those measured after 30s of exposure to SNAP. **D,** Leak-subtracted currents elicited by voltage ramps delivered in the presence of GABA before (black trace) and in the presence of (gray trace) SNAP (500µM). Scale bars are 100pA and 10mV. All recordings were made with Cs<sup>+</sup>-A internal and TEA-A external in the ruptured-patch configuration.

inhibited in the presence of the NO scavenger carboxy-PTIO, indicating that NO rather than donor end products underlie the GABA<sub>A</sub> current enhancement (Fig. 2.1C, p=0.002).
Second, higher concentrations of NO (hundreds of nanomolar to low micromolar, NO-bubbled solutions, see Methods) produced a transient (1–3s), GABA-independent inward current (Figs. 2.2, A and C, and 2.7Bi, asterisks). Based on reversal potential measurements, this NO-dependent, GABA-independent, inward current is a cation current ($E_{\text{rev}} = +23.4 \pm 6.6\text{mV}$, $n = 5$).

The third effect of NO is the most dramatic and will form the main focus of this work. We find that brief (1–3s) exposure to higher NO concentrations also produced a several-fold enhancement of the GABA-gated current (Fig. 2.2A) due to a transient positive shift in $E_{\text{GABA}}$ (shift = 24.8 $\pm$ 4.2mV; Fig. 2.2, C–E). The slight inhibition of GABA-gated current amplitude after exposure to air-exposed NO solution (Fig. 2.2B) is most likely due to the acidic nature of these solutions (see Methods) (Huang and Dillon 1999). Note that the time course of recovery for the (higher concentration) NO-induced current enhancement (Fig. 2.2A) is consistent with the recovery time course for $E_{\text{GABA}}$ (Fig. 2.2E). Similar shifts in $E_{\text{GABA}}$ were obtained with high concentrations (2mM) of the NO donor NOC12 (not shown) indicating that the mechanism responsible for the shift in $E_{\text{GABA}}$ can be activated by either NO delivery method as long as the concentration of NO is in the correct concentration range. A small increase in the slope of the I-V relationship for the GABA-gated current (1st NO effect) was also detectable with higher NO concentrations (Fig. 2.2D, inset). The role of soluble guanylate cyclase (sGC) activation in the NO-induced shift in $E_{\text{GABA}}$ was investigated using the sGC inhibitor ODQ. Prolonged (~10min) preincubation with the inhibitor did not block the NO-induced shift in $E_{\text{GABA}}$ indicating that sGC activity is not involved in this mechanism (Fig. 2.2, F and G). The nonsignificant trend toward larger responses in ODQ suggests that basal sGC
activity might have a suppressive effect on the mechanism underlying the shift in $E_{GABA}$.

Finally, the NO-dependent current (2nd NO effect) may be related to the shift in $E_{GABA}$ but does not seem to be an absolute requirement because we have observed the shift in the absence of this current.

**NO-induced Shift in $E_{GABA}$ Is Due to an Elevation in Cytosolic $Cl^-$**

Three possible explanations for the NO-induced shift in $E_{GABA}$ were examined: a change in ion selectivity of the GABA$_A$ receptors, the introduction of another permeant anion into the cell, or an increase in intracellular $Cl^-$ concentration. To determine whether NO increases the selectivity of GABA$_A$ receptors to acetate (the only other anion in the internal solution), internal acetate was substituted with methanesulfonate, a bulky anion unlikely to permeate the receptors. This substitution, however, did not prevent the NO-induced shift in $E_{GABA}$ as indicated by the several-fold enhancement of the GABA-gated current (Fig. 3.3A). This suggests that the NO-induced shift in $E_{GABA}$ is not due to a change in the ion selectivity of the channels.

The shift could be due to the production of nitrate (NO$_3^-$) that occurs when NO reacts with O$_2$ and H$_2$O. Because NO$_3^-$ is quite permeable through GABA$_A$ receptors (Biscoe and Duchen 1985; Bormann et al. 1987), it is possible that trapped cytosolic NO$_3^-$ (from inwardly diffusing NO) contributes to the shift in $E_{GABA}$. Furthermore, although the recording solutions are HCO$_3^-$-free, it is also possible that NO transiently stimulates the production of HCO$_3^-$, another anion that can permeate GABA$_A$ receptors (Bormann et al. 1987). In zero $Cl^-$ internal and zero $Cl^-$ external solutions, no GABA-gated currents were observed either before or after application of NO (Fig. 3.3B).
Figure 2.2: Higher concentrations of NO promote a positive shift in $E_{GABA}$. A and B, top traces. Raw data from ruptured-patch voltage-clamp recordings of GABA-gated currents from a representative cell before and after NO application. GABA pulses (20µM) were 300 ms in duration and are indicated by horizontal bars. A, Whole cell, voltage-clamp recordings (Cs⁺-A internal and TEA-A external) of GABA-gated currents reveal that higher concentrations of NO induce a transient, several-fold enhancement of GABA-gated currents. *, NO-dependent current observed prior to the 2nd GABA application. B, Same experiment as in A, using air-exposed NO solution. Raw data in A and B are from same cell. Scale bars are 150pA, 1s. C, Amacrine cell is held at the predicted $E_{GABA}$. GABA is applied for 300 ms during each trace. No GABA-gated currents are observed until application of NO. *, NO-dependent current. Scale bars are 25pA, 5s. D, Voltage ramps in GABA were delivered before and after addition of NO. Leak-subtracted currents reveal a shift in $E_{GABA}$ after NO application (gray trace). Inset: subtraction of the NO-induced shift in reversal potential reveals an increase in the slope of the GABA-gated current-voltage relationship after NO injection (gray trace). Scale bars are 100pA, 20mV. E, Mean $E_{GABA}$ values are plotted over time. F, Representative GABA-gated currents from voltage ramps delivered after a 11-min treatment with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 2µM). Black trace, before NO injection; Gray trace, after NO injection. G, ODQ did not block the NO-induced shift in $E_{GABA}$ ($p = 0.83, n = 5$).
Figure 2.3: The shift in $E_{GABA}$ is due to an NO-induced increase in cytosolic $Cl^-$. A, Substituting methanesulfonate for acetate in the Cs+ internal (MES internal) solution does not prevent NO-induced enhancement. The NO-dependent current (asterisk) is also observed with this internal solution. Experiment conducted as in Fig. 2 A and B, with horizontal bars indicating time course of GABA application. B, Ruptured-patch, voltage-clamp recordings of an amacrine cell held at $-70\text{mV}$ with $0Cl^-$ internal and external solutions. GABA is applied for 300ms in each trace (horizontal bars). Time scale applies only to current traces (start-to-start intervals are 5s). An NO-dependent current was recorded immediately after the NO application (not shown). Scale bars (A and B) are 10pA, 1s. C, Leak-subtracted currents from voltage ramps delivered in GABA (20mM) and glycine (20mM) before (black trace) and after (gray trace) addition of NO. (Cs+-A internal and TEA-A external solutions). D, Current-clamp recordings from a different cell ($V_{rest} = -65\text{ mV}$) show the voltage responses to GABA and glycine before (black trace) and after (gray trace) exposure to NO. An action potential occurs at the beginning of the glycine-induced depolarization. Scale bars are 10mV, 500ms (normal internal and normal external solutions).
This argues against the possibility that the shift is due to an increase in intracellular NO$_3^-$, or HCO$_3^-$, or any other permeant anion.

If the effect of NO is to raise internal Cl$, then a similar shift in reversal potential should also be observed when glycine receptors are activated. Agonist-gated currents were elicited with pulses of 20µM glycine or 20µM GABA delivered during voltage ramps. Addition of NO caused a shift in the reversal potential to more positive values for both agonists (Fig. 2.3C; GABA shift: 33.6 +/- 11.5mV; glycine shift: 30.0 +/- 14.0mV; p = 0.67; n = 5), indicating that NO is stimulating a redistribution of Cl$ in these cells. An increase in the slope of the glycine-gated current was also observed, indicating an enhancing effect of NO on glycine receptor function. The ability to change the internal Cl$ concentration in the face of diffusion from the pipette may be unexpected, but it is not unprecedented. Cl$ transport has been shown to be effective in opposing diffusion from the recording pipette (Staley et al. 1996) and changes in cytosolic Cl$ have been measured by optical methods in the ruptured-patch recording configuration (Isomura et al. 2003). It is also possible that the redistribution of Cl$ is not a global event, but one that occurs locally, possibly in neuronal processes.

Current-clamp recordings were made to determine how the NO-induced shift in $E_{Cl^-}$ affects GABA- and glycine-dependent changes in membrane potential (Fig. 2.3D). Resting membrane potentials were -60.1 +/- 13.2mV (n = 5). Under control conditions, application of GABA or glycine hyperpolarized these cells to -70.9 +/- 2.7mV and -68.7 +/- 5.3mV, respectively. For the first agonist pulse after NO application, GABA depolarized the cells to -31.8 +/- 25.2mV and glycine depolarized the cells to -34.4 +/- 18.4mV. One cell responded to both GABA (not shown) and glycine (Fig. 2.3D) with a
single action potential followed by a steady depolarization. This is the typical voltage response for these cells given sufficient depolarization (Gleason et al. 1993). It is important to note, however, that action potentials are not required for Ca\(^{2+}\)-dependent neurotransmitter release from these cells (Gleason et al. 1993). These current-clamp recordings demonstrate that NO can change the sign of whole-cell voltage responses to GABA and glycine.

**NO-induced Shift in E\(_{Cl^-}\) Occurs at Synapses**

To examine the influence of NO specifically at retinal amacrine cell synapses, dual perforated-patch recordings were made from synaptically-connected pairs of cultured GABAergic amacrine cells. It is well established that GABAergic amacrine cell synapses are normally inhibitory, both in the retina (Lagnado 1998) and in these cultures (Gleason et al. 1993). Recordings from isolated pairs of amacrine cells demonstrate that brief exposure to NO can transiently change the sign of GABAergic synapses (Fig. 2.4). With both pre- and postsynaptic cells in the voltage-clamp configuration, a depolarizing voltage step in the presynaptic cell produced a small inward chloride movement (outward current) in the postsynaptic cell. After NO, postsynaptic currents become inward indicating an efflux of chloride out of the cell and an excitatory effect on the postsynaptic cell (Fig. 2.4A, gray trace). The relatively small, noisy postsynaptic currents are consistent with the low release rates described for these synapses in culture (Gleason et al. 1993) and for GABAergic amacrine cell synapses in the intact retina (Zheng et al. 2004). When the postsynaptic cell is switched to current clamp, the postsynaptic response is barely detectable because under control conditions, the resting potential of the cell (-67mV) is very near the calculated E\(_{Cl^-}\) (-70mV). After
NO, the postsynaptic voltage response is depolarizing (Fig. 2.4B, gray trace). NO-dependent, excitatory synaptic responses were detected in all pairs examined (n = 5).

Figure 2.4: NO inverts the sign of GABAergic synapses. A, Both pre- and postsynaptic cells were voltage clamped. Only data from the postsynaptic cell are shown. Under control conditions, depolarization of the presynaptic cell from -70mV to -10mV elicits a small outward current in the postsynaptic cell (black trace, $V_{\text{hold}} = -66$ mV). After NO, depolarization of the presynaptic cell produces a large inward current in the postsynaptic cell (gray trace). Traces immediately after NO application were contaminated by the NO-dependent current. The trace shown was collected ~15s after NO. NO exposure time is 2–3s (see Methods) Scale bars are 200pA, 50ms. B, Current-clamp recordings from the same postsynaptic cell as in A. Depolarization of the presynaptic cell (to 0mV, in voltage clamp) produces only a slight hyperpolarization in the postsynaptic cell (black trace) because the resting potential of this cell (-67mV) is near the predicted $E_{\text{Cl}}$ (-70mV). A record collected ~20s after NO exposure shows that the postsynaptic response is now depolarizing (gray trace). Scale bars are 5mV, 100ms. Records were collected with normal internal containing amphotericin B and TEA-A external solutions.

Postsynaptic responses to subsequent presynaptic depolarizations were omitted from Fig. 2.4 for clarity, but they show that recovery from the NO-dependent shift in $E_{\text{Cl}}$ occurs over a similar time frame as observed in recordings of whole cell GABA-gated
currents in single amacrine cells (see Fig. 2.2, A and E). It is possible that NO has other, as yet uncharacterized effects on amacrine cell synapses (Ahern et al. 2002; Holscher 1997), but the key observation here is that the shift in $E_{Cl^{-}}$ found in whole cell recordings also occurs at synapses.

**NKCC Does Not Mediate the Increase in Cytosolic $Cl^{-}$**

What mechanism underlies the increase in cytosolic $Cl^{-}$? The plasma membrane $Na^{+}$-$K^{+}$-$Cl^{-}$ co-transporter (NKCC) and the $K^{+}$-$Cl^{-}$ co-transporter (KCC2) are $Cl^{-}$ co-transport mechanisms known to regulate the distribution of $Cl^{-}$ across neuronal plasma membranes (Kakazu et al. 2000; Russell 2000). It is now well established that changes in $Cl^{-}$ cotransporter expression determine the effects of GABA during development (for review, see Payne et al. 2003). In the adult retina, $E_{Cl^{-}}$ varies among cell types and subcellular locations, and this correlates with the expression pattern of NKCC and KCC2 (Vardi et al. 2000). Furthermore, the response properties of retinal starburst amacrine cells are dependent on the activity of NKCC and KCC2 (Gavrikov et al. 2003). Given the established role of these transporters in determining $E_{Cl^{-}}$, we investigated the role of NKCC and KCC2 in the NO-induced shift in $E_{Cl^{-}}$.

NKCC typically transports 2 $Cl^{-}$, 1 $Na^{+}$, and 1 $K^{+}$ into the cell and the neuron specific $K^{+}$-$Cl^{-}$ co-transporter, KCC2, transports 1 $Cl^{-}$ and 1 $K^{+}$ out of the cell. Thus the NO-induced shift in $E_{GABA}$ could be due to an increase in NKCC activity or a decrease in KCC2 activity. To determine if these mechanisms were involved, perforated-patch recordings were made with $Cl^{-}$-impermeant gramicidin in the pipette and the co-transport inhibitors, bumetanide ($300\mu M$) and furosemide ($300\mu M$) were used to block NKCC and KCC2 activity. Both inhibitors shifted $E_{Cl^{-}}$ to more negative potentials,
indicating a dominant inhibitory effect on NKCC (Fig. 2.5, A and C). Neither blocker, however, inhibited the NO-induced shift in $E_{Cl^-}$ (Fig. 2.5, B and D). To assess the

![Figure 2.5: Inhibition of Na$^+$-K$^+$-Cl$^-$ co-transporter (NKCC) and K$^+$-Cl$^-$ co-transporter (KCC) Cl$^-$ co-transporters does not block the NO-induced shift in $E_{Cl^-}$. A and C, Leak-subtracted currents from voltage ramps delivered in the presence of GABA under control conditions (no NO), in the presence of inhibitor (bumetanide (B) or furosemide (F)) and after NO exposure in bumetanide- or furosemide-containing solutions (gray trace). Records were obtained in the perforated-patch configuration with gramicidin in the pipette (Cs$^+$-B internal, TEA-A external). B and D, Cl$^-$ co-transporter inhibitors do not significantly (bumetanide $p = 0.66$; furosemide $p = 0.17$) reduce the NO-induced shift in $E_{Cl^-}$. NO-induced shifts in $E_{Cl^-}$ in the presence of each inhibitor were compared with control applications of NO in the same cells.

variability of responses under control conditions, the effects of two separate NO applications were also examined and we found no significant difference between the two trials ($p = 0.69$, $n = 5$, not shown). At lower concentrations ($10\mu$M), bumetanide is selective for NKCC (Russell 2000). In similar experiments, bumetanide at $10\mu$M was also ineffective in blocking the shift in $E_{Cl^-}$ (not shown).
Removal of the externally required co-transported ions shifted $E_{\text{Cl}^-}$ to more negative potentials. Furthermore, for those cells that were exposed to both zero $K^+$ and zero $Na^+$ solutions, $E_{\text{Cl}^-}$ was considerably more negative in zero $K^+$ solutions than in zero $Na^+$ solutions (Fig. 2.6A). In some cases, switching to zero $K^+$ shifted $E_{\text{Cl}^-}$ by tens

Figure 2.6: Removal of co-transported ions does not block the NO-induced shift in $E_{\text{Cl}^-}$. A–E, Perforated-patch voltage-clamp recordings made with gramicidin (Cs⁺-B internal, TEA-A external). Voltage ramps were delivered in the presence of GABA for each condition and leak-subtracted currents are shown. A, $E_{\text{Cl}^-}$ is negatively shifted in external solutions lacking $K^+$ or $Na^+$. Overall, the shift from normal solutions was greater in solutions with 0 external $K^+$ than in solutions with 0 external $Na^+$. B, Switching from normal to 0$K^+$ external solutions can produce large negative shifts in $E_{\text{Cl}^-}$. Dotted gray trace shows estimated $E_{\text{Cl}^-}$ in 0 external $K^+$. Zero $K^+$ external solution was applied for 15s before collecting the 0$K^+$ trace. C–E, Bathing the cells in 0$Na^+$, 0$K^+$, or 0$Na^+/K^+$ external solutions did not prevent the NO-induced shift in $E_{\text{Cl}^-}$ (gray traces). F, Average NO-induced shifts in $E_{\text{Cl}^-}$ for cells bathed in 0$Na^+$ or 0$K^+$ external solutions are not significantly different from control (0$Na^+$, $p = 0.17$; 0$K^+$, $p = 0.26$).
of millivolts in as few as 15s (Fig. 2.6B). These results are consistent with the prediction that zero external K\(^+\) would prevent inward Cl\(^-\) transport through NKCC and promote outward Cl\(^-\) transport through KCC2. Despite these effects, none of these manipulations blocked the NO-induced shift in E\(_{Cl^-}\) (Fig. 2.6, C–E). Figure 6F shows that removal of co-transported ions did not cause a statistically significant (p = 0.26; 0.17) change in the magnitude of the NO-induced shift in E\(_{Cl^-}\). Interestingly, a nonsignificant trend toward larger shifts was observed in the absence of co-transported ions. This may relate to an enhanced Cl\(^-\) gradient between the cytosol and an internal compartment (see following text). The roles of two other known plasma membrane Cl\(^-\) transport mechanisms have also been investigated. Neither 4,4\(^{'}\)-diisothiocyanatostilbene-2,2\(^{'}\)-disulfonic acid (DIDS, 40 \(\mu\)M, n = 5) a HCO\(_3^-/Cl^-\) transport inhibitor, nor ethacrynic acid (10 \(\mu\)M, n = 4), an inhibitor of ATP-dependent, Na\(^+\)-independent Cl\(^-\) transport, blocked the shift in E\(_{Cl^-}\) (not shown).

**Shift in E\(_{Cl^-}\) Is Independent of Extracellular Cl\(^-\)**

The lack of involvement of known plasma membrane Cl\(^-\) transporters indicates that NO is either stimulating an unexamined Cl\(^-\) transport mechanism or releasing Cl\(^-\) from inside the cell. Endosomal compartments are known to maintain relatively high Cl\(^-\) concentrations to offset the membrane potentials generated by inward proton pumping and endosomal acidification (Faundez and Hartzell 2004; Sonowane and Verkman 2003). To determine whether NO was releasing Cl\(^-\) from an internal compartment, external Cl\(^-\) was reduced to match the internal Cl\(^-\) concentration (14.2mM). NO was applied while cells were held at the reversal potential for Cl\(^-\). Under these conditions, subsequent records collected during pulses of GABA demonstrated that the NO-
induced redistribution of Cl\textsuperscript{-} persisted in the absence of an electrochemical gradient for chloride across the plasma membrane (Fig. 2.7A, see also Fig. 2.2C). To determine whether external Cl\textsuperscript{-} was required at all, NO was also applied in the absence of external Cl\textsuperscript{-}. For these experiments (Fig. 2.7B), control ramps (Bi) and the NO applications (Bii) were conducted in zero Cl\textsuperscript{-} external solution. The arrival of NO in zero Cl\textsuperscript{-} was marked by the presence of the NO-dependent inward current (asterisk). After switching to normal Cl\textsuperscript{-} external solution (TEA-A), so that the reversal potential of the current could be measured, another set of ramps was delivered (Biii). Values for the reversal potentials of the GABA-gated currents after NO application (mean shift: 26.6 +/- 9.9mV, n = 9) indicate that an increase in internal Cl\textsuperscript{-} occurred in the absence of external Cl\textsuperscript{-}. Control experiments were performed in the same manner but without NO application (Fig. 2.7, D and E). Only slight fluctuations (1–2mV) in reversal potential resulted from just switching between Cl\textsuperscript{-} external and normal external solutions.

The switch back to normal external Cl\textsuperscript{-} concentration before measuring the GABA-gated current reversal potentials leaves open the possibility that rapid Cl\textsuperscript{-} influx could occur just prior to measuring the reversal potential. To avoid this possibility, we also examined the large NO-induced change in the amplitude of the GABA-gated currents at -70mV that results from the positive shift in reversal potential. In this way, the effects of NO can be evaluated without re-introducing external Cl\textsuperscript{-}. In this experiment, both NO and GABA are applied in zero external Cl\textsuperscript{-}. Substantial increases (2.9 +/- 0.7-fold) in the GABA-gated current amplitude were observed in all cells tested (n = 5; Fig. 2.8A). Because external Cl\textsuperscript{-} is absent for both the NO injection and the
Figure 2.7: External Cl⁻ is not required for the NO-induced shift in $E_{Cl^-}$. A, Representative cell held at $E_{Cl^-}$ (0 mV) and pulsed with GABA (horizontal bars) before and after NO application (Cs⁺-A internal, low Cl⁻ external, ruptured patch). Increase in leak current after NO application is due to contamination from the NO-dependent current (not shown). Scale bars are 200pA, 100ms. Time scale applies only to current traces shown (start-to-start time, 6s). B, top, Voltage protocol for i–iii. The 1st ramp of each pair is for leak subtraction. Ramp pairs were delivered 6 times (start-to-start time, 10s). Scale bars are 2s and 200ms. Bi, GABA-gated current collected in 0Cl⁻ solution. Currents from 3 of the 6 voltage ramps are shown and are numbered in order of collection. The outward currents disappear as external Cl⁻ washes out of the bath. The inward current persists but becomes progressively smaller in amplitude. This is likely due to depletion of internal Cl⁻ during the 6 GABA exposures. Bii, NO-dependent current (asterisk) indicates the arrival of NO in 0Cl⁻ solution. Biii: Currents elicited after NO application in normal Cl⁻ external (gray trace, 2nd ramp pair; black trace, sixth ramp pair). C—E, Control data recorded from a different cell. The voltage protocol shown in B is used but data are shown from the 3rd phase of the experiment only (ramps delivered after switching back to normal external Cl⁻). C, NO-induced shift is elicited after NO application in 0Cl⁻ as in Biii to show that this cell is responsive to NO. D, In the same cell as C, no change in $E_{Cl^-}$ is induced by switching from 0Cl⁻ to normal Cl⁻ alone. E, After the same sequence of events, air-exposed NO produces no shift in $E_{Cl^-}$.
enhancement of the GABA-gated current, the Cl\(^{-}\) must be coming from the inside of the cell. Why, in the absence of internal and external Cl\(^{-}\) (Fig. 2.3B), did the NO-induced release of Cl\(^{-}\) from internal stores not produce an inward GABA-gated current? It is plausible that under Cl\(^{-}\)-free conditions, the Cl\(^{-}\) store becomes depleted. Clearly, much remains to be understood about the dynamics of intracellular Cl\(^{-}\) fluxes. Aside from the removal of both internal and external Cl\(^{-}\), the only manipulation that blocked the NO-

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**Figure 2.8: NO promotes release of Cl\(^{-}\) from an internal source.** A, Voltage clamp recording is shown from a representative amacrine cell held at -70mV (normal internal, TEA external). In the control panel, the external solution was switched to 0Cl\(^{-}\) ~90s before the pulse of GABA. In this experiment, the rapid perfusion method usually used to apply GABA (Methods) was used to switch between normal and 0 external Cl\(^{-}\). The return to normal external Cl\(^{-}\) reduces the size of the current due to the reduction in driving force on Cl\(^{-}\). In the same cell, the protocol was repeated with an NO injection delivered into 0Cl\(^{-}\) external 15s before the onset of GABA delivery. The NO-dependent current was observed but is not plotted. The amplitude of the GABA-gated current is enhanced ~3.5-fold, consistent with a substantial positive shift in \(E_{Cl}^{-}\). B, GABA-gated currents recorded with high Cl\(^{-}\) internal (TEA-B external, predicted \(E_{Cl}^{-} = 0\)mV) before (black trace) and after (overlapping gray trace) NO.
induced shift in Cl\(^-\) distribution was to raise the internal Cl\(^-\) concentration to 114mM. Under these conditions, application of NO produced virtually no shift in the reversal potential of the GABA-gated currents (mean shift = 0.5 +/- 0.2mV, n = 5, Fig. 2.8B). This result is consistent with internal Cl\(^-\) release if the 114mM internal Cl\(^-\) reduces the gradient for Cl\(^-\) between an internal Cl\(^-\) compartment and the cytoplasm.

**NO-induced Shift in E\(_{Cl^-}\) Is Not Confined to the Avian Retina**

Does the NO-induced shift in induced shift in Cl\(^-\) distribution rely on an amacrine cell-specific mechanism or is the underlying mechanism more widely expressed? To explore this, we made similar recordings from cultured rat hippocampal neurons. We find that NO also induces a shift in E\(_{Cl^-}\) in hippocampal neurons (Fig. 2.9A; mean shift 38 +/- 19.6mV, n = 6). An inward GABA-independent/NO-dependent current was also observed in hippocampal neurons (Fig. 2.9C, asterisk). To determine whether the shift in E\(_{Cl^-}\) was also due to internal release of Cl\(^-\), we repeated the experiment shown in Fig. 2.7B. Removal of extracellular Cl\(^-\) during NO application did not inhibit the shift in E\(_{Cl^-}\), suggesting that, as for amacrine cells, NO stimulates the release of Cl\(^-\) from an internal store in hippocampal neurons (Fig. 2.9D).

**Discussion**

These results indicate that NO can modulate GABAergic signaling through two distinct mechanisms. Prolonged and moderate (~100nM) release of NO from donors enhances GABA-gated currents by modifying receptor activity. Brief pulses of higher concentrations of NO (hundreds of nanomolar to low micromolar, NO-bubbled solutions) produce sGC-independent increases in intracellular Cl\(^-\) and shift E\(_{GABA}\) to more positive potentials. Furthermore, we show that the shift in the Cl\(^-\) reversal potential
Figure 2.9: NO-induced shift in $E_{\text{Cl}}$ occurs in rat hippocampal neurons. A, GABA-gated currents recorded from a hippocampal neuron during voltage ramps before (black trace) and after (gray trace) NO application. (ruptured-patch, normal hippocampal external and internal solutions). B–D: current records elicited by the voltage protocol depicted in Fig. 7B. B, GABA-gated current collected in 0 external $\text{Cl}^-$. C, NO-dependent current (asterisk) recorded in 0 chloride indicates timing of NO arrival. Scale bar, 2s. D, After returning to normal (TEA-H) external solution, a shift in $E_{\text{GABA}}$ is revealed. The gray trace is recorded 30s after return to normal $[\text{Cl}^-]_o$, and the black trace is recorded 1min after return to normal $[\text{Cl}^-]_o$. Recordings in A–D are from the same cell. Recordings were made in the ruptured-patch configuration with TEA-H or 0Cl-H external solutions and Cs$^+$-H internal solution.

occurs at synaptic sites and is sufficient to promote excitation at GABAergic synapses. Finally, our results indicate that the redistribution of $\text{Cl}^-$ underlying this change results from a release of $\text{Cl}^-$ from an internal compartment.

Role of NO in the Retina

Measurements indicate that the concentration of NO at the cell in NO-bubbled solutions is in the hundreds of nanomolar to low micromolar range. Is this range of concentrations relevant to the levels of NO generated in the retina? NO electrode measurements at the inner retinal surface yield values ranging from 6 to 15$\mu$M (Donati et al. 1995; Groppe et al. 2003). Consistent with the electrode measurements, a biochemical assay (the Griess method) also shows retinal NO production well into the micromolar range (Heiduschka and Thanos 1998). These intraocular measurements
indicate that NO concentrations can be substantial and that the doses of NO used in our experiments are within the physiological range.

Recently, NO electrode measurements have been made near the surface of an individual cell in the ganglion cell layer of the turtle retina (Eldred and Blute 2005). An NO concentration of ~200nM was detected. It is important to note, however, that imaging of NMDA-stimulated NO production in the same retina has demonstrated that the NO signals generated are often highly localized to individual cells and even discrete boutons in the inner synaptic layer (Blute et al. 2000). This observation suggests that some of the downstream effects of NO signaling occur primarily in nNOS-expressing cells and their immediate synaptic partners, where NO concentrations would be higher.

How might the NO-induced changes we show for cultured amacrine cells affect GABAergic signaling between amacrine cells in the inner retina? The diversity of synaptic partners and complex synaptic arrangements of amacrine cells in the IPL (Dowling and Boycott 1965; Hartveit 1999; Kolb 1997; Marc and Liu 2000) complicate this issue. The cartoon in Fig. 2.10 depicts a simplified subset of interactions in the inner retina. The cell on the left is a GABAergic or glycinergic, expressing amacrine cell that receives a mixture of excitatory (glutamatergic, from bipolar cells) and inhibitory (GABAergic or glycinergic, from other amacrine cells) inputs. The net effect of these inputs is to determine the strength of the inhibitory output from the cell onto its postsynaptic partners (ganglion cells, bipolar cells, and/or amacrine cells). If the cell is exposed to a relatively high concentration of NO (presumably via its own nNOS activity), the distribution of chloride shifts in that amacrine cell so the balance of its input
Figure 2.10: Model for NO effects on amacrine cell signaling. An nNOS-expressing GABA- or glycinergic amacrine cell receiving both inhibitory and excitatory input generates a moderate inhibitory output (left). After NO, E_{Cl^-} shifts positive (darker shading), all inputs become excitatory, and inhibitory output from the amacrine cell is enhanced. The cells depicted are highly simplified in that amacrine cell synaptic inputs and outputs are not typically segregated.

becomes more excitatory and the inhibitory output of this cell is enhanced. Consistent with this proposal, Wang et al. (2003) demonstrate an NO-dependent suppression of ganglion cell light responses that is due to alterations in presynaptic (probably amacrine cell) input. This observation would be consistent with an NO-dependent shift in the amacrine cell E_{Cl^-} that results in an enhancement of inhibitory output onto ganglion cells.

Potential Source of Internal Cl^-

Our results indicate that the NO-induced redistribution of Cl^- is due to Cl^- release from inside the cell. Endosomal compartments are a potential source of internal Cl^- . Cl^- is thought to enter these compartments as a counter ion for protons being pumped in by vacuolar proton pumps (Sonawane and Verkman 2003). Movement of Cl^- across endosomal membranes is thought to be mediated by the CLC family of Cl^- transport
proteins. ClC-7 are found on intracellular membranes (for review, see Faundez and Hartzell 2004; Jentsch et al. 2002). Of these, ClC3 may be the most relevant because it is highly expressed in the brain and has been shown to be expressed on endosomes, including synaptic vesicles.

Interestingly, knockouts of the CIC3 gene resulted in marked tissue degeneration specifically in the hippocampus and the retina (Stobrawa et al. 2001). Although physiological evidence for a role for CLC transport proteins in the NO-induced shift in $E_{Cl^-}$ awaits further experimentation, we have found that a polyclonal antibody raised against a conserved peptide from CIC3 gives an intense and punctuate labeling pattern that appears to be located intracellularly (McMains and Gleason, unpublished observations). It may be that the efflux of Cl$^-$ from endosomes via CIC3 contributes to the NO-induced shifts in $E_{GABA}$ and $E_{glycine}$ and the transitions between inhibition and excitation. Interestingly, NO has been shown to reversibly inhibit the vacuolar proton pump through S-nitrosylation (Forgac 1999). Although the mechanism by which NO stimulates Cl$^-$ efflux is not yet known, given the established relationship between proton pumping and Cl$^-$ movement across endosomal membranes, it is plausible that proton flux is somehow involved. However the Cl$^-$ efflux is achieved, the demonstration that it does occur and that it may be a broadly expressed mechanism is sure to impact our view of GABA and glycine-dependent synaptic signaling in the CNS.

References


Heiduschka P. and Thanos S. NO production during neuronal cell death can be directly assessed by a chemical reaction in vivo. 1998. Neureport 9: 4051–4057.

Hoffpauir B. and Gleason E. Activation of mGluR5 modulates GABA_\text{A} receptor function in retinal amacrine cells. 2002. Journal of Neurophysiology 88: 1766–1776.


Chapter 3

Cytosolic Acidification Is Both Sufficient and Necessary for NO-induced Internal Chloride Release
**Introduction**

Nitric oxide is a gaseous signaling molecule that is produced by subsets of each cell type in the vertebrate retina. Though there is evidence that nitric oxide (NO) can affect multiple cellular processes in the retina, much remains unknown, especially with respect to its function in the inner retina. NO production was shown to inhibit output from ON ganglion cells and to block output from OFF and ON/OFF ganglion cells in ferret retina (Wang et al 2003). This inhibition was mimicked by blocking glycinergic signaling, suggesting the involvement of AII amacrine cells in this process. Furthermore, dark-adapted nNOS knockout mice displayed diminished light sensitivity (Wang et al 2007). These findings indicate that NO may play a role in shaping normal, physiological retinal output, possibly by increasing the level of presynaptic (amacrine cell) inhibition on retinal ganglion cells.

Previous work in our lab has demonstrated that NO induces a transient positive shift in the equilibrium potential for Cl⁻ ($E_{Cl^-}$) in cultured retinal amacrine cells (Hoffpauir et al 2006). This $E_{Cl^-}$ shift was determined to be a result of an NO-mediated, cGMP-independent, increase in intracellular chloride levels resulting from the movement of Cl⁻ from internal compartments into the cytosol. Importantly, this NO-dependent increase in cytosolic Cl⁻ concentration was shown to be sufficient for transiently converting normally inhibitory GABAergic synapses into excitatory synapses (Hoffpauir et al 2006).

This dramatic, sign-changing action of NO on retinal amacrine cell synapses encourages further inquiry into the mechanism underlying this effect. What is the source of the increased Cl⁻? Though their sensitivity to NO is unexplored, acidic internal compartments containing high concentrations of Cl⁻ are found in most eukaryotic cell
types. Organelles such as endosomes (early and late), lysosomes, and synaptic vesicles are regulated to have both a low luminal pH and high levels of Cl\(^-\) (Faundez and Hartzell 2004). The acidity of these compartments is vital to their cellular function. Low pH is maintained by the activity of the V-type (vacuolar) proton ATPase, (Mellman 1992). Chloride is transported into acidified internal compartments to minimize the electrical gradient across internal membranes and to allow the proton pump to function optimally (Sonawane and Verkman 2003). Members of the CLC family of chloride channels and transporters have been specifically implicated in this process (Hara-Chikuma et al 2005, Gunther et al 2003, Stowbrawa et al 2001). There are nine members of this family known to be present in mammals, and these nine are separated into three subgroups. Group 2 (ClC-3, ClC-4, and ClC-5) and Group 3 (ClC-6 and ClC-7) members are expressed mainly on intracellular membranes (Jentsch et al 2002) and are thought to be electrogenic Cl\(^-\)/H\(^+\) antiporters (Jentsch 2007). The charge mitigating activity of these chloride transporters is the subject of much active research. No one has yet demonstrated regulated transport of chloride out of internal compartments. However, the existence of intracellular compartments with high luminal Cl\(^-\) as well as the presence of Cl\(^-\) transporters on internal membranes provides a compelling starting point for examining NO-mediated internal chloride release in retinal amacrine cells.

Because movement of chloride across internal membranes is highly coupled to proton flux, here, we use a combination of electrophysiology and pH imaging to examine the effect of NO on cellular pH and the relationship between cytosolic pH and intracellular Cl\(^-\) concentrations in cultured chick amacrine cells. We also explore the expression and activity of CLC transporters in these cells.
Methods

Cultures

Retinal cultures were prepared as previously described (Hoffpauir and Gleason, 2002). Briefly, retinas from eight-day chick embryos were dissected and separated from the pigment epithelium and vitreous humor. Retinal tissue was mechanically and enzymatically dissociated and plated on poly-L-ornithine-treated 35mm culture dishes to achieve a final cell concentration of approximately $1.25 \times 10^5$ cells per dish. For ion imaging and immunocytochemistry experiments, cells were plated on acid-washed, poly-L-ornithine treated glass coverslips placed in culture dishes. One day after plating, the extracellular media was replaced with a Neurobasal and 1% B27 neuronal nutrient medium (Invitrogen, Carlsbad, CA). Cells were fed with this media every other day until the cultures were no longer viable for experimentation.

Electrophysiology

Electrophysiology experiments were performed on isolated amacrine cells after 6-14 days in culture. Culture dishes were mounted on an Olympus IX70 inverted microscope with Hoffman Modulation Contrast optics. Whole cell, voltage clamp recordings were made using an Axopatch 1-D amplifier, Digidata 1322A data acquisition board, and Clampex 9.2 software (Axon Instruments, Union City, CA). A reference Ag/AgCl pellet in 3M KCl was connected to culture dishes via an agar bridge containing 3M KCl. Patch electrodes were pulled from thick walled borosilicate glass using a Flaming/Brown Puller set to pull electrodes with tip resistance values from 3-8 MΩ. All recordings were made at room temperature.
For ruptured patch recordings, electrodes with tip resistance values of 5-8 MΩ were used. Ruptured patch internal solutions were supplemented with an ATP regeneration system containing: 50U/ml creatine phosphokinase, 1mM ATP disodium, 3mM ATP dipotassium, 20mM creatine phosphate, and 2mM GTP disodium. For perforated patch recordings using gramicidin D, 10mg/ml stocks were prepared in 100% ethanol, stored in the refrigerator, and used that day. Gramicidin stocks were diluted to 10ug/ml in internal solution. Data recorded from cells that did not reach a stable series resistance value after attaining a 1 GΩ seal were discarded. Recordings with high series resistance values (>100 MΩ) were also discarded.

**Solutions**

Cells were continuously perfused with external solution during recordings. External recording solution contained NaCl (116.7mM), KCl (5.3mM), TEA-Cl (20mM), CaCl₂ (3mM), MgCl₂ (410mM), HEPES (10mM), and glucose (5.6mM). For experiments in which external Na⁺, K⁺, Ca²⁺, and Cl⁻ were removed, chloride and cations were replaced either with NMG-MeSO₄ or sucrose and the osmolarity of the solutions corrected to ~275mosm. For both NMG-MeSO₄ and sucrose externals, HEPES (10mM and 5mM, respectively) was also included. For solutions containing 25mM or 50mM NH₄Cl, NH₄Cl was substituted for NaCl. All external solutions were corrected and maintained at a pH of 7.4. Rapid solution changes were achieved using a tri-barrel square glass assembly attached to a SF-77B Perfusion Fast Step that could be controlled manually or by the computer software. When controlled manually, this apparatus achieved solution changes in 500ms. For experiments requiring brief pulses of GABA, the software switched solutions in as little as 10ms. Where appropriate,
300nM TTX (Alamone) and/or 50uM LaCl$_3$ was included in external solutions to block voltage-gated sodium and calcium channels, respectively.

For ruptured patch recordings, the solution in the recording pipet contained cesium acetate (100mM), CsCl (10mM), CaCl$_2$ (0.1mM), MgCl$_2$ (2mM), HEPES (10mM), and EGTA (1.1mM) and an ATP regeneration system. For 125mM HEPES internal solution, the recording pipet contained HEPES (125mM), CsCl (10mM), CaCl$_2$ (0.1mM), MgCl$_2$ (2mM), and EGTA (1.1mM) and pH and osmolarity were brought up to 7.4 and 210-230mosm respectively with CsOH. For perforated patch recordings, the ATP regeneration system was replaced with 20mM cesium acetate.

**NO Solutions**

NO-bubbled solutions were prepared by initially bubbling purified water with pure argon for 15 minutes and then bubbling with pure NO that has been passed through a column of soda lime to filter out nitric dioxide for 15 minutes. These NO solutions were then tightly sealed, protected from light, and stored at 4°C until use. NO solution was injected directly into the perfusion line with a 50uL glass Hamilton syringe.

Previous measurements with injections of dye-containing solutions indicated that the delay between NO injection and exposure of cells to NO is roughly 2-3 seconds, that exposure is limited to about 2-5 seconds, and that most of the NO reaches the cells in the first 500ms, as a result of the brief half-life of NO in oxygenated solution (400ms-1s).

All reagents, unless specified differently, were obtained from Sigma-Aldrich (St. Louis, MO). Proton movement and chloride channel blockers were obtained from BioMol (Plymouth Meeting, PA), CIC-3, CIC-5, CIC-6 antibodies from Alpha Diagnostics...
International (San Antonio, Texas), CIC-4 antibodies from Abgent (San Diego, CA), and CIC-7 antibodies from Abcam (Cambridge, MA).

**pH Imaging**

Changes in pH were monitored using 5-(and-6)-carboxy SNARF®-1, acetoxymethyl ester, acetate (Invitrogen). Cells were loaded with dye (2uM) for one hour, washed several times with Hanks Balanced Salt solution (Invitrogen), and placed in an open recording chamber. A Leica TCS-SP2 spectral confocal microscope with a 63X oil immersion lens was used to visualize changes in SNARF-1AM fluorescence. Fluorescence intensity over time was measured at two bands of emission wavelengths: 570-600nm and 630-660nm, using a Leica confocal microscope and excitation with the 543nm laser line. An increase in fluorescence at the former and a decrease in the latter set of wavelengths was consistent with cytosolic acidification whereas an increase at 630-660nm and a decrease at 570-600nm was consistent with cytosolic alkalinization. Cells were kept under constant perfusion with normal external solution between drug applications. Fluorescence intensity data were subsequently analyzed using Origin 7.5 software (OriginLab, Northhampton, MA). Data are reported as the ratio of fluorescence intensity at 570-600nm to fluorescence intensity at 630-660nm.

**Western Blotting**

Western blot analyses were conducted following the method of Crousillac et al 2003. Briefly, material for Western blot analyses was obtained from Sprague-Dawley rats (Laboratory Animal Medicine, LSU) sacrificed by decapitation and White Leghorn chickens (Poultry Sciences, LSU) sacrificed by CO₂ exposure followed by decapitation. Whole-cell lysates of chicken and rat brains and chicken retinae were homogenized in
IP buffer (1% Triton X-100, 150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA, 0.5% NP40) with a protease inhibitor cocktail (Roche, Indianapolis, IN). Protein content was then quantified using the BioRad (Hercules, CA) protein assay kit.

Protein preparations were denatured on a 7.5% SDS-PAGE gradient gel. Following electrophoresis, proteins were transferred to nitrocellulose membranes. The membranes were then incubated overnight at 4°C with blocking buffer (1% bovine serum albumin, 0.1% Tween 20, and 2% nonfat dry milk, in Tris-buffered saline). Membranes were placed in primary antibody (all diluted to 1:500) at room temperature for 1h and then incubated with a goat anti-rabbit IgG peroxidase conjugate secondary antibody (1:5000) for 1h at room temperature. The ECL Western Blotting Detection Reagent Kit (Amersham, Piscataway, NJ) was used for visualization of the antibodies.

**Immunohistochemistry**

Cultured retinal amacrine cells grown on glass coverslips were fixed at embryonic equivalent (EE) days 14-15 for 30 minutes in 2% paraformaldehyde. Fixed material was blocked in 5% normal goat serum in dilution solution (1% BSA, 0.1% saponin in PBS) for 30 minutes before incubation with primary antibody (all diluted to 1:250) for one hour at room temperature. Cells were then washed three times with PBS before application of fluorescently-labeled secondary antibody for one hour followed by another PBS wash cycle. Coverslips were mounted on microscope slides in mounting medium (70% glycerol, 28% PBS, 2% n-propyl gallate) before viewing (Sen et al 2006).

**PCR**

Reagents were purchased from Invitrogen unless otherwise indicated. A population of amacrine cells was harvested from cell cultures using glass suction electrodes with a tip.
resistance between 3-5 MΩ. Electrodes were filled with lysis buffer containing 5U/100µl of ScriptGuard™ RNAse inhibitor (Epicenter). The tip of the electrode was broken into 1.5mL RNAse-free microcentrifuge tubes containing 100µl of Lysis/Binding buffer (Dynabeads Oligo (dT)25 kit) with 5U of RNAse inhibitor. Tubes were then immediately placed on dry ice and stored at -80°C. The reaction mixture for PCR contained Reaction Mix, primers (Integrated DNA Technologies), SuperScript™III RT/Platinum®Taq Mix, ScriptGuard™ RNase Inhibitor (Epicenter). Two primers were designed against Gallus gallus ClC7 (Accession # NM_001030644). ClC7-L was TACCGTGTGGTGAAGGACAA and ClC7-R was GATCTTCACGCCATTGAGGT, with an expected PCR product size of 189bp. The [Mg2+] was optimized for each primer pair, and the PCR was carried out in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc.) programmed with the appropriate temperature transitions. PCR products were combined with BlueJuice™ gel loading buffer and run out on an agarose gel (100 V, 1 hr) along with a 100 bp DNA ladder.

**miRNA-mediated RNA Silencing**

Single-stranded DNA oligonucleotide sequences encoding pre-miRNAs specific for chicken ClC5 (Accession # XM_420265.2 and top strand: TGCTGTTTCAGAGATCAGCTCACTGGTGTTTTGGCCACTGACTGACACCAGTGATGATCTCTGAA, bottom strand: CCTGTTCAGAGATCATCACTGGTGTCAGTCAGTGGCCAAAACACCAGTGAGCTGATCTCTGAAC) were designed with the aid of the BLOCK-iT™ RNAi Designer (Invitrogen) and annealed into double stranded oligos. Double stranded oligos were ligated into the pcDNA™6.2-GW/EmGFP-miR™ expression vector (Invitrogen). One Shot Top10 chemically competent E. coli were transformed with the pre-miRNA
expression vector and grown overnight at 37°C. Plasmid DNA was purified using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA), and sequencing (Macrogen USA, Baltimore, MD) verified the correct insertion of the construct. Retinal cells were nucleofected with the pre-miRNA expression vector using the Amaxa Nucleofector II and the Chicken Neuron Kit (Amaxa Inc., Gaithersburg, MD) and then plated on 35mm culture dishes. Transfection was verified by analysis of GFP expression and knockdown of ClC5 protein was evaluated through immunocytochemistry.

**Results**

Previous work has shown that moderate concentrations of nitric oxide (NO, hundreds of nanomolar to low micromolar) induce a transient, neurotransmitter-independent, current (Hoffpauir et al 2006). To characterize this “nitric oxide-dependent current” (NDC) further, we performed whole cell, ruptured patch recordings of cultured retinal amacrine cells clamped at -70mV in the presence and absence of NO. NO resulted in a transient, inward current at -70mV that lasted for 1-3 seconds (Fig. 3.1A). NO-bubbled solutions are acidic and could initiate nonspecific effects due to transient exposure to low pH and low osmolarity conditions. To demonstrate that the NDC is not due to the low pH and osmolarity of the NO-bubbled solutions, amacrine cells were exposed to equi-volume pH 2.4, low osmolarity solutions. This treatment did not result in an inward current. Instead there was a small outward current with a longer time course (Fig. 3.1B), indicating that the NDC is indeed NO-specific. The reversal potential of this current was measured by applying a voltage ramp during activation of the NDC. After leak subtraction, the resulting current revealed that the NDC reversed positive to 0mV ($E_{rev} = 23.4 \pm 6.6$ mV, n = 5), indicating that the current is carried by cations (Fig.
3.1C). NO potentiation of acid-sensing ion channels (ASICs), which are nonspecific ion channels, has been demonstrated in cultured dorsal root ganglion cells (Cadiou et al.

**Figure 3.1: Nitric oxide induces a transient cation current.** A, Whole cell recording of a cell held at -70mV. NO produces an inward current, designated the "NO-dependent current", (NDC, scale bar: 50pA, 5s). B, Exposing the same cell to an equal volume of acidic solution does not activate an inward current. C, Leak-subtracted NO-dependent current recorded during a voltage ramp. The reversal potential of the current suggests that the current is carried by cations. D, Left, Whole cell recording of a cell clamped at -70mV. NO resulted in an inward current despite the absence of permeant external cations (Scale: 50pA, 5s). Right, In a different cell, removal of external Na⁺, K⁺, and Ca²⁺ during NO exposure did not abolish the NDC. (Scale: 100pA, 5s)
We determined that ASIC channels are not mediating the NDC because substitution of permeant cations with N-methyl glucamine (NMG) did not abolish the current (Fig. 3.1D Left). Furthermore, the NDC persisted when external Na\(^+\), K\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) were replaced with sucrose (Fig. 3.1D Right). This lack of sensitivity to removal of external Na\(^+\), K\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) suggests that this current is somehow related to proton flux. Previous work has determined that the NDC is not due to the movement of Cl\(^-\) out of the cell because this current is not blocked when internal anions are replaced by methane-sulfonate, a bulky anion unlikely to pass through ion channels (Hoffpauir et al 2006).

To examine the involvement of protons in the nitric oxide dependent current, whole cell, voltage clamp recordings of the NDC were made in the presence of several pharmacological agents that affect the movement of protons (see Table 3.1). The protonophore, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), allows protons to flow across membranes down their electrochemical gradient. It has no effect on cytosolic pH in retinal amacrine cells (Sen et al 2007), but has been used to disrupt proton gradients across internal membranes. Treatment with FCCP (1\(\mu\)M) for one minute, a concentration and application time known to not cause ATP depletion in amacrine cells (Medler and Gleason 2002), induced an inward current in cultured amacrine cells held at a voltage of -70mV (Fig. 3.2A top). This current is due to activation of the electrogenic plasma membrane Na\(^+\)/Ca\(^{2+}\) exchanger in the FCCP-induced absence of mitochondrial Ca\(^{2+}\) uptake (Medler and Gleason 2002). In the same cell, though the presence of FCCP did not abolish the nitric oxide-dependent current,
Table 3.1: Experimental plan for examining the nitric oxide-dependent current

<table>
<thead>
<tr>
<th>Question</th>
<th>Treatment</th>
<th>Result</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is NDC due to NO-mediated activation of ASIC channels?</td>
<td>Replace external cations with NMG</td>
<td>NDC persists despite removal of external cations</td>
<td>No. NO does not activate ASIC channels.</td>
</tr>
<tr>
<td>Is NDC related to proton flux?</td>
<td>Replace external ions with sucrose</td>
<td>Multiple NDC “spikes”</td>
<td>Yes. Intact proton gradients required for monophasic NDC.</td>
</tr>
<tr>
<td></td>
<td>Dissipate proton gradients with FCCP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Block proton extrusion across plasma membrane with amiloride</td>
<td>Slower recovery of NDC</td>
<td>Yes. Na⁺/H⁺ exchanger involved in turning off NDC</td>
</tr>
<tr>
<td></td>
<td>Block V-type proton ATPase-mediated transport of protons into internal compartments with bafilomycin A1</td>
<td>Slower recovery of NDC</td>
<td>Yes. V-type proton ATPase involved in turning off NDC</td>
</tr>
<tr>
<td>Does NO activate cytosolic acidification?</td>
<td>pH imaging with SNARF-1AM</td>
<td>NO induces cytosolic acidification</td>
<td>Yes. NDC is related to proton flux</td>
</tr>
</tbody>
</table>

NO induced multiple NDC current “spikes” (asterisks, Fig. 3.2A bottom), indicating that the current initiated by NO is sensitive to the disruption of intracellular pH gradients.

Plasma membrane Na⁺/H⁺ exchangers remove cytosolic acid loads by moving protons across membranes against their electrochemical gradient. Amiloride is a specific inhibitor of Na⁺/H⁺ exchangers. Six-minute treatment with 600µM amiloride did not result in the development of a current in amacrine cells held at -70mV (not shown). NO delivered in the presence of amiloride induced an inward NDC (Fig. 3.2B). An initial, fast component resembled the NDC under control conditions, but the presence of a
Figure 3.2: Proton transport inhibitors alter the time course of the nitric oxide-dependent current. **A,** top, Exposure of a voltage-clamped amacrine cell to FCCP (1µM) induced an inward current at -70mV. **Bottom,** in the same cell, NO resulted in a multiphasic current super-imposed on the FCCP-dependent current. When FCCP was removed, NO resulted in a typical NDC (Scale bars: 100pA, 50s). **B,** NO application in the presence of amiloride (600µM) resulted in a biphasic current (Scale bars: 25pA, 5s). **Inset,** Same record showing full time course of recovery (Scale bars: 25pA, 20s). **C,** Whole cell, voltage clamp recording of a cultured amacrine cell pre-treated with 1µM bafilomycin A1 for two hours. At -70mV, NO induced a biphasic NDC with an initial, fast component and a secondary, slower component, (Scale bars: 25pA, 5s).
slowly recovering secondary component suggests that amiloride treatment had interfered with the machinery necessary to turn off the current (Fig. 3.2B, n=3).

The acidification of internal compartments is accomplished through the activity of the vacuolar (v-type) proton ATPase. V-type ATPases have also been found to operate on the plasma membrane in several types of neurons and glia (Jouhou et al 2007; Pappas and Ransom 1993). In both cases, these pumps work to actively transport protons against their concentration gradient. Cells were pre-treated with the specific vacuolar proton ATPase inhibitor, bafilomycin A1 (1µM, 2hr, Bowman et al 1988, Fig. 3.2C). As with amiloride, the initial, fast component was similar to control NDCs, but a secondary, slow component was visible in all cells tested (n=5), indicating the involvement of proton pumps in the NDC recovery machinery. Bafilomycin treatment alone did not result in the development of a current (data not shown). The preceding data support the involvement of protons in the nitric oxide dependent current. If inhibiting cellular machinery that removes protons from the cytosol prolongs the NDC, is it possible that this current is correlated with a NO-mediated change in proton flux? To test this idea, NO responses were observed in cultured amacrine cells loaded with the ratiometric pH indicator dye, SNARF1-AM. SNARF1-AM fluorescence emission increases at wavelengths centered on 590nm and decreases at wavelengths centered around 640nm in the presence of an acid load. When proton concentrations decrease, emission at 640nm increases and 590nm emission decreases (Buckler and Vaughn-Jones 1990). The 640nm/590nm emission ratio provides cell-volume and dye-fading independent information on pH changes in the cytosol. In Figure 3.3A, application of
NO induced a transient decrease in the ratio of the fluorescence intensities of the alkaline sensitive and acidic sensitive emission wavelengths of SNARF1-AM, consistent with a NO-mediated cytosolic acidification. Equal volume, low pH solutions did not change cytosolic pH, indicating that the NO-induced acidification was a NO-specific effect (Fig. 3.3A). Treatment with 25mM NH₄Cl resulted in a cellular alkalinization as basic NH₃ crossed the plasma membrane leaving charged NH₄⁺ and protons behind. When NH₄Cl was removed from the bath, NH₃ in the cytosol diffused out of the cell, resulting in a cytosolic acidification (Fig. 3.3B, Boron and de Weer 1974). Amiloride (300µM) exposure also acidified amacrine cells to a similar level as NO (Fig. 3.3C). These data demonstrate that SNARF1-AM is an accurate and effective measure of cytosolic pH changes and that NH₄Cl and amiloride can be used to reliably manipulate cytosolic pH.

Figure 3.3: Nitric oxide application transiently decreases cytosolic pH. A, Data from an amacrine cell loaded with the ratiometric pH indicator dye, SNARF1-AM. The normalized ratio of SNARF1-AM fluorescence intensity at 640nm and at 590nm is shown. An NO-induced decrease in the 640/590 ratio indicates that NO application transiently acidified the cell. B, Data from a different SNARF1-AM-loaded amacrine cell. Exposure to 25mM NH₄Cl resulted in cytosolic alkalinization. When the NH₄Cl was removed, the cytosol was acidified. C, In the same cell, amiloride (300µM) reversibly decreased cytosolic pH.
We have previously shown that NO transiently increases cytosolic chloride levels in retinal amacrine cells and that the source of this increased chloride is intracellular (Hoffpauir et al 2006). The movement of chloride and protons across internal membranes is highly coupled. Are NO-induced acidification and the NO-dependent increase in cytosolic chloride functionally linked? To address this question, we first asked whether changing cellular pH would alter cytosolic chloride concentration as revealed by measurements of the reversal potential of GABA-gated currents. Our cultured retinal amacrine cells express GABA_A receptors; therefore, GABA-gated currents are carried by Cl\(^{-}\) (Hoffpauir et al, 2006). In the ruptured patch configuration, currents recorded during voltage ramps applied to cultured amacrine cells demonstrate that under control conditions, the \(E_{Cl^-}\) is very stable for several minutes (mean \(E_{Cl^-}\) shift = 0.83+/-1.47mV, Fig. 3.4A and D). In the same cell, two minute applications of the alkalinizing agent NH\(_4\)Cl (see Fig. 3.3B) shifted the \(E_{Cl^-}\) to the left, indicating that a basic cellular environment resulted in a decrease in internal Cl\(^{-}\) (mean \(E_{Cl^-}\) shift = -1.83+/-1.70mV, Fig. 3.4B and D). In contrast to this result, 300\(\mu\)M amiloride, which acidifies cytosolic pH (Fig. 3.3C), shifted the \(E_{Cl^-}\) to the right, indicating an increase in cytosolic chloride under acidic cytosolic conditions ((mean \(E_{Cl^-}\) shift = 6.10+/-0.83, Fig. 3.4C and D). The decrease in the slope of the current was due to a direct inhibition of GABA_A receptors by amiloride (Fig. 3.4C, Fisher 2002).

To confirm that altering the pH of the cytosol is sufficient for changing cytosolic Cl\(^{-}\) concentration, we repeated the experiments in Fig. 3.4 in the gramicidin perforated-patch recording configuration where the physiological cytosolic buffering conditions and chloride concentration of the clamped cell are preserved. As in the ruptured patch
Figure 3.4: Changing cytosolic pH changes cytosolic chloride concentration. A, Leak-subtracted GABA currents recorded at the beginning (black) and end (red) of a 2 min. series of voltage ramps in normal external solution. B, In the same cell, GABA currents recorded at resting pH (black) and after alkalinization with NH₄Cl (red) show that a rise in pH induced a negative shift in the E₇Cl⁻. C, Amiloride (300µM) shifted the E₇Cl⁻ to a more positive value. D, Mean E₇Cl⁻ shifts in the presence of NH₄Cl and amiloride are significantly different from variations in the resting ECl⁻ (NH₄Cl: p=.0274; amiloride: p=.0001; n=6).

recordings, the E₇Cl⁻ was very stable over the same time frame of the pH manipulations under control conditions (Mean shift: -1.46+/−1.21mV; Fig. 3.5A and D). Cytosolic alkalization (50mM NH₄Cl, 6 minutes) decreased internal [Cl⁻] while cytosolic acidification (600µM amiloride, 6 minutes) increased internal [Cl⁻] (Mean shift in NH₄Cl: -6.68+/−4.03mV; Mean shift in amiloride: 6.30+/−1.85mV; Fig. 3.5B-D). Gramicidin perforated-patch recordings of NH₄Cl and amiloride mediated E₇Cl⁻ shifts using the same concentration and treatment time of these agents as the ruptured patch experiments showed similar results (data not shown). These data further support the finding that
changing the pH of retinal amacrine cells is sufficient for altering cytosolic chloride levels. Is it necessary?

**Figure 3.5: Under intact cytosolic conditions, altering cellular pH changes intracellular chloride concentration.**  
A, Leak-subtracted GABA gated currents recorded during voltage ramps under control conditions (black trace) and six minutes later (grey trace).  
B, In the same cell, GABA-gated current before (black trace) and after six minutes of treatment with NH$_4$Cl (50mM, grey trace).  
C, Six minute treatment with 600µM amiloride shifted E$_{Cl^-}$ in the positive direction.  
D, Mean pH-induced shifts in the E$_{Cl^-}$ are statistically significant (NH$_4$Cl: p=.0006; amiloride: p<.00001, n=5).

To address this question, we compared E$_{Cl^-}$ measurements from amacrine cells with a typical low concentration of HEPES buffer (10mM) in the recording pipet to those from cells with a high concentration of HEPES (125mM) in the internal recording solution. Whole cell, leak-subtracted GABA-gated currents recorded during voltage ramps under both conditions before and after NO application revealed that a high level of pH buffering limits the ability of NO to shift the E$_{Cl^-}$ to a more positive value (Control E$_{Cl^-}$ shift = 31.53 +/- 9.50mV; 125mM HEPES shift = 13.86 +/- 9.50mV; Fig. 3.6). This
suggests that a NO-induced acidification is not only sufficient, but also necessary to drive a NO-mediated increase in cytosolic Cl\(^-\) levels.

**Figure 3.6: Buffering internal pH with HEPES inhibits NO-induced internal chloride release.**

A, NO shifts the E\(_{\text{Cl}^-}\) in an amacrine cell in the positive direction under control conditions. B, Whole cell, voltage clamp recording of GABA currents before (black trace) and after (red trace) NO effects in a cell containing a high (125mM) concentration of HEPES buffer. The NO-induced E\(_{\text{Cl}^-}\) shift is decreased with respect to control (A). C, Statistical comparison between NO-induced E\(_{\text{Cl}^-}\) shifts in control (10mM HEPES) and highly buffered (125mM HEPES) retinal amacrine cells demonstrates that cytosolic buffering inhibits the NO-dependent release of Cl\(^-\) by (n=10 buffered, 10 control, p=.00059)

If a pH change consistent with a cytosolic acidification is necessary to permit a NO-mediated increase in intracellular chloride, then we would expect that altering internal pH would affect NO-induced shifts in the E\(_{\text{Cl}^-}\). To explore this idea, E\(_{\text{Cl}^-}\) measurements were made in cultured amacrine cells that had been either alkalinized by NH\(_4\)Cl or acidified by treatment with amiloride. Ruptured-patch recordings of GABA-gated currents before and after application of NO under basic cytosolic conditions demonstrate a significant decrease in the NO-induced E\(_{\text{Cl}^-}\) shift (Mean E\(_{\text{Cl}^-}\) shift = 12.71mV\(\pm\)8.71mV, Control shift = 36.38mV\(\pm\)9.61mV, Fig. 3.7B and D). Cytosolic acidification did not significantly alter NO-induced shifts in the E\(_{\text{Cl}^-}\), though there was a non-significant trend towards an increase in shift amplitude in the presence of amiloride (Mean E\(_{\text{Cl}^-}\) shift = 44.63mV\(\pm\)4.47mV, Fig. 3.7C and D). These results are consistent
Figure 3.7: NO-induced shifts in $E_{Cl}$- are altered by manipulating cytosolic pH. A-C, Leak-subtracted GABA-gated currents recorded during voltage ramps. A, NO shifts the $E_{Cl}$ +40mV under control conditions. B, Alkalinization with NH4Cl decreases the amplitude of the NO-induced $E_{Cl}$-shift. C, In the same cell, the NO-induced shift in the $E_{Cl}$- in amiloride is larger (50mV) than the control. D, Mean NO-induced $E_{Cl}$- shifts recorded from 6 cells demonstrate that NH4Cl significantly decreases the amplitude of NO responses ($p=.0085$, $n=6$) and that there is a non-significant trend for larger amplitude NO responses in amiloride ($p=.1148$, $n=6$).

with a causal link between decreases in pH, increases in $[Cl^-]$, and the mechanism underlying NO-mediated changes in postsynaptic excitability in retinal amacrine cells.

To examine the involvement of intracellular chloride transport mechanisms in NO-mediated internal Cl$^-$ release, we performed whole-cell, voltage clamp recordings before and after NO treatment in the presence of three different Cl$^-$ transporter and channel blockers. The presence of DIDS (100µM), NPPB (50µM) or niflumic acid (300µM) in the recording pipet did not abolish the NO-induced shift in the $E_{Cl}$- (Fig. 3.8).
Figure 3.8: Cl\(^-\) channel blockers do not inhibit the NO-dependent redistribution of Cl\(^-\). Data are shown from 4 different amacrine cells. A, GABA-gated currents are measured during voltage ramps in an amacrine cell before (black trace) and after (grey trace) NO exposure. B, In a different cell, inclusion of DIDS (100 µM) in the recording pipet did not abolish the NO-induced shift in the E_{Cl^-}. Similarly, the E_{Cl^-} shift persisted in the presence of NPPB (C, 50 µM) and niflumic acid (D, 300 µM) in the patch pipet. The effects of NO (gray traces) were measured 5 minutes after rupturing the patch.

Chloride is transported into acidified internal compartments mainly via members of the CLC family of chloride channels and transporters to minimize the electrical gradient across internal membranes and to allow the proton pump to function optimally (Jentsch 2007). As CLC transporters are known to be resistant to inhibitors of all other chloride transport blockers, including those used in Figure 3.8, these proteins might be good candidates for effectors of NO-induced internal chloride release. The charge mitigating activity of these chloride transporters is the subject of much active research, but the possibility of these proteins working in reverse to release Cl\(^-\) into the cytosol is completely unexplored.

Are intracellular CLC transporters involved in the NO-induced release of Cl\(^-\) from internal compartments? To begin to explore this possibility, I first examined the expression patterns of internally located members of the CLC family of Cl\(^-\) channels and transporters in cultured retinal amacrine cells. Antibodies specific for ClC3-7 were obtained and their labeling patterns were examined (Fig 3.9-Fig 3.13).
Figure 3.9: ClC3 is expressed in cultured retinal amacrine cells. A, Paraformaldehyde-fixed cultured amacrine cells labeled with an antibody specific for ClC3. Labeling was observed in both cell bodies and, more discretely, in cell processes, (Scale bar = 15µm). B, Western blot of chicken retina (CR) and rat brain (RB) protein (175µg/lane). ClC3 antibody is localized to a band at 160kD, which probably represents binding to ClC3 dimers.

Figure 3.10: ClC4 is expressed in cultured retinal amacrine cells. A, Anti-ClC4 labeling in amacrine cells. Antibody signal was mainly confined to regions of the cell body and was very diffuse in cell processes, (Scale bar = 15µm). B, ClC4 antibody labeling of a band between 75kD and 105kD in a blot of chicken brain (CB) protein (100µg/lane) is consistent with labeling of ClC4 protein.

The specificity of antibodies for ClC3-6 in avian tissue was confirmed through Western blot analysis (Fig 3.9-3.12). ClC5 appeared to be the most strongly expressed member of the CLC family in cultured amacrine cells. Antibody labeling was observed in both the cell body and in processes. Labeling in the processes was punctate and was
particularly strong in growth cones (Fig. 3.11A). ClC3 and ClC6 were also highly expressed and displayed a similar, punctate labeling pattern but without strong labeling

Figure 3.11: ClC5 is expressed in cultured retinal amacrine cells. A, ClC5 antibody labeling is robust in cell bodies and in cell processes. Labeling in the processes is punctate and seems elevated in growth cones (arrow), (Scale bar = 15\(\mu\)m). B, Western blot of CR and RB protein. Antibodies for ClC5 localized at a band at 105kD in chicken retina and at a band between 75kD and 105kD in rat brain consistent with the size of the ClC5 protein in chicken and rat, respectively (50\(\mu\)g/lane CR, 200\(\mu\)g/lane RB).

in growth cones (Fig. 3.9A and 3.12A). ClC4 expression appeared much weaker in comparison to other CLC proteins and was primarily located in the cell body, though some diffuse antibody labeling in the processes was observed (Fig 3.10A). Though anti-CIC7 displayed moderate labeling in the cell body and proximal processes (Fig 3.13A), we were not able to resolve a band at an appropriate molecular weight for CIC7 in chicken tissue. However, PCR amplification of chicken CIC7 confirmed the presence of CIC7 transcripts in cultured amacrine cells (Fig. 3.13B). These results support the presence of a diverse mixture of CLC transporters in amacrine cells. Because examination of the physiological role of CLC transporters in the processes investigated by the current study is confounded by the absence of specific pharmacological inhibitors for these proteins as well as by their internal cellular location, a complete investigation
Figure 3.12: ClC6 is expressed in cultured retinal amacrine cells. A, ClC6 specific antibody labeling in two amacrine cells. Antibody signal was observed in both cell bodies and processes, but at a lower level than ClC5 labeling, (Scale bar = 15µm). B, The ClC6 antibody detects protein close to the 160kD marker in protein from chicken and rat brain, which is near to the appropriate molecular weight for ClC6 dimers (100µg/lane).

Figure 3.13: ClC7 is expressed in cultured retinal amacrine cells. A, Amacrine cell labeled with a ClC7 specific antibody. Anti-ClC7 labeled the cell body as well as discrete areas of the proximal processes, (Scale bar = 15µm). B, ClC7 mRNA transcripts are amplified from cultured retinal amacrine cells. Actin amplification and RT\(^-\) represent positive and negative controls, respectively. (PCR data courtesy of Scott Crousillac).

of their involvement will require the use of other loss-of-function methods. To begin to address this issue, preliminary work using RNA silencing techniques has been done. Since ClC5 was found to be the most highly expressed CLC transporter in cultured amacrine cells, we chose this protein as our first target.
RNA silencing knocks down protein expression at the mRNA level and does not affect protein that has already been translated. Therefore, it was important first to determine the longevity of ClC5 protein in cultured amacrine cells. Figure 3.14 compares ClC5 expression in cells that have been treated with the protein synthesis inhibitor, cyclohexamide, for five days in culture with that of control cells. ClC5 expression in cyclohexamide-treated cells was almost completely abolished, indicating that knock-down of ClC5 protein is feasible in the time-frame that we maintain our cultures (Fig. 3.14B).

**Figure 3.14**: ClC5 expression in amacrine cells is markedly turned over after 7 days in culture. A, ClC5 expression in retinal amacrine cells after a week in culture. B, ClC5 expression after one week in amacrine cells that were treated with cyclohexamide (50µM) two days after plating. (Scale bars = 20µm).

Initial attempts to silence ClC5 expression using small, double-stranded RNA sequences specific for ClC5 mRNA proved unsuccessful. Despite a certain level of amplification from the activity of the RISC complex, siRNAs do not persist indefinitely in transfected cells (Chiu and Rana 2002). For this reason, along with the relatively long lifespan of ClC5 proteins in cultured amacrine cells, we have begun to focus our attention on vector-based delivery of primary miRNA (pri-miRNA) sequences that specifically target ClC5. Endogenous pri-miRNAs are processed by the enzyme,
Drosha, into smaller pre-miRNAs sequences. These sequences are then targeted by a ribonuclease called DICER and made into siRNAs (Kim 2005). Vectors expressing synthetic pri-miRNAs have been demonstrated to be more successful and efficient at knocking down target mRNAs than synthetic siRNAs and shRNAs (Boden et al 2004, Siolas et al 2005). Vector-based delivery of pri-miRNA constructs also afford the additional benefit of allowing investigators to co-express fluorescent reporters to confirm successful transfection and track transfection efficiency. Though this work is preliminary, we have had some success expressing pri-miRNA constructs in our culture system. Figure 3.15 shows EmGFP expression in cultured amacrine cells transfected with a vector expressing EmGFP and a pri-miRNA specific for ClC5. Sequencing confirmed correct insertion of the pri-miRNA construct into this EmGFP vector.

Discussion

These results demonstrate that nitric oxide induces a transient cytosolic acidification in cultured retinal amacrine cells, and that this acidification is not only sufficient, but also necessary to increase intracellular chloride concentrations. The NO-mediated acidification co-occurs with a membrane current that does not require influx of Na\(^+\), K\(^+\), or Ca\(^{2+}\) or efflux of Cl\(^-\) ions. Intracellularly-located members of the CLC family of chloride transporters and channels are expressed in amacrine cells, at varying levels and locations depending on transporter identity. The most robustly expressed CLC transporter protein, ClC5, is expressed as early as embryonic equivalent day 10.
Figure 3.15: A vector expressing emGFP and a primary miRNA targeting CIC5 can be successfully introduced into cultured retinal amacrine cells. A, Phase contrast image of a paraformaldehyde-fixed amacrine cell in culture. Cells were nucleofected with a vector designed to express emGFP and a primary miRNA targeting CIC5. (Scale bar = 20µm). B, CIC5 and GFP expression are colocalized in the cell in A. C, CIC5 antibody labeling in the same cell displayed a similar pattern as CIC5 expression in nontransfected cells (Fig. 3.11). D, GFP expression in the same amacrine cell confirmed successful transfection of cell with emGFP miRNA vector.

Fig. 3.16 depicts a model summarizing our findings. Under resting conditions or low levels of NO production, the cell in Fig. 3.16A contains low levels of cytosolic Cl⁻ and a near neutral pH. In Fig. 3.16B, the same cell is stimulated to increase internal Ca²⁺ concentrations leading to stimulation of nNOS and NO production. NO induces proton movement into the cytosol either across the plasma membrane or out of organelles or both. The resulting acidification allows for the transport of Cl⁻ out of internal compartments, shifting the $E_{Cl^{-}}$ to a more positive value. A previous study from our lab showed that NO is capable of transiently changing inhibitory GABAergic synapses into excitatory synapses (Hoffpauir et al 2006, see Chapter 2). Our findings
from the current study suggest that this synaptic sign change is dependent on altered chloride and proton gradients across internal membranes.

**Figure 3.16: Working model of NO-induced, pH-dependent internal Cl⁻ release.** A, Simplified drawing of a cell with one endosomal compartment. Under resting conditions, the cytosol displays a near-neutral pH and a low [Cl⁻]. B, In the presence of NO, the cytosol becomes more acidic, inducing the release of Cl⁻ from the compartment.

**NO and Acidification**

Though this is the first study to connect NO with changes in both pH and cytosolic Cl⁻, NO-induced intracellular acidification is not unprecedented in the literature. In cultured hippocampal neurons, NO initiates programmed cell death (PCD). Brief NO exposure induces a cytosolic acidification in these cells which in turn activates the acid-sensitive endonucleases responsible for mediating PCD (Vincent et al 1999). NO has also been demonstrated to be involved in mediating changes in neuronal excitability. In the rat cerebellum, spreading depression and intracellular acidification is enhanced by NO donors and diminished by NOS inhibitors (Chen et al 2001). NO-induced acidification is not limited to neuronal cells. NO acidifies cardiac myocytes by blocking Na⁺/H⁺ exchangers via a cGMP-dependent mechanism (Ito et al 1997). Intracellular pH changes modulated and/or mediated by NO are therefore implicated in a diverse number of cellular processes and second messenger pathways.
Protons and Chloride

Chloride has been determined to be the counter-ion responsible for dissipating electrical gradients across internal membranes caused by active transport of protons into internal compartments (Faundez and Hartzell 2004). This coupling of proton and chloride movement across internal membranes has been revealed by investigators measuring luminal pH in CLC knockouts in which Cl transport into internal compartments is eliminated (Stowbrawa et al 2001, Gunther et al 2003). More directly, the finding that CLC transporters, the proteins responsible for electrically shunting internal proton gradients, are actually electrogenic H/Cl exchangers demonstrates pH-Cl coupling at the molecular level (Piccolo and Pusch, Accardi and Miller, Scheel et al 2005). These transporters are therefore able to constantly monitor ambient luminal pH levels and move Cl across the membrane accordingly (Jentsch 2007). To date, there is no evidence of chloride transport machinery on internal membranes being activated to work in reverse. The current study provides no direct evidence of a connection between CLC-mediated Cl transport and NO-mediated internal Cl release. However, the pH dependency of both processes and the experimental elimination of other potential candidates encourage further investigation into their possible relationship.

For proton-sensitive cellular machinery to increase internal Cl\(^-\) to levels high enough to produce the effects observed in this and the previous study, elevated [Cl\(^-\)] must be sensed by GABA\(_A\) receptors at synaptic sites. The physiological relevance of internal Ca\(^{2+}\) stores is well established. Locally high concentrations of Ca\(^{2+}\) in retinal amacrine cells have been demonstrated near mitochondria (Sen et al 2007), indicating that ion microdomains relevant to signaling exist in these cells. It is reasonable to
predict that any ion highly concentrated in and actively transported into internal compartments might participate in cytosolic signaling, especially in cell types, such as neurons, that have exceptionally limited free cytosolic space. Signaling in amacrine cells occurs mainly in cell processes, which have a much smaller diameter (1-2 µm) than the soma. In addition, organelles such as the nucleus, mitochondria, and acidic compartments such as endosomes, lysosomes, and synaptic vesicles account for a large proportion of total amacrine cell volume. It is likely that local elevations of chloride relevant to signaling could be produced in this environment. For example, synaptic vesicles make up more than half of the volume of amacrine cell presynaptic boutons (Cao and Eldred 2001). On average, synaptic vesicles contain ~45mM Cl⁻ (Faundez and Hartzell 2004). To produce $E_{\text{Cl}^-}$ shifts of the magnitude seen in this study would require an intracellular Cl elevation of ~15mM. Not only would it be theoretically possible for synaptic vesicles to release enough Cl⁻ to mediate such changes, but due to the local nature of amacrine cell signaling, it is conceivable that local NO production could bring about changes at some synaptic sites and not others.

References


Chapter 4

Conclusions
I have presented evidence in chapter 2 that NO can transiently convert typically inhibitory synapses into excitatory synapses and that this synaptic conversion is activated by an increase in intracellular chloride concentration. I have further demonstrated that this increase in cytosolic chloride levels is due to a NO-mediated release of chloride from cytosolic compartments and not from chloride entry across the plasma membrane. In chapter 3, I’ve shown that altering cytosolic pH is both sufficient and necessary for changing cytosolic chloride levels.

Inhibition and excitation are not solely determined by neurotransmitter and neurotransmitter receptor phenotype. The distribution of chloride across the plasma membrane is also an important determinant of synaptic sign. One well-known example of this is the developmental shift from GABAergic excitation to inhibition in many CNS neurons (Ben-Ari et al 2004). Early in development, plasma membrane NKCC cation-chloride cotransporters move chloride into the cytosol, shifting the $E_{Cl^-}$ in the positive direction and making GABAergic inputs excitatory. In mature neurons, KCC cation-chloride cotransporters are preferentially expressed. KCC transporters move chloride out of the cytosol, making the $E_{Cl^-}$ more negative and rendering GABAergic inputs inhibitory. The developmental shift in cytosolic chloride levels reminds us that excitability is determined not only by membrane channels and transporters, but also by ionic gradients. My work supports a new angle to this concept—that rapid changes in plasma membrane conductance can be brought about not only by changes in ionic permeability, but also by fast alterations in cytosolic ion concentrations. Though rapid changes in cytosolic ion concentration have been observed under pathological activation of plasma membrane ion channels (Choi et al 1987; Staley et al 1995), my
work is the first to demonstrate a change in neuronal excitability due to transport of ions across internal membranes. The results presented in this dissertation also represent the first example of the involvement of NO in this sort of process.

Though the work presented here was conducted entirely in cultures of dissociated neurons that were exposed to exogenous NO, the potential relevance of these novel mechanisms to signaling in the inner retina must be emphasized. Concentrations of NO in the high nanomolar to low micromolar range have been observed in retinal tissue (Donati et al 1995, Heiduschka and Thanos 1998, Groppe et al 2003) and high levels of NO production are predicted to exist in subsets of retinal amacrine cells (Blute et al 2005). We used similar concentrations of NO in the experiments described in this dissertation (see Chapter 2). My results demonstrate that such levels of NO can transiently convert inhibitory postsynaptic inputs into excitatory inputs. GABAergic amacrine cells are involved in multiple horizontal signaling pathways in the inner retina. If subsets of amacrine cells can be stimulated to produce NO in sufficient quantity to invert amacrine cell synaptic sign, the impact of NO on retinal output could be extensive and complex. Indeed, in the ferret retina, application of the NO precursor, L-arginine, diminished light responses in retinal ganglion cells (Wang et al 2003), a finding consistent with an increased level of presynaptic inhibition, possibly via amacrine cells. A later study by the same group examined retinal ganglion cell light responses in nNOS knockout mice. To the authors’ surprise, nNOS knockout mice responded similarly to wild type when exposed to optimal light stimuli. However, the light sensitivity of retinal ganglion cells was significantly diminished in nNOS knockouts (Wang et al 2007). The presence of endothelial nitric oxide synthase (eNOS) in the
mammalian retina as well as nNOS might account for this finding (Wang et al 2007). Together, these results suggest that NO modulates retinal visual processing at multiple levels. Future work is necessary for elucidating what role NO-induced, amacrine cell synaptic sign inversion may play in determining retinal output. In light of this, we plan to begin examining the effect of endogenous NO production in amacrine cells on cytosolic chloride levels in retinal slices.

In Chapter 2, I demonstrated a NO-mediated release of Cl$^-$ from internal compartments in cultured rat hippocampal neurons. Though I did not investigate synaptic signaling or pH changes in these cells, the finding that NO releases cytosolic Cl$^-$ from internal stores in the mammalian brain is provocative. Is the necessary machinery for NO-induced synaptic sign inversion ubiquitous? What if the activation of this machinery depends solely on the availability of sufficient levels of NO production and/or cytosolic acidification? I have not yet established a physiological link between NO-mediated internal Cl$^-$ release and activation of CLC transporters. However, the expression of these transporters in amacrine cells and the pH sensitivity of the NO-dependent changes explored in this dissertation merit further investigation. It is interesting to note that CLC transporters and other necessary components of vesicular acidification machinery are present in most CNS cell types in which their distribution has been examined. Intriguingly, genetic disruption of the genes expressing CIC3 and CIC7 were found to result in the degeneration of the retina and of the hippocampus (Stowbrawa et al 2001; Kaspar et al 2005).

There is evidence of high intracellular chloride levels leading to excitatory GABA responses in CNS neurons (Ben-Ari et al 2007) as well as support for NO-modulated,
acidification-induced changes in neuronal excitability (Chen et al. 2001) however, our work is the first to uncover a connection between these two phenomena. If this potentially ubiquitous pathway can mediate such striking synaptic modifications, why have no other investigators come across it? One explanation is that most studies of NO effects in neuronal cells employ spontaneous NO donors instead of NO-bubbled solutions. In Chapter 2, we determined that NO donors, in the concentrations most commonly used, produce 10-100 fold smaller concentrations of NO than NO-bubbled solutions. We also demonstrated that these two levels of NO mediated different effects in retinal amacrine cells, and that higher concentrations of NO donors mimicked the effects of NO-bubbled solutions. It is possible that previous investigations have simulated the effects of NO that has diffused some distance from the cells in which it was produced whereas our methods replicate events in the NO-producing cell. Interestingly, our findings support opposing NO concentration-dependent effects in retinal amacrine cells, with low concentrations of NO enhancing inhibition of the cell (by increasing conductance through GABA_A receptors) and high concentrations of NO increasing excitation (by increasing [Cl^-]). Another common practice in NO studies is to use analogues and antagonists of cGMP to probe NO signaling. We have found that the NO-mediated processes described in this dissertation are cGMP-independent. Finally, investigations of GABAergic excitation in central neurons have focused on internal Cl^- elevations induced by changes in the activity of plasma membrane KCC and/or NKCC chloride transporters. Often, authors rely solely on changes in KCC and NKCC expression at the mRNA and protein level to confirm involvement of these transporters in mediating GABAergic excitation without using pharmacological blockers.
and/or ion substitution to explore whether these proteins are truly physiologically involved. We were not able to demonstrate a physiological connection between plasma membrane chloride-cation cotransporters and NO-induced elevation of internal Cl⁻ (Hoffpauir et al 2006).

Much more work must be done before we can form a clear picture of the effectors and physiological relevance of pH-dependent, NO-induced internal Cl⁻ release in central neurons. It is difficult to speculate on which specific signaling and synaptic pathways may be modulated by either physiological or pathological NO production at this level. However, if the necessary components for the NO synaptic switch are found to be truly widespread in the central nervous system, then the eventual relevance of NO for research purposes could be quite vast. NO, at high enough concentrations, could be used to target specific inhibitory interneurons and briefly render them excitatory, a potentially less-invasive means for studying synaptic networks. Many human nervous system disorders, such as epilepsy (Ben-Ari et al 2007), have their basis in altered excitability of GABAergic interneurons. It would be interesting to examine the involvement of NO in experimental models of these diseases.

References


Heiduschka P and Thanos S. NO production during neuronal cell death can be directly assessed by a chemical reaction in vivo. 1999. Neuroreport 9: 4051–4057.


Appendix 1:

Letter of Authorization to Reprint
May 19, 2008

Ms. Emily McMain
Department of Biological Sciences
Louisiana State University
Life Sciences Building Room 107
Baton Rouge, LA 70803

Dear Ms. McMain:

The American Physiological Society grants you permission to use the following Journal of Neurophysiology article in your doctoral dissertation for Louisiana State University:

Brian Hoffpaur, Emily McMains, and Evanna Gleason
Nitric Oxide Transiently Converts Synaptic Inhibition to Excitation in Retinal Amacrine Cells

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Appendix 2:

Chapter 2 Appendix
In the preceding study, we demonstrated that higher concentrations of NO increases intracellular Cl\(^-\) levels in cultured amacrine cells by releasing Cl\(^-\) from an internal compartment. We also discussed evidence from the literature describing nNOS expression and NO production in amacrine cells in the retina. Because we conducted all of our experiments on cells in culture, it is also important to address whether nNOS is expressed and NO produced in cultured amacrine cells.

To address this issue, we performed immunocytochemical and NO imaging experiments. Labeling with an antibody specific for nNOS (Crousillac et al 2003) demonstrated nNOS expression in the cell bodies and processes of nearly all cultured amacrine cells (Fig. A.1A). Cultured cells loaded with DAF-FM diacetate, a fluorescent cell permeant dye sensitive to changes in NO levels, revealed an increase in fluorescence intensity following both exposure to NMDA and to exogenously-applied NO (Fig. A.1B). This result indicates that cultured amacrine cells possess all of the cellular machinery necessary for NO production and therefore provide a valuable model system for determining the sorts of signals that generate NO in these inner retina neurons.

The preceding work demonstrated that NO enhances GABA\(_A\) receptor currents through two different mechanisms. Moderate concentrations of NO (250\(\mu\)M NO donor \(\sim\)100nM NO) enhanced GABA-gated currents without changing the \(E_{\text{Cl}^-}\) and higher concentrations of NO (NO-bubbled solutions \(\sim\)high nanomolar to low micromolar) enhanced GABA-gated currents to a much greater extent by inducing a positive shift in the \(E_{\text{Cl}^-}\). These findings led us to propose that the effects of NO are concentration-dependent. If this were true, we should be able to use NO donors to activate a shift in
Figure A.1: Cultured amacrine cells express nNOS and can be stimulated to produce NO. A, An amacrine cell labeled with a polyclonal antibody raised against a C-terminal peptide of rat nNOS. Scale bar is 20µm. B, Data from an amacrine cell loaded with the NO indicator DAF-FM diacetate. NMDA causes an increase in DAF-FM fluorescence consistent with the NMDA-induced activation of nNOS and production of NO. As expected, addition of NO produces an increase in DAF-FM fluorescence.

the $E_{Cl^-}$ as well, as long as concentration of NO released by the donors is in the high nanomolar to low micromolar range. The amount of NO released by NO donors is directly related to the concentration and half-life of the donor solution. To test whether high concentrations of donor solutions can induce a shift in the $E_{Cl^-}$, we performed the experiment shown in Figure A.2. Application of NOC12 (2mM) within 30 minutes of placing it in solution resulted in a positive shift in the $E_{Cl^-}$, demonstrating that the shift is independent of the source of NO, requiring only that the concentration is high enough.

In Chapter 2, we demonstrated an increase in cytosolic chloride after exposure to NO. We measured this $Cl^-$ elevation by observing changes in the $E_{Cl^-}$ before and after NO application. To determine whether the source of this extra chloride was extracellular or intracellular, we measured $E_{Cl^-}$ changes after NO application in external recording solutions that did not contain $Cl^-$. Because $E_{Cl^-}$ measurements only report whole-cell
Figure A.2: NOC12 induces a positive shift in the $E_{Cl^-}$. Leak-subtracted GABA current recorded during voltage ramp before (black trace) and after (grey trace) NOC12 (2mM) exposure. In the presence of NO donor, the $E_{Cl^-}$ is shifted in the positive direction.

changes in internal Cl$^-$ concentrations, we explored another method, Cl$^-$ imaging, to examine spatially-specific changes in [Cl$^-$]$_i$ in response to NO. Here, we confirm our electrophysiological results and demonstrate the feasibility of chloride imaging in cultured retinal amacrine cells. The Cl$^-$ indicator dye, MQAE, was first employed to track global changes in chloride concentration in amacrine cells. NO application induced a transient, NO-specific increase in cytosolic [Cl$^-$] (Fig. A.3A and B). Removal of extracellular Cl$^-$ did not abolish this NO-induced Cl$^-$ increase (Fig A.3C).

Figure A.3: Nitric oxide increased internal chloride in the absence of external chloride. A, The fluorescence intensity of an MQAE-loaded amacrine cell transiently decreased after NO application, indicating an increase in cytosolic [Cl$^-$]. B, Application of acidic, NO-free solutions did not alter cytosolic [Cl$^-$]. C, In the same cell, removal of Cl$^-$ from the bath solution did not prevent the NO-induced elevation of cytosolic Cl$^-$. 
Vita

Emily Ann McMains was born to Thyre and Jay McMains in Baton Rouge, Louisiana in October, 1978. She received her primary education in Baton Rouge and then attended high school at the Hotchkiss School in Lakeville, Connecticut, graduating in June 1996. She enrolled at Brown University in Providence, Rhode Island, in 1996 and graduated with a Bachelor of Arts in biology in May 2000. She began work towards her doctorate in August 2003 under the guidance of Dr. Evanna Gleason and will complete all the requirements for the Doctor of Philosophy degree in December 2008.