2017

A Presynaptic Role for Nitric Oxide at a GABAergic Synapse

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A PRESYNAPTIC ROLE FOR NITRIC OXIDE AT A GABAERGIC SYNAPSE

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

John Wesley Maddox
B.S., Louisiana Tech University, 2007
M.S., Louisiana Tech University, 2010
August 2017
I dedicate this work
to my parents, William and Barbara Maddox.
Without their compassion, love, and support,
this would have been impossible.
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## TABLE OF CONTENTS

**ACKNOWLEDGEMENTS** ........................................................................................................ iii

**LIST OF FIGURES** ................................................................................................................ vii

**LIST OF ABBREVIATIONS** .................................................................................................. viii

**ABSTRACT** .......................................................................................................................... xi

**CHAPTER 1: INTRODUCTION** ............................................................................................ 1

- Retinal amacrine cells ........................................................................................................... 2
- Retinal amacrine cell signaling ............................................................................................. 4
- Nitric oxide in the retina ......................................................................................................... 5
- Regulation of nNOS and eNOS ............................................................................................. 7
- Nitric oxide targets ................................................................................................................ 8
- References ............................................................................................................................ 9

**CHAPTER 2: NITRIC OXIDE PROMOTES GABA RELEASE BY ACTIVATING A VOLTAGE-INDEPENDENT CA\(^{2+}\) INFLUX PATHWAY IN RETINAL AMACRINE CELLS** .................................................................................................................. 15

- Introduction ......................................................................................................................... 15
- Methods ................................................................................................................................. 17
  - Amacrine cell cultures ....................................................................................................... 17
  - Electrophysiology .............................................................................................................. 18
  - Solutions .......................................................................................................................... 19
  - Reagents ........................................................................................................................... 20
  - Calcium imaging ............................................................................................................... 21
  - Data analysis ..................................................................................................................... 21
- Results .................................................................................................................................. 23
  - SNAP increases sPSC frequency ....................................................................................... 23
  - NO-dependent increase of sPSC frequency does not require sGC activity ................. 23
  - NO-increases GABAergic sPSC frequency ..................................................................... 25
  - NO-dependent increase of sPSC frequency is not action potential-dependent ............ 26
  - The role of Ca\(^{2+}\) .......................................................................................................... 29
  - NO increases evoked GABAergic autaptic transmission ............................................. 33
  - The effect of NO on sPSCs does not involve L-type VGCCs, AMPARs or NMDARs .... 34
  - Clemizole blocks the NO-dependent increase in sPSC frequency and intracellular Ca\(^{2+}\) .................................................................................................................. 36
- Discussion ............................................................................................................................. 39
  - VGCC-independent GABA release .................................................................................. 40
  - NO and GABA release* ................................................................................................. 41
  - Role of spontaneous GABA release ............................................................................... 42
TRPCs in ACs ............................................................................................................. 42
References ..................................................................................................................... 44

CHAPTER 3: TRPC5 IS REQUIRED FOR THE NO-DEPENDENT INCREASE IN
DENDRITIC CA$^{2+}$ AND GABA RELEASE FROM RETINAL AMACRINE
CELLS ............................................................................................................................. 50
Introduction ..................................................................................................................... 50
Methods ............................................................................................................................ 52
Plasmid construction ........................................................................................................ 52
Amacrine cell cultures and transfections ......................................................................... 53
Solutions ........................................................................................................................... 54
Reagents .......................................................................................................................... 55
Electrophysiology ............................................................................................................. 55
Calcium imaging ............................................................................................................... 56
Immunocytochemistry ....................................................................................................... 56
Immunohistochemistry ..................................................................................................... 57
Western blot ....................................................................................................................... 58
TRPC5 gene mutation analysis ......................................................................................... 59
Data analysis ....................................................................................................................... 60
Results ............................................................................................................................... 61
PLC activity is required for the NO-dependent GABA release from retinal
ACs ................................................................................................................................. 61
The NO-dependent increase in sPSC frequency is sensitive to La$^{3+}$ .................................. 62
ML204 can block the NO-dependent increase in cytosolic Ca$^{2+}$ .................................... 64
TRPC5 is expressed in ACs in culture and retina ................................................................. 67
CRISPR/Cas9-mediated dsDNA break of the TRPC5 gene reduces
TRPC5 expression .............................................................................................................. 67
TRPC5 is required for the NO-dependent increase in cytosolic Ca$^{2+}$
and GABA release ........................................................................................................... 69
Discussion ......................................................................................................................... 71
TRPC5 in the retina and brain ......................................................................................... 72
Identification of NO-dependent activation of TRPC5 ..................................................... 74
Effects of NO on amacrine cells ...................................................................................... 75
Multiple effects of NO on amacrine cell synapses .......................................................... 76
References ......................................................................................................................... 78

CHAPTER 4: CONCLUSIONS ......................................................................................... 82
References ......................................................................................................................... 86

APPENDIX: LETTER OF PERMISSION ......................................................................... 87

VITA .................................................................................................................................. 88
LIST OF FIGURES

1.1 Schematic of cellular and synaptic organization in the vertebrate retina...........2
1.2 Enzymatic NO production.................................................................................6
2.1 NO increases frequency of spontaneous postsynaptic currents (sPSCs).......24
2.2 NO-dependent increase of sPSCs does not require soluble guanylate cyclase (sGC) activity .........................................................................................................................25
2.3 NO-induced increase in GABAergic sPSC frequency does not require action potentials .................................................................................................................................27
2.4 NO increases intracellular Ca\textsuperscript{2+} .................................................................................................................................28
2.5 The NO-dependent increase of sPSC frequency is dependent upon cytosolic Ca\textsuperscript{2+} elevations ..............................................................................................................30
2.6 NO-dependent increase of intracellular Ca\textsuperscript{2+} is independent of intracellular stores ........................................................................................................................................32
2.7 NO-dependent increase of sPSC frequency is independent of Ca\textsuperscript{2+} stores.......33
2.8 NO increases evoked autaptic GABAergic postsynaptic currents..................35
2.9 L-type voltage-gated Ca\textsuperscript{2+} channels (VGCCs), AMPARs or NMDARs are not required for the NO-dependent Ca\textsuperscript{2+} influx.................................................................................................37
2.10 Clemizole blocks the NO-dependent increase in sPSC frequency and intracellular Ca\textsuperscript{2+} .......................................................................................................................................39
3.1 PLC activity is required for the NO-dependent increase in sPSC frequency.....63
3.2 The NO-dependent increase in sPSC frequency is sensitive to La\textsuperscript{3+} ...........64
3.3 The TRPC4/C5 inhibitor ML204 can block the NO-dependent increase in cytosolic Ca\textsuperscript{2+} and sPSC frequency at higher concentrations .......................................................66
3.4 Amacrine cells in culture and adult retina express TRPC5 ............................68
3.5 CRISPR/Cas9-mediated TRPC5 mutagenesis reduces TRPC5 expression in amacrine cell processes .............................................................................................................................70
3.6 Knockdown of TRPC5 eliminates the NO-dependent increase in cytosolic Ca\textsuperscript{2+} and sPSC frequency ..................................................................................................................72
3.7 NO has pre- and postsynaptic effects in ACs....................................................77
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>2-APB</td>
<td>2-Aminoethoxydiphenylborane</td>
</tr>
<tr>
<td>AC</td>
<td>amacrine cell</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAPON</td>
<td>carboxy-terminal PDZ ligand of nNOS</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR associated protein 9</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>clemizole</td>
<td>1-[(4-Chlorophenyl)methyl]-2-(1-pyrrolidinylmethyl)-1H-benzimidazole hydrochloride</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-Cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CPTIO</td>
<td>2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3- oxide</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>D-AP5</td>
<td>D-(−)-2-Amino-5-phosphono-pentanoic acid</td>
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<td>DAPI</td>
<td>2-(4-amidinophenyl)-1H-indole-6-carboxamidine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma amino butyric acid</td>
</tr>
<tr>
<td>GCL</td>
<td>ganglion cell layer</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>hDNA</td>
<td>heteroduplexed DNA</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>inositol trisphosphate</td>
</tr>
<tr>
<td>IPL</td>
<td>inner plexiform layer</td>
</tr>
<tr>
<td>KD</td>
<td>knock down</td>
</tr>
<tr>
<td>mGluR5</td>
<td>metabotropic glutamate receptor 5</td>
</tr>
<tr>
<td>ML204</td>
<td>4-Methyl-2-(1-piperidinyl)quinoline</td>
</tr>
<tr>
<td>nifedipine</td>
<td>1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOSIP</td>
<td>nitric oxide synthase interacting protein</td>
</tr>
<tr>
<td>NOSTRIN</td>
<td>eNOS traffic inducer</td>
</tr>
<tr>
<td>ODQ</td>
<td>1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one</td>
</tr>
<tr>
<td>OGB</td>
<td>Oregon Green 488 BAPTA-1, AM</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ONL</td>
<td>outer nuclear layer</td>
</tr>
<tr>
<td>OPL</td>
<td>outer plexiform layer</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylate cyclase</td>
</tr>
<tr>
<td>SKF 96365</td>
<td>1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole hydrochloride</td>
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<tr>
<td>SNAP</td>
<td>S-nitroso-N-acetylpenicillamine</td>
</tr>
<tr>
<td>sPSC</td>
<td>spontaneous postsynaptic current</td>
</tr>
<tr>
<td>T7E1</td>
<td>T7 endonuclease I</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>tdTomato</td>
<td>tandem dimer of dTomato fluorescent protein</td>
</tr>
<tr>
<td>TEA-Cl</td>
<td>tetraethylammonium chloride</td>
</tr>
<tr>
<td>TG</td>
<td>thapsigargin</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TRPC</td>
<td>transient receptor potential canonical</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage-gated calcium channel</td>
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</table>
ABSTRACT

Amacrine cells are a class of retinal interneurons that process the visual signal in the inner retina. Several subtypes of amacrine cells express nitric oxide synthase and produce nitric oxide (NO), making NO a possible regulator of amacrine cell function. My dissertation research tests the hypothesis that NO alters amacrine cell GABAergic synaptic output. To investigate this, I made whole-cell voltage clamp recordings of cultured chick amacrine cells receiving synaptic input from other amacrine cells and Ca$^{2+}$ imaging of amacrine cell dendrites, which can be presynaptic. I find that NO-dependent increases in GABAergic spontaneous postsynaptic current (sPSC) frequency are independent of soluble guanylate cyclase and action potentials. Removal of extracellular Ca$^{2+}$ and buffering of cytosolic Ca$^{2+}$ both inhibit the response to NO. In Ca$^{2+}$ imaging experiments, I confirm that NO increases dendritic Ca$^{2+}$ by activating a Ca$^{2+}$ influx pathway. Neither NO-dependent dendritic Ca$^{2+}$ elevation nor increase in sPSC frequency are dependent upon Ca$^{2+}$ release from stores. NO also enhances evoked GABAergic responses, and because voltage-gated Ca$^{2+}$ channel function is not altered by NO, the enhanced evoked release is likely due to the combination of voltage-dependent Ca$^{2+}$ influx and the voltage-independent, NO-dependent Ca$^{2+}$ influx. Insight into the identity of the Ca$^{2+}$ channel involved in the NO response was provided by characteristics unique to the transient receptor potential canonical (TRPC) channel subunits 4 and 5: the NO-dependent increase in sPSC frequency was dependent on downstream activity of PLC, blocked by 2 mM La$^{3+}$ and enhanced by 10 µM La$^{3+}$. The TRPC inhibitor ML204, which preferentially blocks TRPC4, had no effect on the NO response at 10 µM, but 20 µM ML204 blocked the NO response. The TRPC inhibitor
clemizole, which preferentially blocks TRPC5, blocked NO-dependent dendritic Ca\(^{2+}\) elevations and the increase in sPSC frequency. Genetic knockdown of TRPC5 in cultured amacrine cells using the CRISPR/Cas9 system confirms that TRPC5 mediates NO-dependent dendritic Ca\(^{2+}\) elevations and the increase in sPSC frequency. These results suggest that NO-dependent activation of TRPC5 at amacrine cell presynaptic sites will enhance vesicular GABA release and increase inhibition onto postsynaptic cells.
CHAPTER 1
INTRODUCTION

The vertebrate retina is the photosensory organ responsible for the detection of light in vision (See Figure 1.1). On the input side of the retina, also called the outer nuclear layer (ONL), visual signals from the environment are initiated by the patterns of photons (for example, the different photon patterns of the text you are reading here) interacting with photoreceptors, which are the cells responsible for converting the arrival of photons into neural signals. On the output side of the retina, ganglion cells in the ganglion cell layer (GCL) project their axons to the brain via the optic nerve. Ultimately, the visual signals are sent to the visual cortex in the brain for further processing and interpretation. Before being sent to the brain, the visual signal is processed within the retina itself. There are 3 classes of interneurons (bipolar cells, horizontal cells and amacrine cells) between the photoreceptors and ganglion cells that communicate with each other to process the visual signal. The cell bodies of all interneurons lie in the inner nuclear layer (INL). Bipolar cells complete the vertical pathway of the visual signal between photoreceptor and ganglion cells by receiving synaptic input from photoreceptors in the outer plexiform layer (OPL) and sending synaptic output to ganglion cells in the inner plexiform layer (IPL). Horizontal cells interact with photoreceptors, bipolar cells and other horizontal cells in the OPL to adjust the receptive fields of bipolar cells. Amacrine cells (ACs) have synaptic interactions in the IPL with bipolar cells, ganglion cells, and other ACs. The most common synaptic partner of ACs, however, are other ACs (Marc and Liu 2000), and these are the synapses of interest in my dissertation.
Figure 1.1. Schematic of cellular and synaptic organization in the vertebrate retina. Light enters the eye and transverses through all layers of the retina. Cell bodies of rod photoreceptors (R) and cone photoreceptors (C) lie in the outer nuclear layer (ONL) and convert the arrival of photons into a neural signal. The cell bodies of bipolar cells (B), horizontal cells (H) and amacrine cells (A) lie in the inner nuclear layer (INL). Cell bodies of ganglion cells and displaced amacrine cells lie in the ganglion cell layer (GCL). Synaptic interactions between photoreceptors, bipolar cells and horizontal cells occur in the outer plexiform layer outer plexiform layer (OPL). Synaptic interactions between bipolar cells, amacrine cells and ganglion cells occur in the inner plexiform later (IPL). Ganglion cell axons form the optic nerve that projects to the brain.

Retinal amacrine cells

Retinal ACs are the cells of interest in our lab because they form majority of synapses in the IPL (Marc and Liu 2000). Therefore, ACs must have an important role in shaping retinal signals. There are ~40 morphologically different types of ACs in the vertebrate retina. Different AC morphologies suggest different functions, and understanding their contribution is essential to understanding how visual information is conveyed and shaped in the inner retina. AC somas are located within the INL, as well as some that are displaced to the GCL, and send their processes into the IPL where
they form reciprocal, serial, and/or lateral synapses with bipolar cells, ganglion cells, and other ACs. Even though the functions of majority of ACs remain unknown, the functions of some subtypes have been identified.

Dopaminergic ACs are wide-field (extend their dendrites laterally > 500 µm) and synthesize dopamine, a neurotransmitter which works in a paracrine manner in the retina. Under scotopic (low) light, horizontal cells are electrically coupled via gap junctions, which increases sensitivity to light but decreases visual resolution. As photopic (bright) light range is reached, dopaminergic ACs release dopamine, which in turn acts on the dopamine receptor 1 (DR1) on horizontal cells (Nguyen-Legros et al. 1999). The DR1 is a G-protein coupled receptor that activates adenylyl cyclase, which subsequently activates a cAMP-sensitive protein kinase (Lasater 1987). This pathway leads to horizontal cell uncoupling, which enhances visual resolution by reducing the receptive field of bipolar cells (Shen et al. 2003). The light-dependent release of dopamine also leads to All AC uncoupling (see below).

All (A2) ACs (Vaney et al. 1991) are narrow-field (extend their dendrites vertically), glycinergic cells that serve to integrate signals arising from the rod photoreceptor pathway into the cone photoreceptor pathway (Demb and Singer 2012). The rod pathway is thought to have evolved after the cone pathway because rod bipolar cells “piggyback” onto the cone pathway by synapsing onto All ACs, which in turn synapese onto OFF-cone bipolar cells and form gap junctions with ON-cone bipolar cells (Demb and Singer 2012; Strettoi et al. 1992). All ACs also form gap junctions with other All ACs. This electrical coupling functions to amplify the rod pathway by increasing the receptive field beyond the dendritic field of All ACs (Mills and Massey 1995).
Starburst amacrine cells (SBACs) are direction-selective, wide-field ACs. SBACs are both GABAergic and cholinergic and function in direction selectivity by differentially inhibiting direction-selective ganglion cells (Taylor and Smith 2012). SBACs are the only retinal cell type known to release acetylcholine.

These three types of ACs play an important role in shaping the visual signal that ganglion cells send to visual regions of the brain. The functions just among these three types of ACs are very diverse, suggesting that the theme of diverse functions for ACs is likely to persist as we learn more.

**Retinal amacrine cell signaling**

Neurons are generally considered to be highly polarized cells that receive synaptic input from other neurons at their dendrites, transform the synaptic input into action potentials that propagate down their axon, and then generate synaptic output from the axonal synaptic terminal (e.g. motor neurons). Many types of ACs, however, do not have formal axons. Instead, ACs form complex microcircuits within dual function dendrites: synaptic input is received and synaptic output can be generated within the same dendrite (Chavez et al. 2010; Grimes et al. 2010; Grimes et al. 2015; Tsukamoto et al. 2001). AC microcircuits formed by these mixed-use dendrites allow for highly-localized synaptic signaling in dendrites, which can also have localized Ca\(^{2+}\) elevations (Euler et al. 2002). AC dendritic Ca\(^{2+}\) elevations are initiated by presynaptic bipolar cell glutamate release. Activation of AC glutamate receptors alone is sufficient to trigger GABA release at localized reciprocal synapses (Chavez and Diamond 2008; Chavez et al. 2006; Grimes et al. 2015). At more lateral AC synapses, action potentials are involved in GABA release (Chavez et al. 2010; Cook and McReynolds 1998; Cook and
Werblin 1994; Shields and Lukasiewicz 2003). Voltage-gated L-type Ca\(^{2+}\) channels are localized at narrow-field AII AC output synapses (Habermann et al. 2003) and mediate neurotransmitter release in wide-field GABAergic ACs (Vigh and Lasater 2004).

AC intracellular Ca\(^{2+}\) stores can also play a role in synaptic signaling by enhancing evoked and spontaneous release of GABA through Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) via IP\(_3\) receptors (IP\(_3\)Rs) (Warrier et al. 2005) and/or ryanodine receptors (RyRs) (Chavez et al. 2006). One study, recording from cultured ACs that express both IP\(_3\)Rs and RyRs, demonstrated that CICR through IP\(_3\)Rs, not RyRs, enhances GABA release (Warrier et al. 2005). However, another study recording from A17 ACs in rat retinal slices demonstrated that CICR through RyRs, not IP\(_3\)Rs, enhanced depolarization-independent, but glutamate-dependent GABA release (Chavez et al. 2006). It is unclear, however, if IP\(_3\)-R and RyR-pathways are involved in Ca\(^{2+}\) signaling in the same AC or if each pathway is specific to certain AC subtypes (Chavez et al. 2006; Warrier et al. 2005). Any modulation in localized AC Ca\(^{2+}\) signaling at presynaptic sites will alter their synaptic output, which is important for proper processing of the visual signal.

**Nitric oxide in the retina**

Nitric oxide (NO) is a gaseous messenger molecule synthesized by the enzyme nitric oxide synthase (NOS). The substrate for NOS is L-arginine, and the products are L-citrulline and NO (Figure 1.2). Two calcium/calmodulin (Ca\(^{2+}\)/CaM)-dependent isoforms of NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS), and one calcium-independent isoform, inducible NOS (iNOS), have all been shown to be expressed with variable degree in the vertebrae retina (Donovan et al. 2001; Knowles
The isoform nNOS is constitutively expressed in subsets of all cell types in the vertebrate retina, and recently, the isoform eNOS has been shown to be expressed in horizontal, amacrine and Müller cells in the chicken retina (Tekmen-Clark and Gleason 2013). There are at least 15 known types of nNOS-positive, NO-producing neurons in the chicken inner retina, 9 of which are ACs (Cellerino et al. 2000; Fischer and Stell 1999; Wilson et al. 2011).

**Figure 1.2. Enzymatic NO production.** Using L-arginine, the energy from NADPH, and oxygen, nitric oxide synthase synthesizes L-citrulline and NO. NO can then activate its canonical receptor soluble guanylate cyclase (sGC) and increase cGMP production. NO can also alter protein function by binding to a sulfhydryl group on cysteine residues.

In the intact mouse retina, NO synthesis in the IPL is stimulated by both steady and flickering light (Blom et al. 2012). Activation of voltage-gated calcium channels (VGCCS) or Ca^{2+} permeable NMDA receptors (NMDARs) in ACs that express nNOS/eNOS will cause a transient rise of intracellular Ca^{2+}, which can activate NOS and lead to synthesis of NO (Pang et al. 2010). Light-dependent production of NO also leads to S-nitrosylation (see below) of proteins throughout the retina, which could alter protein function (Tooker and Vigh 2015). The increase of NO production via light stimulation suggests that NO has a role in processing the visual signal. A few functions
for NO have been determined including modulation of dopamine release (Sekaran et al. 2005), uncoupling of horizontal cells (Daniels and Baldridge 2011; DeVries and Schwartz 1989; Lu and McMahon 1997; Pottek et al. 1997), blocking All-cone bipolar coupling (Mills and Massey 1995; Xia and Mills 2004), and modulation of light responses (Kurenny et al. 1994; Levy et al. 2004; Sato et al. 2011). However, the full range of NO’s effect on retinal signaling remains unclear.

**Regulation of nNOS and eNOS**

Although nNOS/eNOS activation is dependent on $\text{Ca}^{2+}/\text{CaM}$ association, additional mechanisms regulate nNOS/eNOS activity and subcellular localization. Post-translational modifications such as phosphorylation, acylation and S-nitrosylation alter the function of nNOS/eNOS. Depending on the site, phosphorylation of nNOS/eNOS can either increase or decrease activity (Dimmeler et al. 1999; Michel et al. 1997; Rameau et al. 2003). Acylation (myristoylation and palmitoylation) attaches eNOS to the plasmalemmal caveolae membrane (Garcia-Cardena et al. 1996; Shaul 2002). Depalmitoylation of eNOS is due to prolonged exposure to its substrate, L-arginine, and is potentiated by $\text{Ca}^{2+}/\text{CaM}$ (Yeh et al. 1999). Depalmitoylation results in translocation of eNOS to other intracellular regions; one possible mechanism by which eNOS activity is regulated (Feron and Balligand 2006; Shaul 2002; Yeh et al. 1999). S-nitrosylation, as described below, can act as a negative feedback mechanism by inhibiting the function of NOS.

Protein-protein interactions can also regulate NOS activity. Association with the caveolae scaffolding protein caveolin reversibly inhibits eNOS (Michel et al. 1997). Neuronal NOS can be anchored to the post-synaptic density protein-95 (PSD-95), which
links nNOS to NMDA receptors (Eldred and Blute 2005; Sattler et al. 1999). This can lead to quick activation of nNOS and NO synthesis when NMDA receptors are activated. CAPON, an adaptor protein, can regulate nNOS interactions with Dexras1, a small G protein of the Ras family (Jaffrey et al. 1998). NOSTRIN (eNOS trafficking inducer protein) is an adaptor protein that regulates internalization of eNOS towards intracellular compartments (Icking et al. 2005; Zimmermann et al. 2002). NOSIP (nitric oxide synthase interacting protein) inhibits nNOS production of NO by modifying nNOS localization and activity (Schleicher et al. 2005). Regulation of NOS activity by subcellular localization adds another level of complexity to the diverse functions of NO.

**Nitric oxide targets**

There are several known targets of NO. The most studied target is soluble guanylate cyclase (sGC), also known as the canonical “NO receptor” (Arnold et al. 1977). Soluble guanylate cyclase activation produces cyclic guanosine monophosphate (cGMP) and occurs when NO binds to the heme group of sGC. Cyclic GMP is a secondary messenger molecule that activates a serine/threonine kinase known as protein kinase G (Poulos 2006).

Nitric oxide can also have direct effects on proteins. S-nitrosylation occurs when a NO group is covalently attached to a thiol group located on cysteine residues (Figure 1.2). Much like phosphorylation, S-nitrosylation can alter the function of a protein. Potential targets for S-nitrosylation include ryanodine receptors (Aracena-Parks et al. 2006), G-protein coupled receptors (Estrada et al. 1997), cyclic nucleotide-gated ion channels (Broillet 2000), potassium channels (Asada et al. 2009; Kawano et al. 2009; Nunez et al. 2006), and transient receptor potential channels (Shimizu et al. 2014;
Takahashi et al. 2012; Yoshida et al. 2006). In the retina, light-dependent NO production leads to S-nitrosylation of more than 300 identified proteins (Tooker and Vigh 2015).

Although work in the Gleason lab has been done on the postsynaptic effects of NO in ACs (Hoffpauir et al. 2006; Krishnan and Gleason 2015), the NO-synthesizing enzyme nNOS is located presynaptically at the electron microscopy level, and a presynaptic role for NO on AC neurotransmitter release had remained uninvestigated. Thus, I tested the hypothesis that NO can alter the release of GABA from ACs. The results presented here provide the first direct evidence that NO enhances spontaneous and evoked Ca\(^{2+}\)-dependent GABA release from ACs. In Chapter 2, I explore the mechanism underlying the NO-dependent enhancement of GABA release from ACs. My work in this chapter was published in the Journal of Neurophysiology in 2017. In Chapter 3, I demonstrate that ACs in culture and in the adult retina express TRPC5 a Ca\(^{2+}\)-permeable ion channel, and using a combination of pharmacology and a genetic knockdown strategy, I demonstrate that TRPC5 is required for the NO-dependent increase in dendritic Ca\(^{2+}\) and enhancement of GABA release.

References


Daniels BA, and Baldridge WH. The light-induced reduction of horizontal cell receptive field size in the goldfish retina involves nitric oxide. *Vis Neurosci* 28: 137-144, 2011.


CHAPTER 2
NITRIC OXIDE PROMOTES GABA RELEASE BY ACTIVATING A VOLTAGE-INDEPENDENT CA\textsuperscript{2+} INFLUX PATHWAY IN RETINAL AMACRINE CELLS*

Introduction

Amacrine cells (ACs) are retinal interneurons that shape the visual signal in the inner retina via activity of their primarily glycinergic and GABAergic synapses. Most ACs have mixed-use dendrites because they are sites of both synaptic input and synaptic output. ACs form complex microcircuits by making reciprocal, lateral and serial inhibitory synapses with bipolar cells, ganglion cells and other ACs (Chavez et al. 2010; Grimes et al. 2010; Grimes et al. 2015; Tsukamoto et al. 2001). These microcircuits are localized to AC dendrites, which can also have localized Ca\textsuperscript{2+} elevations (Euler et al. 2002).

Nitric oxide synthase (NOS) is a nitric oxide (NO) synthesizing enzyme that has two Ca\textsuperscript{2+}-sensitive isoforms: endothelial NOS (eNOS) and neuronal NOS (nNOS). Both enzymes have been localized to ACs as well as other retinal cell types (Fischer and Stell 1999; Haverkamp et al. 2000; Kim et al. 1999; Kim et al. 2000; Pang et al. 2010; Tekmen-Clark and Gleason 2013). Neuronal NOS has also been localized to AC presynaptic terminals in the inner turtle retina (Cao and Eldred 2001), suggesting that localized NO synthesis can affect neurotransmitter release.

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The major signaling mechanisms for NO are through the cGMP pathway via activation of the canonical NO receptor, soluble guanylate cyclase, and through the direct chemical modification of proteins via S-nitrosylation. The light-dependent operation of both pathways appears to be widespread in the retina (Blom et al. 2012; Tooker and Vigh 2015). Although NO is typically viewed as diffusible, NO imaging has demonstrated that NO is largely confined to cellular boundaries in multiple retina cell types including ACs (Eldred and Blute 2005; Tekmen-Clark and Gleason 2013). This implies that NO-dependent mechanisms can be localized to NO-producing cells and to their immediate synaptic partners.

The functions of NO in the retina are not fully known, but multiple sites of NO action have been identified. NO production is stimulated under photopic illumination and is known to be involved in light adaptation. For example, NO decouples horizontal cells (HCs) from other HCs (DeVries and Schwartz 1989; Miyachi et al. 1990) and decouples ON bipolar cells from A2 ACs (Mills and Massey 1995). NO can also affect the ON and OFF pathways. In retinal ganglion cells, NO reduces both ON and OFF light responses with the OFF responses being the most sensitive to NO (Wang et al. 2003). The mechanism underlying this inhibition of the pathway was not fully worked out, but the results pointed to an effect on neurons presynaptic to ganglion cells, most likely ACs. Thus, AC synaptic function may be regulated by NO.

To test the hypothesis that NO can alter synaptic output from ACs, we employed whole-cell current recordings and intracellular Ca\(^{2+}\) imaging in cultured chick ACs. We found that the NO donor SNAP increased both spontaneous and evoked GABAergic neurotransmission due to a NO-dependent increase of cytosolic Ca\(^{2+}\). We find that a
likely TRPC-mediated Ca\textsuperscript{2+} influx is required, however, Ca\textsuperscript{2+} release from stores is not required. These data suggest that NO activates a presynaptic Ca\textsuperscript{2+} influx pathway that enhances GABAergic output from AC synapses in the retina.

**Methods**

**Amacrine cell cultures**

The use of chick embryos to prepare retinal cultures was determined to be exempt by the LSU Institutional Animal Care and Use Committee. Eight-day white leghorn (*Gallus gallus*) chick embryo retinas were removed from the eyecup and dissected free of the retinal pigment epithelium. Retinas were initially dissociated in Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free Hank’s solution (Life Technologies, Grand Island, NY). The tissue was centrifuged (950 × g), and the supernatant replaced with 0.125% trypsin (Sigma-Aldrich, St. Louis, MO). The pellet was resuspended and incubated at 37 °C/5% CO\textsubscript{2} for 30 min. After incubation and centrifugation (1,900 × g), the supernatant was replaced with Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies) supplemented with 5% fetal bovine serum (Sigma-Aldrich), penicillin (100 U/mL), streptomycin (100 µg/mL) and glutamine (2 mM) (Life Technologies). Cells were plated at a density of 2.5 × 10\textsuperscript{5} cells/35 mm dish that were pretreated with 0.01% poly-DL-ornithine hydrobromide (Sigma Aldrich). Cultures were fed every other day after initial plating by replacing half of the media with Neurobasal medium supplemented with 1% B-27, penicillin (100 U/mL), streptomycin (100 µg/mL) and glutamine (2 mM) (Life Technologies). Experiments were performed on ACs that had been in culture for 8-13 days (embryonic equivalent days 16 to 21). Over this time in culture, AC-to-AC
GABAergic synapses are functional. ACs were identified on morphological criteria (Gleason et al. 1993).

**Electrophysiology**

Whole-cell voltage clamp recordings were performed using Axopatch 1D amplifier, Digidata 1322A data-acquisition board, and Clampex 10.0 (Molecular Devices, Sunnyvale, CA). Patch pipette electrodes with a tip resistance between 5-10 MΩ for ruptured-patch recordings or 3-5 MΩ for perforated-patch recordings were pulled from thick-walled borosilicate glass (1.5 mm O.D., 0.86 mm I.D.) using a P-97 Flaming/Brown Puller (Sutter Instruments, Novato, CA). A reference Ag/AgCl electrode pellet was placed in 3 M KCl and connected to the culture dish with a 3 M KCl agarose bridge. Recordings were performed at room temperature (22-24°C). A pressurized perfusion system (AutoMate Scientific, Berkeley, CA) was used to deliver external solutions at a flow rate of 1 mL/min. Most recordings were performed on postsynaptic ACs whose processes were in contact with unclamped presynaptic ACs and that had spontaneous postsynaptic currents (sPSCs). Variability of basal sPSC frequency depended on cell culture density. Denser cultures had higher basal sPSC frequency, possibly due to more synaptic sites. Cells that did not have basal sPSCs also did not respond to the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP; Enzo, Farmingdale, NY) and were excluded from the analysis. Autaptic recordings were made from ACs that were not in contact with other ACs to eliminate the possibility that input was coming from unclamped cells.
Solutions

All reagents were purchased from Sigma-Aldrich unless otherwise stated.

Tetraethylammonium (TEA)-Cl external solution (in mM): NaCl (117), KCl (5.3), TEA-Cl (20), CaCl$_2$ (3), MgCl$_2$ (0.41), glucose (5.6), HEPES (10), pH 7.4. LiCl (117) replaced NaCl in evoked autaptic experiments to block the Na$^+$/Ca$^{2+}$ exchanger current that can contaminate the autaptic currents at the end of the voltage step. Rupture-patch high Cl$^-$ internal solution (in mM): CsCl (110), MgCl$_2$ (2), CaCl$_2$ (0.1), EGTA (1), HEPES (10), NaCl$_2$ (1), ATP-2Na$^+$ (3), ATP-2K$^+$ (1), GTP (2), phosphocreatine (20), creatine phosphokinase (50 U/mL), pH 7.4. Perforated-patch high Cl$^-$ internal solution (in mM): CsCl (145), MgCl$_2$ (2), CaCl$_2$ (0.1), EGTA (1), HEPES (10), NaCl (1), pH 7.4, amphotericin B (200 μg/mL). Perforated-patch normal internal solution (in mM): Cs-Acetate (135), MgCl$_2$ (2), CaCl$_2$ (0.1), EGTA (1), HEPES (10), NaCl (1), pH 7.4, Amphotericin B (200 μg/mL). Typical reversal potential for Cl$^-$ ($E_{Cl^-}$) in cultured ACs is ~-80 mV to -60 mV, however, these high Cl$^-$ internal solutions increase the reversal potential of Cl$^-$ to 0 mV, increasing the driving force on Cl$^-$ and improving the resolution of quantal events. High internal Cl$^-$ also blocks the NO-dependent release of Cl$^-$ (Hoffpauir et al. 2006). While we have previously shown that the concentration of SNAP used here (500 μM) does not elicit Cl$^-$ release (Hoffpauir et al. 2006), this internal solution further ensures that the NO-dependent release of Cl$^-$ will not confound our results. For imaging experiments, the following external solutions were used: Normal external solution (in mM): NaCl (137), KCl (5.37), CaCl$_2$ (3), MgCl$_2$ (0.41), glucose (5.6), HEPES (3.02), pH 7.4. Zero Ca$^{2+}$ external solution (in mM): NaCl (141.5), KCl (5.37), MgCl$_2$ (0.41), glucose (5.6), HEPES (3.02), pH 7.4.
Reagents

The NO donor SNAP (500 μM; Enzo), the NO scavenger 2-[4-carboxyphenyl]-4,4,5,5-tetramethyllimidazoline-1-oxyl-3-oxide (CPTIO, 10 μM, Enzo), bicuculline (10 μM), and TTX (300 nM; Abcam, Cambridge, MA) were dissolved directly into the external solution. 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxyethyl ester) (BAPTA-AM, 10 μM, Life Technologies) was prepared as a 10 mM stock in DMSO. The stock was diluted into Hank’s Balanced Salt Solution with NaHCO₃ (HBSS), briefly vortexed and sonicated for 30 s. Cells were incubated with BAPTA-AM at 37 °C/5% CO₂ for 45 minutes prior to recording. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 2 μM; Tocris, Bristol, UK), 2-Aminoethoxydiphenylborane (2-APB, 20 μM; Tocris), 1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole hydrochloride (SKF 96365, 30 μM, Tocris), N-Ethylmaleimide (NEM, 300 μM; Sigma), 1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (nifedipine, 20 μM; Tocris), 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 50 μM), and 1-[(4-Chlorophenyl)methyl]-2-(1-pyrrolidinylmethyl)-1H-benzimidazole hydrochloride (clemizole, 10 μM; Tocris) were prepared as 1000X stocks in DMSO and diluted to 1X in external solution. D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, 10 μM; Tocris) was prepared as a 10 mM stock and diluted in external solution. The sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) pump inhibitor thapsigargin (TG; 2 μM; Enzo) was prepared as a 2 mM stock in DMSO and added directly to the culture media. Cells were incubated with TG at 37 °C/5% CO₂ for 1 hr. The external solution pH was readjusted to 7.4 after the addition of reagents.
Calcium imaging

The fluorescent Ca\(^{2+}\) indicator Oregon Green 488 BAPTA-1, AM (OGB, 2 µM, Life Technologies) was prepared as a 2mM stock in DMSO. OGB stock and Pluronic F-127 (2.5% w/v in DMSO, Life Technologies) were mixed in a 1:1 ratio and sonicated. 2 µL of the OGB/Pluronic mixture was diluted in 1 mL of Hank’s buffered salt solution (HBSS) then briefly vortexed and sonicated for 30 s. Cells were incubated with the HBSS/OGB/Pluronic mixture for 1 hr at room temperature (22-24°C). HBSS/OGB/Pluronic mixture was replaced with normal external solution. Images were taken every 500 ms (200 ms exposure), 3 s (400 ms exposure), or 6 s (200 ms exposure). To obtain data relevant to synaptic function, regions of interest (ROIs) were chosen at sites of contact between processes. Images were captured on an inverted Olympus IX70 microscope (Tokyo, Japan) fitted with a SensiCam QE (Cooke, Kelheim, Germany). Data were collected and analyzed using Slidebook software (Intelligent Imaging Innovations, Denver, CO). In all Ca\(^{2+}\) imaging experiments, the control fluorescence mean was calculated by using the average of the last 10 ROI time points before switching solutions, and the fluorescence means after switching solutions were calculated by using the 10 ROI time points from the middle of each solution application. The control and different solution fluorescence means were used to calculate percent change in fluorescence.

Data analysis

For sPSC electrophysiology experiments, Mini Analysis Program (Synaptosoft, Inc, Fort Lee, NJ) was used to detect and analyze sPSCs. Quantal event kinetics were analyzed using data from cells that were only exposed to SNAP. Multiquantal events
were removed from analysis by determining the mean and SD of the rise time of quantal events from control data where multiquantal events are extremely rare. Quanta with a rise time longer than 3.7 ms (mean rise time plus one SD) were excluded from the analysis. Mean amplitudes (pA), mean rise times (ms) and mean decay times (ms) were determined from each of the 9 recorded cells during control (254 total events) and application of SNAP (319 total events, 489 multiquantal events removed). Mean amplitude, mean rise time and mean decay time from each cell were used for statistical analysis. Charge transfer integration and Ca\(^{2+}\) current leak subtraction was performed using OriginPro (OriginLab, Northampton, MA). Charge transfer was calculated by integrating the area under the post-depolarization autaptic current waveform. The charge transfer integration (pA*s) was then calculated and reported as pC. After linear leak subtraction, activation voltage of voltage-gated Ca\(^{2+}\) channels was measured at the point the Ca\(^{2+}\) current reached 5% of the maximum current amplitude. In autaptic and voltage-ramp experiments, perforated-patch configuration using amphotericin B (Sigma) was used to maintain the native presynaptic environment as much as possible during prolonged recording times. Because amphotericin B introduces an unknown liquid junction potential, voltages were left uncorrected. Statistics were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Repeated Measures-ANOVA with Fisher’s Least Significant Difference (LSD) post hoc analysis was performed on all data unless otherwise indicated. Data are reported as mean ± SE.
**Results**

**SNAP increases sPSC frequency**

To determine whether NO affects spontaneous neurotransmitter release, ACs bearing processes in contact with other (unclamped) ACs were voltage clamped at -70 mV in TEA-Cl external solution. For spontaneous postsynaptic current (sPSCs) recordings, high-Cl\(^{-}\) internal solution was used to increase the driving force on Cl\(^{-}\) (at -70 mV). Under control conditions, sPSCs corresponding to quantal events were observed ranging in frequency from 1.4 to 77.6 events/30 s. When SNAP (500 μM) and the NO scavenger CPTIO (10 μM) were co-applied, no change in sPSC frequency was observed (control, 9.8 ± 3/30 s, SNAP/CPTIO, 10.7 ± 3/30 s, \( P = 0.5, n = 8 \), Figure 2.1). When CPTIO was removed and only SNAP was present, however, there was a 108% increase in sPSC frequency (SNAP/CPTIO, 10.7 ± 2.7/30 s, SNAP, 22.3 ± 5.2/30 s, \( P = 0.03, n = 8 \), Figure 2.1). NO did not affect the quantal event amplitude (control, 11.6 ± 0.8 pA, SNAP, 11.3 ± 1.2 pA, \( P = 0.63, n = 9 \) cells, paired t-test), rise time (control, 2.3 ± 0.1 ms, SNAP, 2.5 ± 0.1 ms, \( P = 0.12, n = 9 \) cells, paired t-test), or decay time (control, 9.55 ± 1.1 ms, SNAP, 9.9 ± 1.2 ms, \( P = 0.86, n = 9 \) cells, paired t-test). Thus, these data indicate that NO, rather than the donor molecule or NO metabolites, increased presynaptic spontaneous vesicle fusion and neurotransmitter release but did not affect the postsynaptic receptors.

**NO-dependent increase of sPSC frequency does not require soluble guanylyl cyclase (sGC) activity**

The canonical receptor for NO is sGC, and NO-activated sGC increases the production of the secondary messenger cGMP. To determine if NO-dependent activation of sGC and cGMP signaling was involved in the increase of sPSC frequency,
Figure 2.1. NO increases frequency of spontaneous postsynaptic currents (sPSCs). A: recording from a representative postsynaptic AC voltage clamped at -70 mV and synaptically connected with other unclamped ACs. Co-application of the NO donor SNAP (500 µM) and the NO scavenger CPTIO (10 µM) had no effect on the sPSC frequency. After removal of CPTIO, a significant increase in sPSC frequency was observed. *Multiquantal events. B: quantified mean event frequency of sPSCs/30 s SE; n = 8. **P < 0.01 (repeated measures-ANOVA).

Cells were pre-incubated with the sGC inhibitor ODQ (2 µM) for 12 minutes prior to recording, and ODQ remained throughout the recording. Even in the presence of ODQ, SNAP caused a 173% increase in sPSC frequency (ODQ, 20.1 ± 4.5/30 s, ODQ/SNAP, 54.9 ± 15.0/30 s, P = 0.03, n = 12, Figure 2.2). Acute application of ODQ alone did not alter sPSC frequency (control, 6.6 ± 3.19/30 s, ODQ, 5.25 ± 2.8/30 s, P = 0.06, n = 4, paired t-test). These data suggest that sGC does not mediate the NO-dependent increase in sPSC frequency.

Because activation of the canonical NO receptor sGC is not required for the increase in sPSC frequency, we investigated the role of another NO signaling pathway, S-nitrosylation that occurs when NO reacts with a cysteine residue thiol group to form a S-nitrosothiol. S-nitrosylation posttranslational protein modification is similar to phosphorylation in that it can alter the function of the targeted protein. N-ethylmaleimide
Figure 2.2. NO-dependent increase of sPSCs does not require soluble guanylate cyclase (sGC) activity. A: Recording from a representative AC held at -70 mV. Cells were exposed to the sGC inhibitor ODQ (2 µM) for at least 12 minutes prior to and throughout the recordings. SNAP increased the frequency of sPSCs in the presence of ODQ. B: Quantified mean event frequency of sPSCs/30 s ± SE, n = 12. *P < 0.05 (paired t-test).

(NEM), a known inhibitor of S-nitrosylation, was used to investigate if S-nitrosylation was involved in the NO-dependent increase in sPSC frequency. NEM (300 µM), however, caused an increase in sPSC frequency from baseline by 411% (control, 24.2 ± 15.26/30 s, NEM, 123.5 ± 26.78/30 s, P = 0.007, n = 7, paired t-test). Unfortunately, NEM has multiple targets including presynaptic proteins (Clary et al. 1990; Peters et al. 1990; Rothman 1996) and has been shown to increase GABAergic spontaneous neurotransmitter release (Kirmse and Kirischuk 2006; Knight et al. 2004) and activate some Ca^{2+} channels (Bindoli and Fleischer 1983; Graham et al. 2010; Pan et al. 2011). These results precluded further experiments using NEM in this study.

**NO-increases GABAergic sPSC frequency**

Cultured ACs used in the present study have been demonstrated to be GABAergic (Gleason et al. 1993). However, to confirm that NO is promoting the release of GABA rather than an additional neurotransmitter, the ionotropic GABA receptor
antagonist bicuculline (10 μM) was added to the extracellular solution during the application of SNAP. SNAP caused a 190% increase in sPSC frequency (control, 7.6 ± 2.0/30 s to SNAP, 22.1 ± 5.7/30 s, $P = 0.02; n = 9$, Figure 2.3A-B). However, bicuculline caused a 99% reduction in NO-dependent sPSC frequency (SNAP, 22.1 ± 5.7/30 s, SNAP/bicuculline, 0.3 ± 0.1/30 s, $P = 0.003; n = 9$, Figure 2.3A-B). These data indicate that NO increased GABAergic sPSCs due to increased presynaptic vesicle fusion and GABA release, but not due to the release of any other neurotransmitter. Previous work from our lab demonstrated a small (~15%) NO-dependent enhancement of whole-cell GABA$_A$ receptor currents, apparently due to an alteration in channel function (Hoffpauir et al. 2006). Here, we see no effect of NO on sPSC current amplitude, suggesting that synaptic and extra-synaptic receptors may be differentially regulated.

**NO-dependent increase of sPSC frequency is not action potential-dependent**

Some ACs are known to fire action potentials (Cook et al. 1998; Heflin and Cook 2007; Watanabe et al. 2003). For the experiments recording sPSCs (Figures. 2.1-3A), presynaptic ACs were unclamped, which allows their membrane potentials to fluctuate and possibly produce action potentials. To determine whether the NO-dependent increase of GABA release was due to presynaptic action potentials in response to NO, the voltage-gated Na$^+$ channel blocker tetrodotoxin (300 nM, TTX) was included in the TEA-Cl external solution. In the presence of TTX, SNAP still elicited a 202% increase in sPSC frequency (TTX, 7.3 ± 2.0/30 s, TTX/SNAP, 22.1 ± 5.7/30 s, $P = 0.03; n = 5$, paired $t$-test, Figure 2.3C-D). This indicates that sPSC frequency was increased by a NO-dependent mechanism that does not require action potentials.
Figure 2.3. NO-induced increase in GABAergic sPSC frequency does not require action potentials. A: Recording from a representative postsynaptic AC held at -70 mV. Application of the NO donor SNAP increased the frequency of sPSCs. Addition of the GABA<sub>A</sub> receptor antagonist bicuculline abolished the NO-dependent increase of sPSCs, indicating that these are GABAergic sPSCs. B: Quantified mean event frequency of sPSCs/30 s ± SE, n = 9. *P < 0.05, **P < 0.01 (repeated measures-ANOVA). C: Voltage clamp recording from a representative postsynaptic AC held at -70 mV. SNAP increased the frequency of sPSCs in the presence of the voltage-gated Na<sup>+</sup> channel blocker TTX (300 nM). D: Quantified mean event frequency of sPSCs/30 s ± SE, n = 5. *P < 0.05 (paired t-test).

Vesicle fusion and neurotransmitter release depends on the local presynaptic Ca<sup>2+</sup> concentration. To determine whether NO affects cytosolic Ca<sup>2+</sup>, cytosolic Ca<sup>2+</sup> levels were monitored using the fluorescent Ca<sup>2+</sup> indicator Oregon Green 488 BAPTA-1 (OGB, 2 μM). Data were collected from regions of neuronal process contacts, potentially synaptic sites. Co-application of the NO scavenger CPTIO and SNAP did not significantly alter intracellular Ca<sup>2+</sup> (P = 0.17, n = 57, Figure 2.4A-B). After removal
Figure 2.4. NO increases intracellular Ca$^{2+}$. A: Calcium imaging of AC processes loaded with Oregon Green BAPTA-1 488, AM, revealed that NO caused an increase of intracellular Ca$^{2+}$ at potential synaptic sites. When the NO donor SNAP (500 μM) and NO scavenger CPTIO (10 μM) were co-applied, there was no significant increase in intracellular Ca$^{2+}$ ($P > 0.15$). Removal of CPTIO was followed by a significant increase in cytosolic Ca$^{2+}$. Note that mean fluorescence in A is larger because data were collected with longer exposure times (400 ms) than all other data (200 ms). B: Quantified mean peak fluorescence ± SE in (A), $n = 57$. ****$P < 0.0001$ (repeated measures-ANOVA). C: Intracellular Ca$^{2+}$ is reduced when extracellular Ca$^{2+}$ is removed (0 Ca$^{2+}_{\text{ext}}$) even in the presence of SNAP. D: Quantified mean peak fluorescence ± SE in (C) showing that SNAP caused a significant increase in fluorescence and that removing extracellular Ca$^{2+}$ (0 Ca$^{2+}_{\text{ext}}$) significantly reduced the fluorescence, $n = 35$. ****$P < 0.0001$ (repeated measures-ANOVA). Fluorescence over time data are plotted as mean fluorescence ± SE.

of CPTIO, however, there was a 32% increase in intracellular Ca$^{2+}$ (CPTIO/SNAP, 26.7± 1.2 AU, SNAP, 35.3 ± 1.6 AU, $p < 0.0001$, $n = 57$, Figure 2.4A-B). Additionally, there was an 11% reduction in cytosolic Ca$^{2+}$ when external Ca$^{2+}$ was removed during the SNAP application, (SNAP, 7.9 ± 0.5 AU, SNAP/0 Ca$^{2+}$, 7.0 ± 0.4 AU, $p < 0.0001$, $n = 35$, Figure 2.4C-D). These results indicated that NO, rather than the donor molecule or
NO metabolites, increased intracellular Ca\(^{2+}\) via an extracellular Ca\(^{2+}\)-dependent mechanism.

**The role of Ca\(^{2+}\)**

Having established that NO can produce Ca\(^{2+}\) elevations in ACs, we asked whether the Ca\(^{2+}\) elevations were driving the NO-dependent increase in sPSCs. BAPTA-AM (10 \(\mu\)M), a cell-permeant fast Ca\(^{2+}\) chelator, was loaded into the cells prior to recordings. In BAPTA-containing cells, sPSC frequency in the recorded cell was not enhanced by SNAP (control, 5.9 ± 1.1/30 s, SNAP, 6.4 ± 1.1/30 s, \(P = 0.62, n = 12\), paired t-test, Figure 2.5B-C). In cells that did not have BAPTA, however, SNAP caused a 172\% increase in sPSC frequency (control, 9.2 ± 1.3/30 s, SNAP, 25.0 ± 2.7/30 s, \(P = 0.0002, n = 12\), paired t-test, Figure 2.5A-C). These results suggest that the NO-dependent increase in intracellular Ca\(^{2+}\) is responsible for the NO-dependent increase in sPSC frequency.

To determine if Ca\(^{2+}\) influx, specifically, is involved in the NO-dependent increase of sPSCs, extracellular Ca\(^{2+}\) was removed during the application of SNAP. Prior to Ca\(^{2+}\) removal, SNAP caused a 61\% increase in sPSC frequency (control, 19.4 ± 8.8/30 s, SNAP, 31.2 ± 12.7/30 s, \(P = 0.03, n = 8\), Figure 2.5D-E). When extracellular Ca\(^{2+}\) was removed during SNAP application, there was a 62\% reduction in sPSC frequency (SNAP, 31.2 ± 12.7/30 s, SNAP/0 Ca\(^{2+}\), 11.9 ± 7.1/30 s, \(P = 0.02, n = 8\), Figure 2.5D-E). These results indicate that Ca\(^{2+}\) influx is required for the NO-dependent increase of sPSC frequency.

Ca\(^{2+}\) stores can play a role in vesicle fusion and GABA release in ACs (Chavez et al. 2010; Chavez et al. 2006; Ke et al. 2010; Warrier et al. 2005). The contribution of
stores to the NO-dependent increase in cytosolic Ca\textsuperscript{2+} was investigated in OGB-loaded ACs that were pre-incubated with the SERCA pump inhibitor thapsigargin (TG, 2µM, 1 hr). The effectiveness of this treatment in emptying the Ca\textsuperscript{2+} store was evaluated by
perfusing caffeine (20 mM) to release store Ca\(^{2+}\). For control cells in zero Ca\(^{2+}\) external solution (0 Ca\(^{2+}\_\text{ext})), caffeine reliably produced rapid increases in cytosolic Ca\(^{2+}\) at contact points between processes (0 Ca\(^{2+}\_\text{ext}, 12.33 \pm 1.21 \text{ AU}, 0 \text{ Ca}^{2+}\_\text{ext} \text{ and caffeine}, 13.52 \pm 1.14 \text{ AU}, P = 0.004, n = 25, Figure 2.6A,C). However, cells pretreated with TG did not produce caffeine-dependent Ca\(^{2+}\) elevations, indicating depletion of the Ca\(^{2+}\) store (Figure 2.6B,D). Using this same TG pretreatment protocol, the effect of SNAP on cytosolic Ca\(^{2+}\) was examined. Under normal external Ca\(^{2+}\) conditions, SNAP elicited a 15\% and 9\% Ca\(^{2+}\) elevation in both control (control, 6.2 \pm 0.4 \text{ AU}, SNAP, 7.1 \pm 0.5 \text{ AU}, p < 0.0001, n = 50, paired t-test, Figure 2.6E,G) and TG-treated (control, 9.4 \pm 1.3 \text{ AU}, SNAP, 10.2 \pm 1.2 \text{ AU}, p < 0.0001, n = 17, paired t-test, Figure 2.6F,H) cells, respectively. TG-treated cells produced no NO-dependent Ca\(^{2+}\) elevations in the absence of extracellular Ca\(^{2+}\) (0 Ca\(^{2+}\), 6.9 \pm 0.3 \text{ AU}, 0 \text{ Ca}^{2+}/\text{SNAP}, 6.8 \pm 0.3 \text{ AU}, P = 0.06, n = 107, Figure 2.6I,J). These data show that under these conditions NO elicits Ca\(^{2+}\) elevations by activating a Ca\(^{2+}\) influx pathway exclusively.

To confirm that Ca\(^{2+}\) stores do not play a role in the NO-dependent increase of sPSCs, voltage clamp recordings were made from ACs pre-incubated in TG. SNAP still caused a 65\% and 171\% increase in sPSC frequency in TG-treated (control, 18.3 \pm 5.3/30 \text{ s}, SNAP, 30.2 \pm 6.1/30 \text{ s}, P = 0.003, n = 8, paired t-test, Figure 2.7B-C) and in control (control, 10.1 \pm 4.1/30 \text{ s}, SNAP, 27.4 \pm 8.3/30 \text{ s}, P = 0.034, n = 4, paired t-test, Figure 2.7A,C) cells, respectively. These results confirm that NO increases GABAergic sPSC frequency by activating a plasma membrane Ca\(^{2+}\) influx pathway that is independent of Ca\(^{2+}\) release from stores.
Figure 2.6. NO-dependent increase of intracellular Ca\(^{2+}\) is independent of intracellular stores. A: In the absence of extracellular Ca\(^{2+}\) (0 Ca\(^{2+}\)\_ext), caffeine (20 mM) produced a rapid and transient increase in intracellular Ca\(^{2+}\) when thapsigargin (TG) was not present. B: For cells pre-incubated in TG (2 μM, 1 hr), no caffeine-dependent store release was observed. C,D: Quantified mean fluorescence ± SE in A (n = 25) and B (n = 37), respectively. E, F: The NO donor SNAP produced an increase in intracellular Ca\(^{2+}\) in both control and thapsigargin-treated AC processes. G,H: Quantified mean fluorescence ± SE in E (n = 50) and F (n = 107), respectively. I: The NO donor SNAP does not increase intracellular Ca\(^{2+}\) in the presence of TG and the absence of extracellular Ca\(^{2+}\). J: Quantified mean fluorescence ± SE in I (n = 107). Images were taken every 500 ms in A-B, and every 6 s in E-F. *P < 0.05, **P < 0.001, ***P < 0.0001, ****P < 0.00001, nsP > 0.57 (repeated measures-ANOVA). Fluorescence over time data are plotted as mean fluorescence ± SE.
Figure 2.7. NO-dependent increase of sPSC frequency is independent of Ca$^{2+}$ stores. 

A,B: Representative recordings from postsynaptic amacrine ACs held at -70 mV without (A) or with (B) pre-incubation with TG (2 µM, 1 hr). The NO-dependent increase of sPSC frequency persisted in the presence of TG. C: Quantified mean event frequency/30 s ± SE. Addition of SNAP increased sPSCs in cells with intact Ca$^{2+}$ stores (No TG, n = 4) and after Ca$^{2+}$ store depletion (+TG, n = 8). *P < 0.05, **P < 0.01 (paired t-tests).

NO increases evoked GABAergic autaptic transmission

In the retina, ACs receive excitatory signals from bipolar cells. Depolarization of ACs leads to evoked neurotransmitter release onto postsynaptic cells, which can be bipolar cells, ganglion cells or other ACs. To determine the role of NO in evoked neurotransmitter release, perforated patch whole-cell recordings were performed on isolated ACs making autapses. Cells were depolarized from -70 mV to -20 mV for 50 ms to activate voltage-gated Ca$^{2+}$ channels (VGCCs) and subsequently cause vesicle fusion and neurotransmitter release. The autaptic currents persisting after the voltage step were quantified. SNAP caused a 51% increase in charge transfer (control, 177.0 ± 47.6 pC, SNAP, 268.1 ± 59.7 pC, P = 0.03, n = 6, Figure 2.8A-B). Bicuculline blocked the NO-dependent increase of the charge transfer (bicuculline, 48.2 ± 13.4 pC,
bicuculline/SNAP, 53.1 ± 16.3 pC, \( P = 0.32, n = 5 \), Figure 2.8C-D). Note that the apparent SNAP-dependent change in the \( \text{Ca}^{2+} \) current amplitude during the voltage-step is due to the contamination from inward GABA-gated current (Figure 2.8A), an effect that is absent in the presence of bicuculline (Figure 2.8C). The sPSC frequency was also elevated during the SNAP application (Figure 2.8A inset). These data suggest that NO increases evoked GABAergic neurotransmission, possibly due to additive effects of two \( \text{Ca}^{2+} \) influx pathways being activated (NO-dependent \( \text{Ca}^{2+} \) influx and voltage-dependent \( \text{Ca}^{2+} \) influx) simultaneously.

The effect of NO on sPSCs does not involve L-type VGCCs, AMPARs or NMDARs

L-type VGCCs have been demonstrated to mediate neurotransmitter release between these cultured ACs (Gleason et al. 1993) and other AC synapses in the retina (Haberrmann et al. 2003; Vigh and Lasater 2004). It has also been demonstrated that NO can activate retinal ganglion cell \( \text{Ca}^{2+} \) channels (Hirooka et al. 2000) and augment neuronal L-type \( \text{Ca}^{2+} \) currents (Tozer et al. 2012). To determine if NO influences the voltage-gated \( \text{Ca}^{2+} \) current or activation voltage, voltage ramps from -90 mV to +50 mV were delivered in the presence of TTX (300 nM) and bicuculline (10 \( \mu \)M) to isolate the \( \text{Ca}^{2+} \) current. In these experiments, perforated-patch normal internal solution and TEA-Cl external solution were used. Addition of SNAP did not change the \( \text{Ca}^{2+} \) current amplitude (control, -268.9 ± 45.1 pA, SNAP, -261.2 ± 43.8 pA, \( P = 0.45, n = 9 \), Figure 2.9A) or the \( \text{Ca}^{2+} \) channel activation voltage (control, -45.36 ± 1.1 mV, SNAP, -45.13 ± 0.73 mV, \( P = 0.69, n = 9 \)). Additionally, the L-type VGCC inhibitor nifedipine (20 \( \mu \)M) did not prevent the NO-dependent increase in sPSC frequency (nifedipine, 20.40 ± 4.2/30 s, nifedipine/SNAP, 37.65 ± 6.07/30 s, \( P = 0.028, n = 5 \), Figure 2.9B-C). The NO-
Figure 2.8. NO increases evoked autaptic GABAergic postsynaptic currents. A: Recording from a representative AC depolarized from -70 mV to -20 mV for 50 ms. Evoked autaptic currents were increased after the addition of the NO donor SNAP. Inset: sPSC frequency was elevated between depolarizations during the application of SNAP. B: Quantified mean charge transfer ± SE, n = 6. *P < 0.05 (paired t-test). C: Recording from a representative AC depolarized from -70 mV to -20 mV for 50 ms. Addition of the GABA\textsubscript{A} receptor antagonist bicuculline blocked the NO-dependent increase of charge transfer. D: Quantified mean charge transfer ± SE. The NO donor SNAP did not significantly alter the evoked autaptic currents in the presence of bicuculline, n = 5. P > 0.3, (paired t-test).

dependent increase in sPSC frequency remained unchanged after removal of nifedipine and leaving only SNAP in the bath (nifedipine/SNAP, 37.65 ± 6.07/30 s, SNAP, 40.3 ±
These results demonstrate that L-type VGCCs are not involved. The possibility that NO could activate AMPA and/or NMDA glutamate receptors (AMPARs and NMDARs) was investigated using Ca\(^{2+}\) imaging. The AMPAR and NMDAR antagonists CNQX (50 \(\mu\)M) and D-AP5 (10 \(\mu\)M), respectively, were applied before and during the application of SNAP. While AMPRs and NMDARs were blocked, NO increased intracellular Ca\(^{2+}\) by 10.8\% (D-AP5/CNQX, 8.1 ± 0.3 A.U., D-AP5/CNQX and SNAP, 8.9 ± 0.4 A.U., \(n = 152\) ROIs, \(p < 0.0001\), Figure 2.9D). When D-AP5 and CNQX were removed, intracellular Ca\(^{2+}\) remained elevated (D-AP5/CNQX and SNAP, 8.9 ± 0.4 A.U., SNAP, 9.1 ± 0.4 A.U., \(n = 152\) ROIs, \(P = 0.1\), Figure 2.9D). These results show that NO does not activate L-type VGCCs, AMPARs or NMDARs. Therefore, NO must be activating another plasma membrane Ca\(^{2+}\) influx pathway to admit extracellular Ca\(^{2+}\) presynaptically and to increase sPSC frequency. Clemizole blocks the NO-dependent increase in sPSC frequency and intracellular Ca\(^{2+}\).

Previous work in our lab demonstrated that these amacrine cells contain mRNA for TRPC subunits 1, 3, 4, 5, 6, and 7 (Crousillac et al. 2003), and TRPC channels can be activated via S-nitrosylation (Yoshida et al. 2006). To explore the possible role of TRPC channels in the NO-dependent increase in sPSC frequency, we used two traditional TRPC inhibitors, 2-APB and SKF 96365. These inhibitors, however, have multiple documented effects on other ion channels, some of which conduct Ca\(^{2+}\) (DeHaven et al. 2008; Hong et al. 1994; Hotta et al. 2005; Leung et al. 1996; Merritt et al. 1990; Prakriya and Lewis 2001; Singh et al. 2010; Usmani et al. 2010; Wang 2003). Bath perfusion of 2-APB (20 \(\mu\)M) alone increased sPSC frequency from baseline by 108\% (control, 52 ± 7.5/30 s, 2-APB, 107.9 ± 15.3/30 s, \(P = 0.01\), \(n = 10\), paired \(t\)-test),
Figure 2.9. L-type voltage-gated Ca\textsuperscript{2+} channels (VGCCs), AMPARs or NMDARs are not required for the NO-dependent Ca\textsuperscript{2+} influx.  

A: Voltage clamp recording from a representative AC ramped from -90 mV to 50 mV. The NO donor SNAP (500 µM) did not affect the activation voltage or the peak of the Ca\textsuperscript{2+} current. Bicuculline and TTX were included in the TEA-Cl external solution to isolate the Ca\textsuperscript{2+} current.  

B: Current recording from a representative amacrine cell voltage clamped at -70 mV. SNAP still increased sPSC frequency in the presence of the L-type VGCC inhibitor nifedipine (Nif, 20 µM). sPSC frequency was unchanged after removal of Nif.  

C: Quantified mean event frequency/30 s ± SE, n = 5. *P < 0.05 (repeated measures-ANOVA). D: The NO-dependent influx of Ca\textsuperscript{2+} still occurred in the presence of the NMDAR inhibitor D-AP5 (10 µM) and the AMPAR inhibitor CNQX (50 µM). Data are Mean fluorescence (A.U.) ± SE, n = 152 ROIs. ****P < 0.0001 (repeated measures-ANOVA).

and application of SKF 96365 (30 µM) alone increased sPSC frequency from baseline by 431% (control, 33.4 ± 7.5/30 s, SKF 96365, 177.4 ± 44.3, P = 0.044, n = 4, paired t-test). To avoid these confounding effects, clemizole (10 µM), a more specific TRPC channel blocker (with highest selectivity for TRPC5), was utilized to investigate TRPC channel involvement in the NO-dependent response. In the presence of clemizole,
SNAP was unable to increase sPSC frequency (control, 17.2 ± 3.7/30 s, clemizole/SNAP, 17.9 ± 2.0/30 s, P = 0.84, n = 10, Figure 2.10A-B). When clemizole was removed, SNAP increased sPSC frequency by 203% (clemizole/SNAP, 17.9 ± 2/30 s, SNAP, 54.1 ± 16.8/30s, P = 0.04, n = 10, Figure 2.10A-B). Additionally, SNAP was unable to increase process intracellular Ca\(^{2+}\) in the presence of clemizole (clemizole, 5.7 ± 0.4 A.U., clemizole/SNAP, 5.7 ± 0.4 A.U., P = 0.45, n = 46 ROIs, Figure 2.10C-D). When clemizole was removed, SNAP increased intracellular Ca\(^{2+}\) by 7% (clemizole/SNAP, 5.7 ± 0.4 A.U., SNAP, 6.1 ± 0.4 A.U., p < 0.0001, n = 46, Figure 2.10C-D). Clemizole is also able to block H1 histamine receptors (Aguilar et al. 1986).

It has been demonstrated that these receptors are functionally expressed in rodent dopaminergic amacrine cells and that their activation can engender Ca\(^{2+}\) elevations (Frazao et al. 2011; Gastinger et al. 2006). Although the amacrine cells in our cultures are GABAergic, we tested whether histamine (10 µM and 100 µM) produced Ca\(^{2+}\) elevations. We found that Ca\(^{2+}\) elevations were not elicited by 10 µM histamine (control, 5.09 ± 0.25 A.U., 10 µM histamine, 5.05 ± 0.26 A.U., P = 0.16, n = 48) or 100 µM histamine (control, 4.05 ± 0.12 A.U., 100 µM histamine, 4.07 ± 0.12 A.U., P = 0.19, n = 216) suggesting that either histamine receptors are not expressed or that their activation is not linked to an increase in cytosolic Ca\(^{2+}\). Together, these results provide evidence that NO increases GABA release from amacrine cells by activating a TRPC-mediated Ca\(^{2+}\) influx.
Figure 2.10. Clemizole blocks the NO-dependent increase in sPSC frequency and intracellular Ca$^{2+}$. A: Current recording from a representative amacrine cell voltage clamped at -70 mV. Application of the TRPC inhibitor clemizole (clem, 10 µM) and co-application of clem and the NO donor SNAP (500 µM) did not alter sPSC frequency. Removal of clem from the bath leaving only SNAP caused an increase in sPSC frequency. B: Quantified mean event frequency/30 s ± SE, n = 10. *P < 0.05 (repeated measures-ANOVA). C: SNAP was unable to increase intracellular Ca$^{2+}$ during the application of clem, however, SNAP increased intracellular Ca$^{2+}$ upon removal of clem. D: Quantified mean fluorescence ± SE, n = 46. ****P < 0.0001, (repeated measures-ANOVA). Fluorescence over time data are plotted as mean fluorescence ± SE.

Discussion

These results demonstrate that NO promotes neurotransmitter release at GABAergic synapses in retinal amacrine cells (ACs). We find that this action of NO is not dependent on presynaptic action potentials or sGC activity, however, it is dependent
upon an elevation in cytosolic Ca$^{2+}$. Both NO-dependent Ca$^{2+}$ elevations and NO-dependent increases in sPSCs were unaffected by store depletion, indicating that Ca$^{2+}$ influx alone mediates the effects of NO. Evoked GABA release was also enhanced in the presence of NO, but the activation of voltage-gated Ca$^{2+}$ channels (VGCCs) was unaffected by NO. Together, these observations imply that during evoked release, the NO-dependent Ca$^{2+}$ elevations can add to the voltage-dependent Ca$^{2+}$ elevations to enhance the evoked release of GABA. This interpretation is consistent with the steep dependence of evoked neurotransmitter release on Ca$^{2+}$ (Augustine and Charlton 1986; Dodge and Rahamimoff 1967; Reid et al. 2003). Blocking TRPC channels using clemizole prevented the NO-dependent increase in sPSC frequency and intracellular Ca$^{2+}$. These results suggest that NO activates a TRPC-mediated Ca$^{2+}$ influx pathway that is sufficient to drive synaptic exocytosis.

**VGCC-independent GABA release**

Other mechanisms of VGCC-independent GABA release have been demonstrated in ACs. Chavez et al. (2006) showed that in the rat retina, reciprocal GABAergic synapses from A17 ACs onto bipolar cells can be directly stimulated by the activation of Ca$^{2+}$-permeable AMPA receptors. Voltage-independent GABA release was also demonstrated for cultured chick ACs via activation of metabotropic glutamate receptor 5 (Warrier et al. 2005). Both mechanisms involved amplification through release of Ca$^{2+}$ from stores. Additionally, a study of unstimulated GABA release from cultured rat ACs demonstrated the involvement of both Ca$^{2+}$ influx and store release (Ke et al. 2010). The NO-dependent increase in sPSCs reported here is novel because our results argue against a contribution from stores.
NO and GABA release

In agreement with our finding in cultured ACs, experiments measuring the levels of a GABA analog loaded in slices of turtle retina showed that NO promoted GABA release from ACs as well as horizontal cells (Yu and Eldred 2005). Significantly, in the inner retina where ACs signal, the NO-dependent increase in GABA release was largely Ca\textsuperscript{2+}-dependent and soluble guanylate cyclase-independent, consistent with what we find for cultured ACs. NO has also been reported to stimulate GABA release from GABAergic cells in the ganglion cell layer (most likely displaced ACs) (Maggesissi et al. 2009) in the intact chicken retina, although the underlying mechanism was not investigated. Previous studies measuring the effects of NO on GABA release at other central synapses have demonstrated both enhancement (Merino et al. 2014; Tarasenko et al. 2014) and inhibition (De Laurentiis et al. 2000; Wall 2003). In studies of the effects of NO on GABAergic sPSC frequency specifically, both increases in sPSC frequency (Li et al. 2004; Li et al. 2002; Xing et al. 2008; Yang and Cox 2007) and decreases in sPSC frequency (Lee 2009) have been reported for central mammalian synapses. Both effects can be dependent upon soluble guanylate cyclase activity (Lee 2009; Yang and Cox 2008: Li et al. 2004). Here, we show that GABAergic sPSC frequency is enhanced by NO via a soluble guanylate cyclase-independent mechanism. Together, these studies establish that the effects of NO on GABAergic signaling can be functionally and mechanistically diverse, indicating that the effects of NO on the synaptic output of any one class of neuron is not predictable.
Role of spontaneous GABA release

There is abundant evidence that spontaneous and action potential (AP) evoked vesicular neurotransmitter release occur by distinct processes that involve different Ca\(^{2+}\) sensitivities, molecular machinery and synaptic vesicle pools (Kavalali 2015; Smith et al. 2012). This existence of a parallel and similarly complex system of neurotransmitter release implies that spontaneous release has distinctive functions. One emerging role is in the regulation of the synapse itself. Most is known about the role of spontaneous glutamate release in regulating synaptic function, however, recent work has demonstrated that spontaneous GABA release regulates synaptic scaling in the embryonic chick spinal cord (Garcia-Bereguaiain et al. 2016). The mechanism underlying this action of GABA depends on the depolarizing effect of GABA at that time in development, and scaling is achieved by adjusting the level of postsynaptic Cl\(^{-}\). We have previously demonstrated that NO can release Cl\(^{-}\) from an internal store and transiently convert inhibitory GABAergic synapses into excitatory GABAergic synapses (Hoffpauir et al. 2006; Krishnan and Gleason 2015). Thus, the ability of GABA to even transiently generate depolarization-dependent Ca\(^{2+}\) elevations opens the possibility that NO-dependent spontaneous activity at AC synapses can further modulate their own synaptic function.

TRPCs in ACs

Our experiments with clemizole suggest that TRPC channels are induced to admit presynaptic Ca\(^{2+}\) by NO. There is additional evidence in the literature suggesting the functional expression of TRPC channels in ACs. In cultured rat ACs, the broad TRPC channel inhibitor SKF96365 reduced GABAergic sPSC frequency, suggesting
TRPC channels play a role in synaptic output (Ke et al. 2010). In our experiments, SKF96365 caused an increase in sPSC frequency. In cultured chick ACs, 2-APB was used to inhibit activation of IP₃ receptors which prevented both a depolarization- and mGluR5-dependent increase in sPSC frequency (Warrier et al. 2005). Here, (in the absence of depolarization or mGluR5 activation) 2-APB caused an increase in sPSC frequency. Having multiple sites of action (DeHaven et al. 2008; Hong et al. 1994; Hotta et al. 2005; Leung et al. 1996; Merritt et al. 1990; Prakriya and Lewis 2001; Singh et al. 2010; Usmani et al. 2010; Wang 2003), SKF96365 and 2-APB are problematic in investigating the involvement of TRPC channels in the NO-dependent increase in intracellular Ca²⁺ and GABAergic sPSC frequency in cultured ACs. The more specific TRPC inhibitor clemizole did not activate spontaneous release on its own, but it was effective in suppressing the effects of NO. Clemizole is most selective for TRPC5 (IC₅₀ = 1.1 μM), however, it can also inhibit TRPC4 (IC₅₀ = 6.4 μM), TRPC3 (IC₅₀ = 9.1 μM), TRPC6 (IC₅₀ = 11.3 μM) and TRPC7 (IC₅₀ 26.5 μM) (Richter et al. 2014). Interestingly, homomeric TRPC1, TRPC5, or heteromeric TRPC1/5 channels can be activated by S-nitrosylation (Yoshida et al. 2006). The effects of the S-nitrosylation inhibitor NEM alone, however, precluded our use of this reagent to assess the role of this modification. Transcripts encoding TRPCs 1, 3-7 have been RT-PCR amplified from pools of 10-20 cultured ACs (Crousillac et al. 2009). There is also immunocytochemical evidence that TRPC1 is expressed by ACs in the chicken retina and can co-localize with nNOS (Crousillac et al. 2003). The TRPC channel (s) involved in the NO-dependent increase in intracellular Ca²⁺ and sPSC frequency remains to be elucidated.
References


Tozer AJ, Forsythe ID, and Steinert JR. Nitric oxide signalling augments neuronal voltage-gated L-type (Ca(v)1) and P/Q-type (Ca(v)2.1) channels in the mouse medial nucleus of the trapezoid body. *PLoS One* 7: e32256, 2012.


CHAPTER 3
TRPC5 IS REQUIRED FOR THE NO-DEPENDENT INCREASE IN DENDRITIC CA\textsuperscript{2+} AND GABA RELEASE FROM RETINAL AMACRINE CELLS

Introduction

Retinal amacrine cells (ACs) play a major role in processing the visual signal in the retina before the signal is sent to the visual cortex of the brain. There are ~40 different morphological AC subtypes, and some subtypes of ACs are involved in dopamine-dependent light adaptation (Mills and Massey 1995; Xia and Mills 2004), rod pathway integration into the cone pathway (Vaney et al. 1991), and direction selectivity (Taylor and Smith 2012). The function of majority AC subtypes have not been identified, including nitric oxide (NO) producing ACs. Our lab has identified two roles NO plays in regulating AC signaling. NO induces Cl\textsuperscript{-} release from internal acidic organelles, which can transiently convert typical inhibitory synapses into excitatory (Hoffpauir et al. 2006; Krishnan and Gleason 2015), and NO enhances the release of Ca\textsuperscript{2+} dependent GABA release by activating a Ca\textsuperscript{2+} influx pathway (Maddox and Gleason 2017).

The enzyme nitric oxide synthase (NOS) synthesizes NO by catalyzing L-arginine into L-citrulline and NO. The two Ca\textsuperscript{2+}-sensitive NOS isoforms, endothelial NOS (eNOS) and neuronal NOS (nNOS), have been localized to ACs, as well as other retinal cell types (Fischer and Stell 1999; Haverkamp et al. 2000; Kim et al. 1999; Pang et al. 2010; Tekmen-Clark and Gleason 2013). Neuronal NOS has been specifically localized to AC presynaptic terminals in the inner turtle retina (Cao and Eldred 2001), and NO stimulates Ca\textsuperscript{2+} dependent GABA release from ACs, independent of the canonical NO receptor soluble guanylate cyclase (Maddox and Gleason 2017; Yu and Eldred 2005). In some tissues, NO is traditionally considered a diffusible signaling
molecule, however, studies that monitored endogenous NO production in retinas loaded with the NO fluorescent indicator DAF-FM have demonstrated that NO is largely confined to cellular boundaries in turtle (Eldred and Blute 2005), mice (Blom et al. 2012), and chicken (Tekmen-Clark and Gleason 2013) retinas, suggesting that NO synthesis may affect neurotransmitter release from NO producing cells or from their immediate synaptic partners.

We previously determined that the NO-dependent increase in dendritic Ca\textsuperscript{2+} and GABA release relied on a likely transient receptor potential canonical (TRPC)-mediated Ca\textsuperscript{2+} influx pathway (Maddox and Gleason 2017). Interestingly, it has been shown that NO can directly activate TRPC channels via S-nitrosylation (Shimizu et al. 2014; Yoshida et al. 2006). S-nitrosylation is a posttranslational modification that can alter the function of a protein and occurs when NO binds to a cysteine thiol group. In the mouse retina, TRPC1, -4, and -5 are present in the inner plexiform layer (IPL), where ACs form synapses, and TRPC3, -6, and -7 is mostly absent (Witkovsky et al. 2008). In the mouse IPL, TRPC5 is the most abundant TRPC subunit (Witkovsky et al. 2008). In the chicken retina, TRPC1 expression has been demonstrated in the IPL and TRPC4 is expressed by Müller cells (Crousillac et al. 2003).

We previously demonstrated that clemizole, an inhibitor for TRPC5, -4, -3, -6, and -7 (IC\textsubscript{50} = 1.1, 6.4, 9.1, 11.3, and 26.5 μM, respectively) (Richter et al. 2014), prevented the NO-dependent dendritic Ca\textsuperscript{2+} elevation and the increase in GABAergic spontaneous postsynaptic current (sPSC) frequency (Maddox and Gleason 2017). To identify the TRPC family member involved in the NO-dependent enhancement of GABA release, we employed whole-cell current recordings, Ca\textsuperscript{2+} imaging,
immunocytochemistry, and a CRISPR/Cas9 protein knockdown strategy. In the present study, we find that downstream activity of phospholipase C (PLC) is required for the NO-dependent increase in sPSC frequency. PLC cleaves phosphatidylinositol-4,5-bisphosphate (PIP₂) into the secondary messengers inositol trisphosphate (IP₃) and diacylglycerol (DAG). DAG can activate TRPC3, -6, and -7, however, it is thought that TRPC4 and -5 are inhibited by PIP₂ but can be activated once PLC cleaves PIP₂ to relieve inhibition (Svobodova and Groschner 2016). We also show that La³⁺ has a biphasic, concentration-dependent effect on sPSC frequency, consistent with TRPC4 and 5 expression. The TRPC4/C5 inhibitor ML204 had no effect on the NO response at 10 µM but blocked the NO response at 20 µM. Using the CRISPR/Cas9 system to target the 2ⁿᵈ exon of the TRPC5 gene, we find that TRPC5 protein expression is required for the NO-dependent Ca²⁺ influx and GABAergic sPSC frequency increase. These findings provide evidence that the NO-dependent activation of TRPC5 can enhance GABA release from amacrine cell synapses, which suggests that NO-dependent TRPC5 activity enhances inhibition in the inner retina.

**Methods**

**Plasmid construction**

pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid #48138) (Ran et al. 2013). Due to the overlapping excitation/emission wavelengths of GFP and the Ca²⁺ indicator Oregon Green 488 BAPTA-1, AM (OGB, Life Technologies, Carlsbad, CA), the GFP gene in pSpCas9(BB)-2A-GFP (PX458) was replaced with tdTomato gene from tdTomato-N1 (Addgene plasmid #54642) to create pSpCas9-2A-tdTomato. tdTomato-N1 was a gift from Michael Davidson and Roger Tsien. The
inverted repeat BbsI restriction sites needed to clone the 20-nt guide RNA sequence into pSpCas9(BB)-2A-GFP also occurs twice within the tdTomato gene. The BbsI restriction sites in pSpCas9(BB)-2A-GFP were replaced with inverted repeat Bsai restriction sites prior to replacing GFP with tdTomato. Oligos used to clone the Bsai restriction sites into pSpCas9(BB)-2A-GFP were Bsai-F: CACCGGAGACCGAGGTCTCC and Bsai-R: AAACGGAGACCTCGGTCTCC. A 20-nt guide sequence (AAAAGGGCACTCCGACCAAG) with a quality score of 95 was designed using the CRISPR design portal (crispr.mit.edu) to target Cas9 to the 2nd exon of TRPC5. Oligos used to clone the 20 bp TRPC5 guide sequence into pSpCas9-2A-tdTomato were sgTRPC5-F: caccgAAAAGGGCACTCCGACCAAG and sgTRPC5-R: aaacCTTGGTCCAGTCCCTTTTc. The 20 bp guide sequence for TRPC5 was cloned into pSpCas9-2A-tdTomato to create pCRISPR-TRPC5. All oligos were synthesized by Integrated DNA Technologies (Coralville, IA).

Amacrine cell cultures and transfections

The use of chick embryos to prepare retinal cultures was determined to be exempt by the LSU Institutional Animal Care and Use Committee. Retinal cell cultures were prepared and maintained as previously described (Maddox and Gleason 2017). Experiments were performed on ACs that had been in culture for 8-13 days (embryonic equivalent days 16 to 21). Over this time in culture, AC-to-AC GABAergic synapses are functional. ACs were identified on morphological criteria (Gleason et al. 1993).

Dissociated retinas (~8 x 10⁶ cells) from 8-day chick embryos were pelleted and resuspended with 100 µL PBS supplemented with 1 mM CaCl, 5 mM glucose, and either 5 µg of pSpCas9-tdTomato (¬sgRNA) or 5 µg pCRISPR-TRPC5 (⁺sgRNA). Cell
suspensions were electroporated using Nucleofector II device, program G-013 (Lonza, Basel, Switzerland). Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) was added to the electroporated cell suspension and allowed to rest for 5 min. Transfected cells used for current recording/Ca\(^{2+}\) imaging experiments and transfected cells used for immunocytochemistry were plated onto tissue culture dishes pretreated with 0.01% poly-DL-ornithine hydrobromide (Sigma Aldrich, St. Louis, MO) and coverslips pretreated with 0.01% poly-DL-ornithine hydrobromide at a density of \(\sim 1 \times 10^6\) cells per transfection, respectively. DMEM was replaced 24 hrs after initial plating with Neurobasal medium supplemented with 1% serum-free B-27, penicillin (100 U/mL), streptomycin (100 µg/mL) and glutamine (2 mM).

Solutions

All reagents were purchased from Sigma-Aldrich unless otherwise stated.

*Tetraethylammonium (TEA)-Cl external solution* (in mM): NaCl (117), KCl (5.3), TEA-Cl (20), CaCl\(_2\) (3), MgCl\(_2\) (0.41), glucose (5.6), HEPES (10), pH 7.4. *High Cl\(^-\) internal solution* (in mM): CsCl (110), MgCl\(_2\) (2), CaCl\(_2\) (0.1), EGTA (1), HEPES (10), NaCl\(_2\) (1), ATP-2Na\(^+\) (3), ATP- 2K\(^+\) (1), GTP (2), phosphocreatine (20), creatine phosphokinase (50 U/mL), pH 7.4. The high Cl\(^-\) content of these internal and external solutions sets the reversal potential for Cl\(^-\) at 0 mV and allows spontaneous presynaptic quantal events to easily be resolved during current recordings in postsynaptic amacrine cells voltage-clamped at -70 mV (Borges et al. 1995; Gleason et al. 1993; Maddox and Gleason 2017). For imaging experiments, the following external solutions were used: *Normal*
external solution (in mM): NaCl (137), KCl (5.37), CaCl₂ (3), MgCl₂ (0.41), glucose (5.6), HEPES (3.02), pH 7.4.

Reagents

The NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 500 μM, Enzo, Farmingdale, NY) and LaCl₃ (2 mM and 10 μM) were dissolved directly into the external solution. 4-Methyl-2-(1-piperidinyl)quinolone (ML 204, Tocris Bioscience, Bristol, UK), 1-[(17β)-3-Methoxyestra-1,3,5(10)-tri-en-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U 73122, Tocris), and 1-[(17β)-3-Methoxyestra-1,3,5(10)-tri-en-17-yl]amino]hexyl]-2,5-pyrrolidinedione (U 73343, Tocris) were prepared as 1000 X stocks in DMSO and diluted to 1 X into the external solution. The external solution pH was readjusted to 7.4 after the addition of reagents.

Electrophysiology

Whole-cell voltage clamp recordings were performed using Axopatch 1D amplifier, Digidata 1322A data-acquisition board, and Clampex 10.0 (Molecular Devices, Sunnyvale, CA). Patch pipette electrodes with a tip resistance between 5-10 MΩ were pulled from thick-walled borosilicate glass (1.5 mm O.D., 0.86 mm I.D.) using a P-97 Flaming/Brown Puller (Sutter Instruments, Novato, CA). A reference Ag/AgCl electrode pellet was placed in 3 M KCl and connected to the culture dish with a 3 M KCl agarose bridge. Recordings were performed at room temperature (22-24°C). A pressurized perfusion system (AutoMate Scientific, Berkeley, CA) was used to deliver external solutions at a flow rate of 1 mL/min. Recordings were performed on postsynaptic ACs whose processes were in contact with unclamped presynaptic ACs and that had spontaneous postsynaptic currents (sPSCs). Transfected cell recordings
were performed on postsynaptic tdTomato-expressing cells that were in contact with other tdTomato expressing cells. Cells that did not have basal sPSCs also did not respond to SNAP and were excluded from the analysis.

**Calcium imaging**

The fluorescent Ca\(^{2+}\) indicator OGB (2 µM) was prepared as previously described (Maddox and Gleason 2017). Images were taken every 3 s (200 ms exposure). To obtain data relevant to synaptic function, regions of interest (ROIs) were chosen at sites of contact between processes. Images were captured on an inverted Olympus IX70 microscope (Tokyo, Japan) fitted with a SensiCam QE (Cooke, Kelheim, Germany). ROIs used to obtain data in transfected cell processes expressing tdTomato were selected in the tdTomato channel (ex: 554, em: 581) prior to Ca\(^{2+}\) imaging (ex: 494, em: 523). Data were collected and analyzed using Slidebook software (Intelligent Imaging Innovations, Denver, CO). In all Ca\(^{2+}\) imaging experiments, the control fluorescence mean was calculated by using the average of the last 10 ROI time points before switching solutions, and the fluorescence means after switching solutions were calculated by using the 10 ROI time points from the middle of each solution application. The control and different solution fluorescence means were used to calculate percent change in fluorescence.

**Immunocytochemistry**

Transfected and non-transfected cells on coverslips were fixed using 2% paraformaldehyde in PBS for 30 min at 4 °C and then washed 4 X with 30 mM glycine in PBS. The last wash contained 0.1% triton X-100 for membrane permeabilization. Fixed cells were immediately blocked with 5% normal goat serum in dilution solution (1%
BSA/0.5% saponin in PBS) for 30 min at 4 °C. TRPC5 primary monoclonal antibody (Sigma-Aldrich, Cat: SAB140977) was diluted in dilution solution (5 µg/mL) and incubated for 2 hrs at room temperature. Goat anti-mouse Dylight-488 (Thermo Fisher Scientific) secondary antibody was diluted 1:500 in dilution solution and incubated for 1 hr at room temperature. Coverslips were mounted on plain glass microscope slides (Thermo Fisher) using ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher) and cured overnight. Labeled cells were viewed on an inverted Olympus IX70 microscope (Tokyo, Japan), fitted with a SensiCam QE (Cooke, Kelheim, Germany). Images were captured and analyzed using Slidebook 5.5 software (Intelligent Imaging Innovations, Denver, CO).

Immunohistochemistry

The use of adult chickens to prepare retinal sections and brain protein preps was approved by the LSU Institutional Animal Care and Use Committee. Adult white leghorn chickens (4-7 weeks old) were sacrificed by intraperitoneal injection of 250 mg/kg sodium pentobarbital (Sigma-Aldrich) and decapitated. Eyes were enucleated and hemisected. The eye cup was fixed in 4% paraformaldehyde for 1 hr at room temperature. The retina was removed and was washed 3 X with PBS containing 1% glycine. Retinas were infused with 15% sucrose for 30 min, 20% sucrose for 1 hr, and 30% sucrose at 4°C overnight. Retinas were placed in 1:1 v/w 30% sucrose and O.C.T. compound (Sakura Finetek, Torrence, CA) for 30 min. Retinas in fresh sucrose/OCT mixture were frozen in a dry ice/isopentane bath. Retinas were cryosectioned at ~15-18 µm on a Leica CM1850 cryostat (Wetzlar, Germany) and mounted on Superfrost Plus Micro Slides (VWR, Radnor, PA).
Sections were washed PBS for 10 min to remove the sucrose/O.C.T. mixture and blocked with 5% normal goat serum in dilution solution at 4 °C for 30 min. The primary TRPC5 antibody (Sigma-Aldrich) was diluted (5 µg/mL) in dilution solution and incubated on blocked sections overnight at 4 °C. Sections were washed 3 X with PBS. The goat anti-mouse Dylight-555 secondary antibody (Thermo Fisher) was diluted 1:500 in dilution solution and incubated in the dark for 1 hr at room temperature. Labeled sections were mounted with #1.5 coverslips using ProLong Diamond Antifade mountant with DAPI (Thermo Fisher). Labeled sections were viewed using an inverted Leica TCS SP8 Spectral confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 40X oil immersion objective (1.25 N.A.). Dylight-555 was observed with white light laser set to 562 nm with HyD detector emission collection from 573-600 nm. Images were captured using the Leica LAS X software package. Each z-plane was an average of 32 line scans with 0.3 µm steps. The brightness of sum intensity projection images of 40 z-planes were adjusted using ImageJ (NIH).

**Western blot**

Adult chickens were sacrificed as described above. Chicken brains were flash frozen with liquid nitrogen and stored at -80°C until use. Brains were homogenized on ice in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8.0) containing phosphatase inhibitor cocktail 2 (Sigma-Aldrich) and protease inhibitor cocktail (Sigma-Aldrich). Homogenates were centrifuged at 17,000 x g for 20 min at 4°C. The supernatant was immediately aliquoted and stored at -80°C. Protein concentration was determined using the BCA Protein Assay Kit from Pierce (Rockford, IL). Prior to
electrophoresis, protein samples were prepared in Laemmli buffer containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue, and 62.5 mM Tris. Protein samples (20 µg) in Laemmli buffer were incubated at 60 °C for 30 min. Proteins were separated on an 8% bis-acrylimide/SDS gel with 10 µL of Precision Plus Protein Dual Color Standard (BioRad, Hercules, CA). Separated proteins were transferred onto nitrocellulose membrane at 4 °C overnight. The membrane was blocked with 1X blocking buffer (Abcam, Cambridge, MA) in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 hr at room temperature. The blocking buffer was replaced with the diluted primary anti-TRPC5 antibody (1:1000) in 1X blocking buffer/TBS-T and incubated overnight at 4 °C. The membrane was washed 3 X with TBS-T. The goat anti-mouse secondary antibody conjugated with horseradish peroxidase (BioRad) was diluted 1:5,000 in 1X blocking buffer/TBS-T. The membrane was incubated with the secondary antibody for 1 hr. The membrane was washed three times with TBS-T. Protein was visualized using the Clarity chemiluminescence kit (BioRad) and the ChemiDoc XRS+ system (BioRad). Western blot images were captured using the Image Lab Software (BioRad).

**TRPC5 gene mutation analysis**

To detect indels (insertions/deletions) in the 2nd exon of TRPC5 by non-homologous end joining induced by Cas9-mediated dsDNA cleavage, genomic DNA (gDNA) from whole dishes containing transfected and non-transfected cells was isolated using Arcturus PicoPure kit (Thermo Fisher). The 2nd exon of TRPC5 was PCR amplified using Q5 Hotstart High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) and primers that were designed to anneal within the introns flanking the
2nd exon (gDNA-TRPC5-F: GGGGTTAGTCTCGTGAGCATT; gDNA-TRPC5-R: GTACCACATTGCTCTGGGC). Correct PCR amplification was verified by sequencing (Eurofins Genomics, Louisville, KY). Purified gDNA PCR products (~721 bp) from pCRISPR-TRPC5 transfections and pSpCas9-tdTomato transfections were used in the T7 Endonuclease I (T7E1) mutation detection assay (NEB), which recognizes and cleaves mismatched dsDNA. PCR products (200 ng) from each gDNA amplification (pCRISPR-TRPC5 transfections and pSpCas9-2A-tdTomato) were denatured and slowly reannealed to allow heteroduplex formation. Each heteroduplexed DNA sample was digested with T7E1 (10 U) for 30 min at 37 °C. DNA (50 ng) from each T7E1 digestion was separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized using the ChemiDoc XRS+ system (BioRad). Images were captured and analyzed using the Image Lab Software (BioRad).

Data analysis

For sPSC electrophysiology experiments, Mini Analysis Program (Synaptosoft, Inc, Fort Lee, NJ) was used to detect and analyze sPSCs. For immunocytochemistry experiments on transfected cells, slidebook software (Intelligent Imaging Innovations) was used to create a threshold mask in the TRPC5 channel based on non-transfected cells labeled with only the secondary antibody. The threshold mask for TRPC5 was applied to images of cells transfected with pSpCas9-tdTomato (Control) and pCRISPR-TRPC5 (TRPC5 KD). Due to their thickness, cell bodies were removed from the TRPC5 mask in all images, and only processes with tdTomato expression were used for analysis. Slidebook software automatically calculated TRPC5 mask descriptive statistics for TRPC5 objects > 5 pixels for total TRPC5 object area, TRPC object size,
TRPC5 object intensity, and tdTomato intensity in TRPC5 object area. Welch’s t-tests were performed on TRPC5 mask descriptive statistics. Repeated Measures-ANOVA with Fisher’s Least Significant Difference (LSD) post hoc analysis was performed on all Ca\(^{2+}\) imaging and electrophysiology data unless otherwise indicated. Statistics were performed using GraphPad Prisim 7 (GraphPad Software, La Jolla, CA). Data are reported as mean ± SE.

**Results**

**PLC activity is required for the NO-dependent GABA release from retinal ACs**

We previously demonstrated that nitric oxide (NO) enhances spontaneous and evoked Ca\(^{2+}\)-dependent GABA release by activating a transient receptor potential canonical (TRPC) channel-mediated Ca\(^{2+}\) influx pathway, without the requirement of Ca\(^{2+}\) stores, action potentials, or activation of the NO receptor sGC (Maddox and Gleason 2017). All TRPC family members (1-7) are known to be activated, albeit by different mechanisms, by downstream phospholipase C (PLC) activity (Svobodova and Groschner 2016). To determine if PLC activity is required for the NO-dependent increase in spontaneous postsynaptic current (sPSC) frequency, the effects of the broad PLC inhibitor U73122 (10 µM) was tested. The NO donor SNAP (500 µM) caused a significant increase in sPSC frequency by 68.3% (control, 24.9 ± 8.4/30 s, SNAP, 41.9 ± 10.3/30 s, \(P = 0.0012, n = 8\), Figure 3.1A,B). Application of the PLC inhibitor U73122 together with SNAP significantly reduced the NO-dependent increase in sPSC frequency by 74.1% (SNAP, 41.9 ± 10.3/30 s, SNAP/U 73122, 10.89 ± 2.5/30 s, \(P = 0.017, n = 8\), Figure 3.1A,B). U73343 (the less active analog of U73122) did not influence the NO-dependent increase in sPSC frequency (SNAP, 38.6 ± 9.8/30 s,
SNAP/U 73343, 37.8 ± 6.0/30 s, P = 0.85, n = 8, Figure 3.1C,D). Additionally, application of U73122 alone significantly reduced basal sPSC frequency by 61.5% (control, 15.81 ± 3.3/30 s, U73122, 6.09 ± 0.92/30 s, P = 0.0096, n = 10, paired t-test, Figure 3.1E,F). U73343, however, did not alter basal sPSC frequency (control, 38.7 ± 12.04/30 s, U73343, 28.96 ± 6.9/30 s, P = 0.23, n = 6, paired t-test, Figure 3.1E,F).

These results suggest PLC activity is required for basal spontaneous GABA release. Although TRPC channels can be activated independent of PLC activity, the NO-dependent increase in spontaneous GABA release cannot overcome the requirement of PLC activity.

**The NO-dependent increase in sPSC frequency is sensitive to La³⁺**

We have previous shown that the NO-dependent increase in cytosolic Ca²⁺ and sPSC frequency is blocked by clemizole, a TRPC channel inhibitor with highest affinity for TRPC5 and TRPC4, respectively (Maddox and Gleason 2017). Additionally, we now show that downstream PLC activity is required for the NO-dependent increase in sPSC frequency. Together, these findings argue for the involvement of a TRPC channel, possibly TRPC4 or TRPC5. Unique to TRPC4 and TRPC5, mM concentrations of lanthanides block these channels currents and µM concentrations potentiate channel activity (Jung et al. 2003). To test whether TRPC4 or TRPC5 are involved, the effects of La³⁺ were evaluated. In 2 mM La³⁺, SNAP was unable to increase sPSC frequency (2 mM La³⁺, 5.4 ± 1.9/30 s, 2 mM La³⁺/SNAP, 4.8 ± 1.4/30 s, P = 0.52, n = 8, Figure 3.2A,B). When 2 mM La³⁺ was removed, SNAP caused a significant increase in sPSC frequency by 639% (SNAP/La³⁺, 4.8 ± 1.4/30 s, SNAP, 35.3 ± 13.4/30 s, P = 0.04, n = 8, Figure 3.2A,B). Conversely, after NO caused a significant increase in sPSC
frequency (control, 20.08 ± 4.97/30 s, SNAP, 32.23 ± 6.26/30 s, \( P = 0.025, n = 9 \), Figure 3.2C,D), 10 µM La\(^{3+}\) enhanced the NO-dependent increase in sPSC frequency by 77.5% (SNAP, 32.23 ± 6.26/30 s, SNAP/10 µM La\(^{3+}\), 57.23 ± 12.96/30 s, \( P = 0.018, n = 9 \), Figure 3.2C,D). This differential effect of La\(^{3+}\) on sPSC frequency suggests that the NO-dependent increase in sPSC frequency is mediated by TRPC4 and/or TRPC5.

Figure 3.1. PLC activity is required for the NO-dependent increase in sPSC frequency. A: representative recording from a postsynaptic AC voltage clamped at -70 mV. The PLC inhibitor U73122 (10 µM) reduced the NO-dependent increase in sPSC frequency. B: quantified mean event frequency/30 s ± SE; \( n = 10 \). *\( P < 0.05 \), **\( P < 0.01 \). C: representative recording from a postsynaptic AC voltage clamped at -70 mV. The less active U73343 (10 µM) did not affect the NO-dependent increase in sPSC frequency. D: quantified mean event frequency/30 s ± SE; \( n = 6 \). *\( P < 0.05 \), **\( P < 0.01 \). E,F: representative recordings from postsynaptic ACs voltage clamped at -70 mV. Perfusion of only U73122 reduced sPSCs frequency (E). Perfusion of only U73343 had no effect on sPSC frequency (F). G,H: quantified mean event frequency/30 s ± SE; \( n = 10 \) (G), \( n = 6 \) (H). **\( P < 0.01 \) (paired \( t \)-test).
Figure 3.2. The NO-dependent increase in sPSC frequency is sensitive to La$^{3+}$. 

A: representative recording from postsynaptic AC voltage clamped at -70 mV. 2 mM La$^{3+}$ prevented the NO-dependent increase in sPSC frequency when exposed to the NO donor SNAP. When 2 mM La$^{3+}$ was removed and SNAP remained, NO increased the sPSC frequency. 

B: quantified mean event frequency/30 s ± SE; $n = 8$. *$P < 0.05$. 

C: representative recordings from postsynaptic ACs voltage clamped at -70 mV. 10 µM La$^{3+}$ enhanced the NO-dependent increase in sPSC frequency. 

D: quantified mean event frequency/30 s ± SE; $n = 9$. *$P < 0.05$, **$P < 0.01$. 

ML204 can block the NO-dependent increase in cytosolic Ca$^{2+}$

ML204 preferentially inhibits TRPC4 channels with an IC$_{50}$ of 1 µM in Ca$^{2+}$ imaging and 3 µM in whole-cell current recordings (Miller et al. 2011). In over expression cell lines, 10 µM ML204 nearly inhibits TRPC4 currents completely and inhibits TRPC5 currents by ~ 68 % (Miller et al. 2011), suggesting that higher concentrations of ML204 are needed to sufficiently block TRPC5. To determine if 10 µM ML204 can block the NO-dependent increase in cytosolic Ca$^{2+}$, changes in cytosolic Ca$^{2+}$ in regions of process contacts (potential synaptic sites) were monitored using the fluorescent Ca$^{2+}$ indicator Oregon Green BAPTA-488 that was preloaded into ACs.
ML204 alone had no effect on cytosolic Ca\(^{2+}\) (control, 8.19 ± 0.59 A.U., 10 µM ML204, 8.09 ± 0.58 A.U., \( P = 0.093, n = 53, \) Figure 3.3A,B). While 10 µM ML204 was still present, the NO donor SNAP caused a significant increase in cytosolic Ca\(^{2+}\) by 6.2% (10 µM ML204, 8.09 ± 0.58 A.U., 10 µM ML204/SNAP, 8.6 ± 0.56 A.U., \( P = 0.001, n = 53, \) Figure 3.3A,B). Cytosolic Ca\(^{2+}\) remained elevated when 10 µM ML204 was removed and only SNAP remained (10 µM ML204/SNAP, 8.6 ± 0.56 A.U., SNAP, 8.78 ± 0.61 A.U., \( P = 0.46, n = 53, \) Figure 3.3A,B). Also, 10 µM ML204 did not block the NO-dependent increase in sPSC frequency (10 µM ML204, 13.06 ± 2.5/30 s, 10 µM ML204/SNAP, 21.78 ± 3.7/30 s, \( P = 0.027, n = 9, \) Figure 3.3C,D). To further test the effectiveness of ML204 on the NO response, the concentration of ML204 was increased to 20 µM. Co-application of 20 µM ML204 and SNAP blocked the NO-dependent increase in cytosolic Ca\(^{2+}\) (20 µM ML204, 6.6 ± 0.3 A.U., 20 µM/SNAP, 6.51 ± 0.3 A.U., \( P = 0.15, n = 102, \) Figure 3.3E,F) and the NO-dependent increase in sPSC frequency (20 µM ML204, 20.38 ± 4.8/30 s, 20 µM ML204/SNAP, 23.78 ± 5.5/30 s, \( P = 0.48, n = 12, \) Figure 3.3G,H). When 20 µM ML204 was removed and only SNAP remained, NO caused a significant increase in cytosolic Ca\(^{2+}\) by 5.3% (20 µM ML204/SNAP, 6.51 ± 0.3 A.U., SNAP, 6.85 ± 0.32 A.U., \( P < 0.0001, n = 102, \) Figure 3.3E,F) and in sPSC frequency by 70% (20 µM ML204/SNAP, 23.78 ± 5.5/30 s, SNAP, 35.35 ± 6.7/30 s, \( P = 0.02, n = 12, \) Figure 3.3G,H). The ability for ML204 to block the NO-dependent response at 20 µM but not 10 µM suggests that
NO is acting on TRPC5, not TRPC4, to activate the NO-dependent Ca\(^{2+}\) influx pathway to enhance Ca\(^{2+}\)-dependent GABA release.

Figure 3.3. The TRPC4/C5 inhibitor ML204 can block the NO-dependent increase in cytosolic Ca\(^{2+}\) and sPSC frequency at higher concentrations. A: co-application of 10 µM ML204 and NO donor SNAP did not prevent the NO-dependent increase in cytosolic Ca\(^{2+}\). Cytosolic Ca\(^{2+}\) remained elevated upon removal of 10 µM ML204 from the bath. B: quantified mean fluorescence ± SE; n = 53. C: current recording from a representative amacrine cell voltage clamped at -70 mV. 10 µM ML204 was not effective at blocking the NO-dependent increase in sPSC frequency. D: quantified mean event frequency/30 s ± SE; n = 13. E: 20 µM ML204 blocked the NO-dependent increase in cytosolic Ca\(^{2+}\). NO increased cytosolic Ca\(^{2+}\) after removing 20 µM ML204 from the bath and SNAP remained. F: quantified mean fluorescence ± SE; n = 102. G: current recording from a representative amacrine cell voltage clamped at -70 mV. 20 µM ML204 blocked the NO-dependent increase in sPSC frequency. NO increased sPSC frequency upon removal of 20 µM ML204 from the bath and SNAP remained. H: quantified mean event frequency/30 s ± SE; n = 12. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns, not significant. Fluorescence over time data are plotted as mean fluorescence ± SE.
TRPC5 is expressed in ACs in culture and retina

Inhibitors for the TRPC channels are not completely specific for a single TRPC family member, however, previous experiments using clemizole, which preferentially inhibits TRPC5 (Maddox and Gleason 2017), blocked NO-dependent dendritic Ca$^{2+}$ elevations and the increase in sPSC frequency. Here, 10 µM ML204 did not block the NO response, however, 20 µM ML204 blocked the NO-dependent increase in cytosolic Ca$^{2+}$ and the increase in sPSC frequency (Figure 3.3). The results using these two inhibitors suggests that NO may be activating TRPC5, not TRPC4, to admit Ca$^{2+}$ into the presynaptic terminal and enhance vesicular GABA release. To determine if ACs in culture and the adult retina express TRPC5, we used a monoclonal TRPC5 antibody raised against amino acids 534 – 603 of the human TRPC5 protein (96% antigen homology with the chicken TRPC5). This antibody recognizes a single band in a western blot around the appropriate molecular weight of TRPC5 (112 kDa) in chicken brain homogenate (Figure 3.4A). The TRPC5 antibody recognizes cell bodies and processes in cultured ACs (Figure 3.4B) and AC bodies (arrowheads, Figure 3.4C) and processes arising from AC bodies into the IPL (arrowheads, Figure 3.4D) in the retina. Additionally, there was TRPC5 labeling in Müller cell bodies in the INL (arrows, Figure 3.4C) and in cell bodies in the GCL (asterisks, Figure 3.4C). The punctate TRPC5 labeling pattern in the IPL, where ACs form synapses, suggests that TRPC5 forms clusters in the IPL, possibly at AC presynaptic terminals.

CRISPR/Cas9-mediated dsDNA break of the TRPC5 gene reduces TRPC5 expression

To confirm that TRPC5 is the Ca$^{2+}$ permeable channel involved in the NO response, a CRISPR/Cas9 system targeting the TRPC5 gene was developed to induce
Figure 3.4. Amacrine cells in culture and adult retina express TRPC5. 

A: Western blot for TRPC5 in chicken brain protein with the monoclonal TRPC5 (1C8 clone) antibody recognizes a single band at the appropriate molecular weight (112 kDa). B: an amacrine cell in culture immunolabeled with the TRPC5 antibody and DNA marker DAPI. C: adult chicken retina immunolabeled with the TRPC5 antibody. Likely Müller cell bodies (arrows) and amacrine cell bodies (arrow heads) within the INL are clearly labeled. Cell bodies in the GCL are also labeled (asterisks). Strong punctate labeling is present in the IPL, suggesting that TRPC5 clusters within processes D: expanded view of the box in C. TRPC5 is present within a process arising from an amacrine cell body (arrow heads). PR, photoreceptors; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

dsDNA breaks in the 2nd exon of the TRPC5 gene (Ran et al. 2013). After dsDNA breaks, the cellular DNA repair machinery will generate insertions/deletions (indels) at the site of the dsDNA break, which will lead to reduced expression or expression of non-functional protein. To confirm that a Cas9-mediated dsDNA break within the 2nd exon of TRPC5 promoted indels, we employed the T7 endonuclease I (T7E1) mutation detection assay (NEB). Heteroduplexed PCR products of the TRPC5 2nd exon from...
cultures transfected with the control plasmid pSpCas9-tdTomato were not digested by T7E1, therefore, no indels were present (Figure 3.5A, left lane, control). Cultures transfected with pCRISPR-TRPC5 contained indels because heteroduplexed PCR products were digested by T7E1 into the predicted sizes based on the Cas9-targeted cut site (Figure 3.5A, right lane, TRPC5 KD).

To confirm CRISPR/Cas9-mediated TRPC5 dsDNA breaks lead to reduced expression of TRPC5, ACs that were transfected with either pSpCas9-tdTomato (control, Figure 3.5B-D & Bi-Di) or pCRISPR-TRPC5 (TRPC5 KD, Figure 3.5E-G & Ei-Gi) were immunolabeled using the same TRPC5 antibody in Figure 3.4. CRISPR/Cas9-mediated dsDNA breaks in the TRPC5 gene reduced total dendritic TRPC5 labeled area (control, 9.67 ± 1.0 µm², n = 30, TRPC5 KD, 1.95 ± 0.2 µm², n = 43, P < 0.0001, Welch’s t-test, Figure 3.5H), TRPC5 object size (control, 125.4 ± 12.41 nm², n = 30, TRPC5 KD, 76.36 ± 3.66 nm², n = 43, P = 0.0006, Welch’s t-test, Figure 3.5I), and TRPC5 object intensity (control, 15019 ± 1419 A.U., n = 30, TRPC5 KD, 11454 ± 789.4 A.U., n = 43, P = 0.03, Welch’s t-test, Figure 3.5J). There was no significant difference in tdTomato fluorescence within the TRPC5 object domain (control, 120103 ± 12018 A.U., n = 30, TRPC5 KD, 101802 ± 7154 A.U., n = 43, P = 0.2, Welch’s t-test, Figure 5K).

TRPC5 is required for the NO-dependent increase in cytosolic Ca^{2+} and GABA release

The CRISPR/Cas9-mediated knockdown of TRPC5 was used to investigate the role of TRPC5 in the NO-dependent Ca^{2+} influx and enhancement of GABA release. In ACs transfected with the control plasmid pSpCas9-tdTomato, NO caused a 3% increase in cytosolic Ca^{2+} (control, 6.28 ± 0.39 A.U., SNAP, 6.5 ± 0.39 A.U., n = 149, P < 0.0001,
Figure 3.5. CRISPR/Cas9-mediated TRPC5 mutagenesis reduces TRPC5 expression in amacrine cell processes. A: T7 endonuclease I (T7EI) indels (insertions/deletions) detection assay. The 2nd TRPC5 exon genomic DNA region from cultures transfected with either pSpCas9-tdTomato (Control) or pCRISPR-TRPC5 (TRPC5 KD) was PCR amplified, denatured, and slowly reannealed to form mismatched heteroduplex DNA (hdDNA). hdDNA was then digested with T7EI, which recognizes mismatched nucleotides. T7EI only recognized and digested hdDNA mismatched nucleotides from TRPC5 KD into predicted sizes (408 bp and 313 bp). B-G, amacrine cells transfected with pSpCas9-tdTomato (Control, B-D) or pCRISPR-TRPC5 (TRPC5 KD, E-G) and immunolabeled for TRPC5. Scale bars, 10 µm. Insets in B-G correspond to Bi-Gi, respectively. Scale bars, 5 µm. H-K, quantification of TRPC5 labeling in processes from pSpCas9-tdTomato (control) and pCRISPR-TRPC5 (TRPC5 KD) transfected cells. TRPC5 KD significantly reduced the total area of TRPC5 immunofluorescence (H), TRPC5 object size (> 5 pixels, I), intensity (int.) of TRPC5 object (> 5 pixels) immunofluorescence (J), and TRPC5 immunofluorescence intensity that falls within tdTomato fluorescence (K). Mean (dotted line) ± SE. All data points are plotted. Control (light grey circles), n = 30. TRPC5 KD (dark grey circles), n = 43. *P < 0.05, ***P < 0.001 ****P < 0.0001 (Welch’s t-test).
Figure 3.6A-B) and a 47.5% increase in sPSC frequency (control, 16.5 ± 4.2/30 s, SNAP, 24.3 ± 4.7/30 s, n = 9, P = 0.009, Figure 3.6C-D). In ACs transfected with pCRISPR-TRPC5, NO did not increase cytosolic Ca^{2+} (control, 4.53 ± 0.23 A.U., SNAP, 4.44 ± 0.23 A.U., n = 255, P < 0.0001, Figure 3.6E-F) and did not increase sPSC frequency (control, 19.84 ± 4.99/30 s, SNAP, 20.11 ± 5.55/30 s, n = 9, P = 0.78, Figure 3.6G-H). These data confirm that TRPC5 is required for the NO-dependent influx of Ca^{2+} into the presynaptic terminal and enhanced Ca^{2+}-dependent vesicular GABA release.

Discussion

To our knowledge, this is the first report demonstrating that a member of the canonical TRP family, TRPC5, mediates NO-dependent vesicular neurotransmitter release at a GABAergic synapse. Here, we find the action of NO on sPSC frequency is dependent on downstream activity of PLC, is blocked with 2 mM La^{3+}, and is enhanced by 10 µM La^{3+}. The requirement of PLC activity and dual effects of La^{3+} are characteristic of TRPC4 and TRPC5 channels. The TRPC4/C5 inhibitor ML204 at 10 µM did not block the NO response, however 20 µM ML204 prevented the NO-dependent increase in dendritic Ca^{2+} and sPSC frequency. CRISPR/Cas9-mediated genetic knockdown of TRPC5 using a guide RNA designed to target Cas9 to the 2^{nd} exon of TRPC5 precluded an NO-dependent increase in dendritic Ca^{2+} and sPSC frequency. Additional evidence that further supports TRPC4 is not involved in the NO-response is provided by previous work in the Gleason lab that demonstrated TRPC4 is not expressed by ACs in culture or by ACs in adult retina (Crousillac et al. 2003). These results confirm that NO enhances voltage-independent vesicular neurotransmitter release.
Figure 3.6. Knockdown of TRPC5 eliminates the NO-dependent increase in cytosolic Ca$^{2+}$ and sPSC frequency. A-D, data from amacrine cells transfected with pSpCas9-tdTomato (Control). A, The NO-dependent increase in cytosolic Ca$^{2+}$ in amacrine cell processes was still present in control cells. B, quantified mean fluorescence ± SE, n = 149. ****P < 0.0001. C, recording from a representative amacrine cell voltage clamped at -70 mV and in contact with other transfected amacrine cells. SNAP still caused an increase in sPSC frequency in cells transfected pSpCas9-tdTomato. D, quantified mean event frequency/30 s ± SE; n = 9. **P < 0.01, paired t-test.

E-F, data from amacrine cells transfected with pCRISPR-TRPC5 (TRPC5 KD). E, The NO-dependent increase in cytosolic Ca$^{2+}$ was absent in amacrine cells transfected with pCRISPR-TRPC5. F, quantified mean fluorescence ± SE; n = 255. G, recording from a representative amacrine cell transfected with the pCRISPR-TRPC5. Cells were voltage clamped at -70 mV and were in contact with other transfected amacrine cells. SNAP was unable to cause an increase in sPSC frequency in cells transfected with pCRISPR-TRPC5. H, quantified mean event frequency/30 s ± SE; n = 9. P = 0.78 (paired t-test).

release at retinal AC GABAergic synapses via a TRPC5-mediated Ca$^{2+}$ influx into the presynaptic terminal.

TRPC5 in the retina and brain

First identified in a Drosophila melanogaster mutant screen for visual defects (Cosens and Manning 1969), the transient receptor potential (TRP) channel was named
due to the shortened response of photoreceptors under intense illumination (Minke 2010; Minke et al. 1975). Almost all members of the TRP channel superfamily are expressed with varying degree in the vertebrate retina, however, the role of TRP channels in visual signal processing in the vertebrate retina remains to be fully explored. Recent work has identified a vertebrate TRP channel of the melastatin subfamily, TRPM1, necessary for depolarizing light responses of retinal ON-bipolar cells (Koike et al. 2010; Morgans et al. 2009). Additionally, patients that suffer from complete congenital stationary night blindness have a TRPM1 gene mutation (Audo et al. 2009; Nakamura et al. 2010). Here, we identified a TRP channel in the canonical subfamily, TRPC5, that may be involved in processing the visual signal by enhancing GABAergic inhibition in the inner retina.

In the brain, studies have identified that TRPC5 has an essential role in dendritic length, patterning, and growth cone morphology in developing hippocampal neurons (Greka et al. 2003; He et al. 2012; Puram et al. 2011). One study found that binding of epidermal growth factor leads to a rapid insertion of readily available vesicles containing TRPC5, and this pathway is needed for proper hippocampal neuron development (Bezzerides et al. 2004). The role of TRPC5 in mature neurons is not fully understood. Two studies, however, have identified separate roles for TRPC5 in mature neurons in the brain including innate fear processing in the amygdala (Riccio et al. 2009) and seizures and seizure-induced cell death in the hippocampus (Phelan et al. 2013). To date, there are no published studies investigating the physiological role of TRPC5 in the retina.
In the mouse retina, TRPC5 expression is inner row of the inner nuclear layer (INL) where AC bodies lie (Gilliam and Wensel 2011). However, a separate study characterizing dopaminergic interplexiform processes in the mouse retina found TRPC5 immunolabeling to be mostly restricted to the synaptic layers, inner plexiform layer (IPL) and outer plexiform layer (OPL) (Witkovsky et al. 2008). The TRPC5 labeling pattern presented here is comparable to the mouse retina and was found to be in cell bodies within the inner half of the INL (likely Müller cells and ACs) and cell bodies in the ganglion cell layer. Additionally, there was extensive punctate labeling within the IPL. The punctate labeling within the IPL suggests that TRPC5 might form clusters at possible synaptic sites, however, synaptic localization of TRPC5 within the retina remains unknown.

**Identification of NO-dependent activation of TRPC5**

PLC downstream activity is known to be an activator of all TRPC channels, however, the PLC activity-dependent activation of each TRPC family member is not fully understood. Two studies have demonstrated that TRPC4 (Otsuguro et al. 2008) and TRPC5 (Obukhov and Nowycky 2004; Trebak et al. 2009) are inhibited by phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and become activated once PLC relieves this inhibition by cleaving PIP$_2$ into inositol trisphosphate (IP$_3$) and diacylglycerol (DAG). A previous study using the global PLC inhibitor U73122 and the disulfide agent 2,2'-dithiobis(5-nitropyridine) demonstrated in an over expression system that direct activation of TRPC5 via cysteine thiol modification did not require PLC activity (Yoshida et al. 2006). The results presented here suggest that that PLC activity is require for the NO-dependent activation of TRPC5 and increase in sPSCs. U73122, however, also
abolished almost all baseline sPSCs. This suggests S-nitrosylation-dependent activation of natively expressed TRPC5 in ACs may not overcome the PLC-dependence of spontaneous GABA release. Of the two known mechanisms in which NO functions (the NO-sGC pathway and S-nitrosylation), we previously established that the NO-sGC pathway is not involved in the NO response. Although the exact mechanism in which NO activates TRPC5 to enhance vesicular GABA release remains unknown, TRPC5 may have a physiological role in the inner retina to enhance inhibition.

Effects of NO on amacrine cells

NO has previously been shown to enhance the release of GABA in all layers of the turtle retina (Yu and Eldred 2005), however, this was indirectly demonstrated by the NO-dependent reduction in labeling of a preloaded GABA analog, γ-vinyl-GABA (GVG) (Yu and Eldred 2005). Interestingly, the NO-dependent reduced GVG labeling was largely dependent on extracellular Ca$^{2+}$ in the IPL, which is where processes from amacrine cells ramify and form synapses (Yu and Eldred 2005). Another study found that NOS-stimulated NO production with its substrate L-arginine dampens both ON and OFF light responses in all major ganglion cell types, with the OFF response being the most sensitive to NO (Wang et al. 2003). Since NO only affected the intrinsic ganglion cell membrane properties of a small population of each ganglion cell type, it was suggested that NO works at a site immediately presynaptic to ganglion cells (likely amacrine cells) to increase inhibition and dampen the light responses (Wang et al. 2003). Incorporating the findings of these two studies with the findings in my dissertation, NO may have reduced ganglion cell light responses (Wang et al. 2003) by
increasing Ca\(^{2+}\)-dependent GABA release (Yu and Eldred 2005) through the NO-dependent TRPC5-mediated Ca\(^{2+}\) influx into amacrine cells.

**Multiple effects of NO on amacrine cell synapses**

We previously demonstrated that NO activates a Ca\(^{2+}\) influx pathway that is sufficient to drive vesicular exocytosis and GABA release without depolarization (Maddox and Gleason 2017). Here, using the TRPC4/C5 inhibitor ML204 and CRISPR/Cas9-mediated knockdown of gene expression, we identify the channel responsible for the NO-dependent Ca\(^{2+}\) influx as TRPC5. Previous work in the Gleason lab determined that higher concentrations of NO (~2 µM) transiently alter inhibitory postsynaptic voltage responses in ACs by releasing Cl\(^{-}\) from internal acidic compartments (Hoffpauir et al. 2006; Krishnan and Gleason 2015). This Cl\(^{-}\) release shifts the reversal potential of GABA-gated currents more positive and changes postsynaptic voltage responses to less inhibitory or even excitatory. Taken together, these results suggest that locally generated NO in the inner retina can affect both pre- and postsynaptic sites: voltage-independent GABA release is enhanced and GABA action on the postsynaptic amacrine cell can lead to depolarization. If these actions co-occur, then they will enhance inhibitory output from the downstream AC (See Figure 3.7). The patterns of NO production under different stimulus conditions will determine the sites of enhanced GABAergic output and thus the impact on the signals that ganglion cells send to visual centers in the brain.
Figure 3.7. NO has pre- and postsynaptic effects in ACs. A: Simplified cartoon of a GABAergic AC (light grey) in the retina receiving glutamatergic input from a bipolar cell (BC) and presynaptic to another AC (Left). NO synthesis in a NOS expressing AC (red) can elicit changes in both pre- and postsynaptic processes (Right). B: Expanded view of the box in A, depicting presynaptic and postsynaptic effects of NO. Top, Activation of a Ca\(^{2+}\)-dependent isoform of NOS (eNOS or nNOS) produces NO (1.). NO activates TRPC5, possibly via S-nitrosylation (S-NO?), to admit Ca\(^{2+}\) into the presynaptic terminal (2.). Ca\(^{2+}\) elevations in the presynaptic terminal lead to increased vesicle fusion events and GABA release (3.) onto its postsynaptic partner. VGCC, voltage-gated Ca\(^{2+}\) channel; GluR, ionotrophic glutamate receptor. Bottom, NO diffuses into the postsynaptic terminal and induces Cl\(^{-}\) release from acidic organelles (4., Krishnan and Gleason, 2015). Increased [Cl\(^{-}\)] in the postsynaptic terminal reverses the Cl\(^{-}\) gradient, converting typical GABAergic inhibition into depolarization (5., Hoffpaurir et al. 2006). Depolarization (+) leads to activation of VGCCs in an adjacent presynaptic site in the same process (6.). Ca\(^{2+}\) influx through activated VGCCs leads to enhanced GABA release (7.). The postsynaptic partner that is downstream from the targets of NO will experience increased inhibition (8.).
References


CHAPTER 4
CONCLUSIONS

In Chapter 2, I provide direct evidence that NO enhances neurotransmitter release at retinal AC GABAergic synapses, independent of the canonical NO receptor, sGC, and presynaptic action potentials. Ca\(^{2+}\) imaging at regions of process contacts and whole-cell current recordings of spontaneous and evoked GABA release revealed that NO activates a Ca\(^{2+}\) influx pathway, independent of Ca\(^{2+}\) release from stores. The TRPC inhibitor clemizole, which preferentially blocks TRPC5, blocked the NO-dependent Ca\(^{2+}\) elevation and the increase in sPSC frequency. Since NO is endogenously produced in vertebrate retinas (Blom et al. 2012; Eldred and Blute 2005; Tekmen-Clark and Gleason 2013), these findings suggest that NO enhances inhibition by activating a TRPC-mediated Ca\(^{2+}\) influx and enhancing Ca\(^{2+}\)-dependent vesicular GABA release from ACs.

In Chapter 3, I determined that the Ca\(^{2+}\) permeable, nonselective cation channel TRPC5 mediates the NO-dependent Ca\(^{2+}\) influx and increase in GABA release. The first pieces of evidence that suggested the involvement TRPC5 was that downstream PLC activity is required and that 2 mM La\(^{3+}\) blocks and 10 µM La\(^{3+}\) enhances the NO-dependent response. The requirement of PLC activity and the dual effects of La\(^{3+}\) on the NO response are unique characteristics of TRPC4 and TRPC5 channel activity. ML204, another TRPC inhibitor that preferentially blocks TRPC4, had no effects on the NO-dependent Ca\(^{2+}\) elevations or increase in sPSC frequency, suggesting the involvement of TRPC5 and not TRPC4. Previous work in the Gleason lab demonstrated that TRPC4 is not expressed by ACs in culture or in the adult retina, further suggesting that TRPC4 is not involved. Immunolabeling experiments in my dissertation revealed
that TRPC5 is expressed by ACs both in culture and in the adult retina. Using the CRISPR/Cas9 system, genetic knockdown of protein expression allowed me to correctly identify the involvement of TRPC5 in the NO response. Reduced TRPC5 expression in transfected ACs precluded the NO-dependent Ca\(^{2+}\) elevation and the NO-dependent increase in sPSC frequency. Since ACs in the retina can produce NO and express TRPC5, these findings suggest that NO enhances GABAergic inhibition in the inner retina by activating TRPC5.

TRPC5 has been shown to be directly activated by S-nitrosylation (Yoshida et al. 2006). In my experiments, the mechanism in which NO activates TRPC5 remains unknown. To admit Ca\(^{2+}\) into the presynaptic terminal and promote Ca\(^{2+}\)-dependent GABA release, NO may directly activate TRPC5 via S-nitrosylation, may act on an unidentified regulator of TRPC5 activity, or a combination of both. A western blot using immunoprecipitated TRPC5 after exposure to NO and probed for S-nitrosylation using “S-NO” antibodies, which recognize cysteine S-nitrosothiols, would reveal if native TRPC5 expressed in ACs becomes S-nitrosylated. To investigate if TRPC5 is being activated via S-nitrosylation, experiments using a mutated version of TRPC5 lacking the cysteine resides needed for S-nitrosylation could be overexpressed in these cells to see if the NO-dependent Ca\(^{2+}\) influx absent. This would reveal if S-nitrosylation is the mode in which NO activates the TRPC5-mediated Ca\(^{2+}\) influx.

Previously, it has been indirectly demonstrated that NO enhances Ca\(^{2+}\) dependent GABA release in the turtle inner retina due to NO-dependent reduced labeling of the preloaded GABA analog GVG (Yu and Eldred 2005). Additionally, another study found that NOS-stimulated NO production reduced ganglion cell ON and
OFF light responses, with the OFF response being the most sensitive to NO (Wang et al. 2003). The reduced light responses were not due to intrinsic changes in ganglion cell membrane properties, suggesting that NO acts on a presynaptic site to enhance inhibition onto the ganglion cells. Because they are the only cells presynaptic to ganglion cells that provide inhibitory signals, ACs are likely the site in which NO acts. Combining the findings of these two studies with findings in my dissertation, NO may have reduced ganglion cell light responses (Wang et al. 2003) by increasing Ca\(^{2+}\)-dependent GABA release (Yu and Eldred 2005) through the NO-dependent TRPC5-mediated Ca\(^{2+}\) influx in ACs.

The physiological role of TRPC channels in the retina remains unclear. Future experiments using transgenic knockout models must be performed to validate this pathway by recording from cells in retinal slices that are postsynaptic to ACs (bipolar cells, ganglion cells or other ACs). The use of nNOS knockout mice to investigate this pathway would not produce reliable results. In a previous study, ganglion cell light responses in nNOS knockout mice required brighter light stimuli compared to normal mice, suggesting NO produced by nNOS is important for phototransduction in photoreceptors (Wang et al. 2007). Experiments using commercially available TRPC5 knockout mice would confirm if ACs expressing TRPC5 are responsible for the NO-dependent enhanced inhibition, without interrupting other NO-dependent mechanisms due to the loss of nNOS expression. If this nNOS-NO-TRPC5 pathway enhances GABAergic inhibition, ganglion cell light responses in TRPC5 knockout mice will be enhanced due to the loss of NO-dependent activation of TRPC5.
This enhanced NO-dependent GABAergic signaling at AC synapses may be highly localized. Activation of NMDA ionotropic glutamate receptors (NMDARs), coupled to a Ca\(^{2+}\)-dependent NOS isoform (nNOS or eNOS), initiates NO production in the turtle retina (Blute et al. 2000). In the chicken retina, NMDAR activation increases NO production predominately in the INL and IPL (Tekmen-Clark and Gleason 2013). AC synaptic terminals can have multiple presynaptic sites, and in the turtle retina, nNOS can be present at one presynaptic site and absent at an adjacent presynaptic site within the same AC synaptic terminal (Cao and Eldred 2001; Eldred and Blute 2005). Light-dependent and -independent activation of ON bipolar cells and OFF bipolar cells, respectively, provides glutamatergic input onto ACs. Activation of both pathways leads to increases NO production in the mouse retina (Blom et al. 2012) and salamander retina (Eldred and Blute 2005). Since the NO-dependent release of GABA is voltage-independent, NO produced at one AC presynaptic site would eliminated the need for depolarization by diffusing to adjacent presynaptic sites and enhance NO-dependent GABA release from multiple presynaptic sites within the same AC synaptic terminal. This would enhance GABAergic inhibition of postsynaptic cell (s). Furthermore, if sufficient NO is produced and the postsynaptic cell receiving increased NO-dependent GABAergic input is another AC, NO would release intracellular Cl\(^-\), invert sign of the GABAergic input, and drive depolarization-dependent GABA release from the postsynaptic AC. This NO-dependent amplification of GABA release may be a mechanism needed to provide sufficient inhibition to properly process visual signals during prolonged light stimulation.
References


APPENDIX: LETTER OF PERMISSION

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