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Roles Of Gaseous Neuromodulators NO And CO In Determining Neuronal Electrical Activity And Growth Cone Motility

Stephen Estes

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ABSTRACT

Throughout neuronal development, bouts of spontaneous electrical activity are critical for the proper wiring of neuronal connections. Alterations in firing activity can affect growth cones, which tip developing and regenerating neurites and are responsible for the integration of extracellular guidance cues into pathfinding behaviors. While growing evidence implicates gaseous signaling molecules, nitric oxide (NO) and carbon monoxide (CO), as modulators of neuronal firing activity, less is understood about how they affect growth cone motility. Therefore, in this dissertation, I focus on how NO and CO affect electrical activity of developing and regenerating neurons and how these effects translate into changes at the growth cone level. The specific goals of this dissertation were to investigate 1) the neuron-type-specific effects of NO on growth cone...
motility; 2) the role of CO in the regulation of neuronal firing activity and excitability; and 3) the role CO plays in the regulation of growth cone motility.

Using the well-established developmental model, *Helisoma trivolvis*, neurons were isolated in single-cell culture allowing for the maximal control over environmental conditions for the direct characterization of NO and CO. In the study of NO, differences in B5 and B19 growth cone responses to NO were due to neuron-type-specific differences in action potential duration. Moreover, the non-responsive B19 growth cones could be made responsive to NO treatment upon the pharmacological broadening of its action potentials. While NO has been found to increase firing activity, the study of CO revealed that CO had the opposite effect on electrical activity, silencing spontaneous firing activity and decreasing neuronal excitability. The study of CO on growth cone motility showed that CO increased growth cone filopodial length through a soluble guanylyl cyclase/protein kinase G/ryanodine receptor mediated pathway without inducing robust increases in growth cone calcium concentration. Taken together, this dissertation reveals new insight into how NO and CO regulate electrical activity and growth cone motility, providing evidence for these gases as important signaling messengers during for the development and regeneration of nervous system.

INDEX WORDS: Carbon monoxide, Nitric oxide, Calcium, Electrical activity, Development, Neuron, Growth cone, Filopodia
ROLES OF GASEOUS NEUROMODULATORS NO AND CO IN DETERMINING NEURONAL ELECTRICAL ACTIVITY AND GROWTH CONE MOTILITY

by

STEPHEN ESTES

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

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Georgia State University

2015
ROLES OF GASEOUS NEUROMODULATORS NO AND CO IN DETERMINING NEURONAL ELECTRICAL ACTIVITY AND GROWTH CONE MOTILITY

by

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College of Arts and Sciences
Georgia State University
August 2015
DEDICATION

I dedicate my dissertation work to my family, whose influence has led me on my path of scientific intrigue and discovery.
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I would first like to acknowledge and thank my PhD advisor, Dr. Vincent Rehder, for his guidance throughout my PhD journey. Dr. Rehder has been an integral player in not only developing me as a scientist but also as an individual. I appreciate the patience that he has shown me as I navigate life and science simultaneously. I cannot thank him enough for all of his support. Without him I surely would not have been able to accomplish so much.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... v

LIST OF TABLES ..................................................................................................................... xiv

LIST OF FIGURES .................................................................................................................. xv

LIST OF ABBREVIATIONS ..................................................................................................... xvii

CHAPTER 1 GENERAL INTRODUCTION ............................................................................... 1
  1.1 Specific aims of dissertation .......................................................................................... 1
  1.2 Neuronal development ................................................................................................ 4
    1.2.1 Neuronal growth cone structure .......................................................................... 4
    1.2.2 Guidance Factors ................................................................................................. 6
    1.2.3 Calcium signaling ................................................................................................. 8
    1.2.4 Electrical activity in development ...................................................................... 13
  1.3 Gaseous neuromodulators ............................................................................................. 15
    1.3.1 Nitric oxide ........................................................................................................... 16
    1.3.2 Carbon monoxide ................................................................................................. 17
  1.4 Helisoma trivolvis a model for neuronal development .................................................. 19

2 THE ROLE OF ACTION POTENTIALS IN DETERMINING NEURON-TYPE-SPECIFIC RESPONSES TO NITRIC OXIDE ................................................................. 21
  2.1 Acknowledgements ...................................................................................................... 21
  2.2 Abstract ......................................................................................................................... 21
2.3 Introduction ........................................................................................................... 22

2.4 Methods ................................................................................................................. 24

2.4.1 Neuronal culture ............................................................................................... 24

2.4.2 Electrophysiology .............................................................................................. 25

2.4.3 Calcium imaging ............................................................................................... 26

2.4.4 Growth cone filopodial dynamics ..................................................................... 27

2.4.5 Pharmacological agents ..................................................................................... 28

2.4.6 Statistical analysis .............................................................................................. 28

2.5 Results .................................................................................................................... 29

2.5.1 Neuronal firing frequency sets [Ca\(^{2+}\)]\(_i\) in B5 growth cones .................. 29

2.5.2 Evoked APs lead to a frequency-dependent elevation of [Ca\(^{2+}\)]\(_i\) in growth cones of B19 neurons .......................................................................................... 30

2.5.3 Longer AP durations increase the activity-dependent growth cone calcium concentration .............................................................................................................. 31

2.5.4 NO-donor NOC-7 transiently increases firing frequency and [Ca\(^{2+}\)]\(_i\) in growth cones of B5 neurons .......................................................................................... 32

2.5.5 NO acts through an increase in spiking activity to elevate [Ca\(^{2+}\)]\(_i\) and elongate filopodial ............................................................................................................. 33

2.5.6 NO elevates the spiking frequency and growth cone [Ca\(^{2+}\)]\(_i\) in B19 neurons ...................................................................................................................... 35
2.5.7  Widening of action potentials increases sensitivity to NO and results in
filopodial elongation in B19 neurons ............................................................. 35

2.6  Discussion ..................................................................................................... 37

2.6.1 Electrical activity sets growth cone calcium ........................................... 38

2.6.2 Intrinsic electrical properties determine effects of NO on growth cone
morphology ......................................................................................................... 40

2.6.3 Modulating neuronal responses through intrinsic AP properties: the role of
K+ channels ......................................................................................................... 41

2.6.4 Implications for the role intrinsic AP properties play in development .... 42

2.7  Figures and tables ......................................................................................... 44

3  REGULATION OF ELECTRICAL ACTIVITY AND NEURONAL
EXCITABILITY IN HELISOMA TRIVOLVIS BY CARBON MONOXIDE .... 56

3.1  Acknowledgments ......................................................................................... 56

3.2  Abstract ......................................................................................................... 56

3.3  Introduction .................................................................................................... 57

3.4  Methods ......................................................................................................... 59

3.4.1 Neuronal culture ....................................................................................... 59

3.4.2 Electrophysiology ..................................................................................... 59

3.4.3 Pharmacological agents ........................................................................... 62

3.4.4 Statistics ..................................................................................................... 62

3.5  Results ........................................................................................................... 62
3.5.1 Carbon monoxide hyperpolarizes $V_m$ and silences spontaneous firing activity ........................................................... 62

3.5.2 CO inhibits a persistent sodium current to hyperpolarize the membrane potential .......................................................... 64

3.5.3 CO inhibits neuronal excitability ............................................................ 65

3.5.4 CO inhibits neuronal excitability through the inhibition of VGCCs ................. 66

3.5.5 CO similarly modulates neuronal firing activity of B19 neurons ................. 67

3.6 Discussion ........................................................................................................ 68

3.6.1 $I_{NaP}$ regulation of the resting membrane potential ........................................ 69

3.6.2 VGCC regulation of neuronal excitability ..................................................... 70

3.6.3 Implications of CO in CNS function and development ............................ 71

3.7 Figures ............................................................................................................. 74

4 THE MODULATION OF GROWTH CONE CALCIUM AND FILOPODIA BY CARBON MONOXIDE ................................................................. 84

4.1 Acknowledgements .......................................................................................... 84

4.2 Abstract ............................................................................................................ 84

4.3 Introduction ...................................................................................................... 85

4.4 Methods ........................................................................................................... 87

4.4.1 Neuronal culture .......................................................................................... 87

4.4.2 Electrophysiology ....................................................................................... 88
4.4.3 Calcium imaging ................................................................. 89
4.4.4 Growth cone filopodia dynamics ........................................... 89
4.4.5 Pharmacological agents ....................................................... 90
4.4.6 Statistical analysis ............................................................. 91

4.5 Results ..................................................................................... 92
4.5.1 CO increases filopodia length in B5 and B19 neurons ............... 92
4.5.2 CO increases filopodia length through a soluble guanylyl cyclase pathway .......................................................... 94
4.5.3 CO increases filopodia length through activation of protein kinase G ..... 95
4.5.4 CO increases filopodia length through activation of ryanodine receptors. 96
4.5.5 CO-induced changes in filopodia occur through undetectable changes in calcium .......................................................................................................................... 97

4.6 Discussion ................................................................................. 99
4.6.1 CO modulates growth cone filopodial length .............................. 99
4.6.2 CO signaling: a sGC/PKG/RyR mediated pathway that modulates filopodial length ......................................................................................................................... 100
4.6.3 CO and NO: implications on growth cone motility ....................... 103

4.7 Figures ...................................................................................... 105

5 GENERAL DISCUSSION AND CONCLUSIONS ................................. 113
5.1 Nitric oxide: a gaseous effector molecule of neuronal development ........ 114
5.2 Generating specific growth cone behaviors: electrical activity ............... 117

5.3 Carbon monoxide: a novel regulator of neuronal electrical activity ....... 120

5.4 Carbon monoxide: a novel modulator of growth cone motility ............. 123

5.5 Special considerations for neural regeneration: CO as a therapeutic ...... 128

5.6 Conclusions ........................................................................................................... 129

REFERENCES .................................................................................................................... 131
LIST OF TABLES

Table 2.1 Neuron-type specific action potential characteristics. .................................................. 55
LIST OF FIGURES

Figure 1.1 Phase contrast image of cultured B5 neuronal growth cone. .......................... 6

Figure 2.1 Correlation of firing frequencies and growth cone calcium concentrations in B5 neurons. .................................................................................................................. 44

Figure 2.2 Correlation of firing frequencies and growth cone [Ca$^{2+}$]$_i$ in B19 neurons. ... 46

Figure 2.3 Action potential duration affects the magnitude of activity-dependent growth cone [Ca$^{2+}$]$_i$. .......................................................................................................................... 48

Figure 2.4 Nitric oxide increases the firing frequency and growth cone [Ca$^{2+}$]$_i$ in B5 neurons. .......................................................................................................................... 50

Figure 2.5 APs are necessary for NO to modulate growth cone filopodia. ....................... 51

Figure 2.6 Nitric oxide increases firing frequency and growth cone calcium in B19 neurons. .......................................................................................................................... 52

Figure 2.7 Nitric oxide causes filopodial elongation in B19 neurons after broadening of their APs.......................................................................................................................... 53

Figure 3.1 CO donor, CORM-2, silences spontaneous firing activity of B5 neurons. ..... 74

Figure 3.2 CO inhibition of $I_{NaP}$ results in the hyperpolarization of the RMP and silencing of spontaneous firing activity................................................................. 76

Figure 3.3 CO decreases neuronal excitability. ................................................................. 78

Figure 3.4 CO inhibits voltage gated calcium channels. ................................................. 80

Figure 3.5 CO hyperpolarizes B19 RMP and decreases neuronal excitability. .............. 82

Figure 3.6 Summary for CO modulation of electrical activity ........................................ 83

Figure 4.1 CO effects on filopodia. ................................................................................ 105

Figure 4.2 CO acts through sGC .................................................................................. 107
Figure 4.3 CO acts through PKG................................................................. 108
Figure 4.4 CO acts through RyR................................................................. 109
Figure 4.5 CO decreases calcium............................................................... 110
Figure 4.6 CO proposed model................................................................. 112
Figure 5.1 Schematic of NO and CO filopodial signaling pathways............... 126
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHP</td>
<td>Afterhyperpolarization</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>ARC</td>
<td>Arachidonic acid regulated calcium</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BK</td>
<td>Large conductance Ca-activated K</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>[Ca²⁺]ᵢ</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>CRAC</td>
<td>Calcium release-activated calcium</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium induced calcium release</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>CORM-2</td>
<td>Carbon monoxide releasing molecule-2</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>HVA</td>
<td>High voltage activated</td>
</tr>
<tr>
<td>IₙNaP</td>
<td>Persistent sodium current</td>
</tr>
<tr>
<td>IP₃/IP₃R</td>
<td>Inositol triphosphate/Inositol triphosphate receptors</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>LGN</td>
<td>Lateral geniculate nucleus</td>
</tr>
<tr>
<td>LVA</td>
<td>Low voltage activated</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-d-glucamine</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>RMP</td>
<td>Resting membrane potential</td>
</tr>
<tr>
<td>ROC</td>
<td>Receptor-operated channels</td>
</tr>
<tr>
<td>Ry/RyR</td>
<td>Ryanodine/Ryanodine receptor</td>
</tr>
<tr>
<td>SK</td>
<td>Small-conductance calcium-activated potassium</td>
</tr>
<tr>
<td>SOC</td>
<td>Store operated calcium</td>
</tr>
<tr>
<td>STIM1</td>
<td>Stromal interaction molecule-1</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
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<tr>
<td>VGCC</td>
<td>Voltage-gated calcium channel</td>
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CHAPTER 1 GENERAL INTRODUCTION

1.1 Specific aims of dissertation

The goal of this dissertation is to examine the interaction between electrical activity and growth cone intracellular concentration ([Ca\(^{2+}\)]\(_i\)) during neuronal development. Moreover, this dissertation aims to characterize how two gaseous neuromodulators, nitric oxide (NO) and carbon monoxide (CO), regulate these interactions to affect growth cone motility and to determine the ion channel targets and intracellular signaling pathways that mediate these effects. Experiments use the highly identifiable neurons from the buccal ganglia of the fresh water snail, *Helisoma trivolvis*, as a model. A brief description of each specific aim is listed below.

**Specific Aim 1 (Chapter 2): How does the electrical activity of a neuron determine the response of growth cones to NO?**

Developing axons and dendrites, collectively termed neurites, encounter a variety of molecular signals produced by the surrounding cellular environment that aide in their navigation to potential synaptic targets. The selective response of a neuron to these guidance cues enables the assembly of defined neural networks, which in turn provide the circuitry for distinct behaviors. Determining how a growth cone discriminates between signals is an important step in understanding how the nervous system is wired and is still not fully understood.

Developing buccal neurons, B5 and B19, display different growth cone morphological responses to NO exposure; in which, NO increases growth cone [Ca\(^{2+}\)]\(_i\) and growth cone filopodial length in B5 neurons only (Van Wagenen and Rehder, 2001). The electrical activity of a neuron can affect growth cone [Ca\(^{2+}\)]\(_i\), which in turn has been shown to affect growth cone guid-
ance behaviors (Neely and Nicholls, 1995, Spitzer, 2006, Zheng and Poo, 2007). With developing B5 and B19 neurons displaying different spontaneous firing activity 2 days in culture, this study aimed to characterize how electrical activity regulated growth cone [Ca^{2+}] in both neuron-types. In addition, previous studies have shown that NO increases the firing activity of B5 (Artinian et al., 2010) and B19 neurons (Zhong et al., 2013c); therefore, this study also aimed to understand why B5 neurons showed changes in growth cone filopodial length following NO treatment and why B19 neurons did not. Findings from this study provide important insight into how intrinsic electrical properties of a neuron serve as major determinants of the response of a neuron to signaling molecules.

**Specific Aim 2 (Chapter 3): How does CO regulate electrical activity and neuronal excitability?**

Since the first discovery of CO as an endogenously produced gas in humans (Sjostrand, 1949) numerous species have been shown to produce CO including bacteria (Engel et al., 1972), plants (Shekhawat and Verma, 2010, Vreman et al., 2011), and animals (Maines, 1997, Wu and Wang, 2005). While CO is often and rightfully associated with being a toxic gas that kills thousands of people worldwide every year, it has been found to serve important physiological functions as a signaling molecule. In the nervous system, CO has been shown to affect circadian rhythms (Artinian et al., 2001), learning and memory (Cutajar and Edwards, 2007), nociception (Steiner et al., 2001), olfaction (Zufall and Leinders-Zufall, 1997), and neuronal migration (Knipp and Bicker, 2009). The mechanisms underlying the physiological effects of CO are diverse (Maines, 1997, Peers et al., 2014) and are still not fully understood. In recent years, CO has gained increasing evidence as a regulator of ion channel activity (Wilkinson and Kemp, 2011b,
Peers et al., 2014). With the composition and activity level of ion channels in the plasma membrane determining the firing activity and excitability of a neuron, this study aimed to assess the role CO played in both the spontaneous and evoked firing activity and to determine the ion channel targets that mediate these effects. Findings from this study will provide new insight into the modulatory role that CO can play in the regulation of neuronal firing activity.

*Specific Aim 3 (Chapter 4): How does the regulation of electrical activity by CO affect growth cone calcium dynamics and growth cone motility?*

The enzyme responsible for producing CO, heme oxygenase (HO), is prevalent throughout the nervous system and its development (Maines, 1997). Surprisingly, little is known about the role CO plays in development except that it can function as an inhibitory signal slowing neuronal migration (Knipp and Bicker, 2009). With CO modulating a number of ion channels (Wilkinson and Kemp, 2011b, Peers et al., 2014), it is likely that CO affects neuronal firing activity and growth cone \([Ca^{2+}]_i\) as well. Changes in growth cone \([Ca^{2+}]_i\) affect a variety of growth cone behaviors ranging from growth cone turning (Robles et al., 2003, Henley and Poo, 2004, Wen et al., 2004), neurite outgrowth (Gomez and Spitzer, 2000, Trimm and Rehder, 2004) and filopodial motility (Rehder and Kater, 1992, Van Wagenen et al., 1999, Tornieri and Rehder, 2007, Welshhans and Rehder, 2007, Zhong et al., 2013b). Therefore, in this aim, the role of CO in affecting the activity-dependent growth cone \([Ca^{2+}]_i\) is assessed. With filopodia being crucial to the ability of the growth cone to steer during pathfinding (Marsh and Letourneau, 1984, Bentley and Toroian-Raymond, 1986, McCaig, 1989, Robles et al., 2003, Geraldo and Gordon-Weeks, 2009) and with changes in filopodial length being one of the earliest morphological indi-
cators of changes in growth cone $[\text{Ca}^{2+}]_i$, another goal of this aim is to determine how CO modulates filopodia length and the pathway by which it mediates these effects. Findings from this study will expand our knowledge in the role CO plays as a developmental signaling molecule as well as a general modulator of $[\text{Ca}^{2+}]_i$.

1.2 Neuronal development

Neuronal development occurs in multiple stages that involve the proliferation, differentiation, migration and synapse formation of a neuron and its processes. While each stage contributes significantly to the establishment of a functional nervous system, the guidance and formation of synaptic connections ultimately determines the functionality of a given neural circuit. Newly formed neurons will send out neurites to navigate through a dense, dynamic cellular environment to locate synaptic targets. Disruption of this navigational process can lead to a lack of synaptic connectivity and/or inappropriate synaptic connections (Catalano and Shatz, 1998, Hanson and Landmesser, 2004) resulting in aberrant behaviors and neurodevelopmental disorders (Gepner and Feron, 2009, Mitchell, 2011). Decades of research have been spent understanding the structures and general mechanisms involved in the guidance and establishment of synaptic connections; however, a clear understanding of all the players involved in the wiring of the nervous system still remains to be determined.

1.2.1 Neuronal growth cone structure

The growth cone, located at the tip of developing and regenerating neurites, functions as a “GPS” for the neurite, integrating guidance cues produced by the surrounding cellular environment into pathfinding behaviors that collectively navigate the neurite to its synaptic target. The
structure of the growth cone [Fig. 1.1] is characterized by 3 regions: the central (C), transitional (T), and peripheral (P) domains. The C-domain is comprised of stable microtubule bundles that extend from the axonal shaft and contain organelles and vesicles that are important for growth cone motility. The T-domain is the transitional zone between microtubules and actin filaments found in the P-domain. The P-domain is the dynamic region of the growth cone that contains both lamellipodia and filopodia; these two structures are rich in filamentous actin and contain receptors embedded in the plasma membrane that detect guidance factors found in the environment. Upon binding of a guidance factor, the growth cone cytoskeleton rearranges to produce a pathfinding behavior, such as: turning, changes in the rate of outgrowth, filopodial elongation, and growth cone collapse (Mattila and Lappalainen, 2008, Lowery and Van Vactor, 2009). Extending from the leading edge of the growth cone, the long finger-like filopodia act as sensory-motor antennae (Davenport et al., 1993, Mattila and Lappalainen, 2008) and determine the exploratory radius of the growth cone, enabling the growth cone to traverse areas of low substrate adhesivity to continue pathfinding (Hammarback and Letourneau, 1986). Furthermore, by extending out in front of the growth cone, filopodia act as the initial point of contact with potential target cells and thus play an important role in the determination of synapse formation (Shen and Cowan, 2010). The disruption of filopodial actin dynamics results in abnormal growth cone steering in response to guidance signals (Bentley and Toroian-Raymond, 1986, Zheng et al., 1996) as well as slower rates of neurite outgrowth and reduced terminal axonal branching (Dwivedy et al., 2007). Taken together, these findings suggest that the structures of the P-domain, particularly filopodia, are critical for the motility of a growth cone and for its pathfinding ability.
Figure 1.1 Phase contrast image of cultured B5 neuronal growth cone.

The growth cone is divided into 3 regions: the central domain (indicated by the “C”), the transitional domain (indicated by the “T”), and the peripheral domain (indicated by the “P”). The P-domain contains an actin mesh called the lamellipodium (indicated by the “L”) as well as long finger-like projections called filopodia (indicated by the “F”).

1.2.2 Guidance Factors

Guidance factors, produced by the surrounding cellular environment, provide the developmental framework and steering instructions that help navigate developing and regenerating neurites to their target locations. They are commonly classified into two groups: (1) adhesion molecules and (2) chemotropic guidance cues. (1) Adhesion molecules are either presented directly on the cell surface, referred to as cell adhesion molecules (CAMs; examples: cadherins, contactin, Axonin-I, neuronal CAMs), or are a part of the extracellular matrix (ECM; examples: laminin, fibronectin, and heparin sulfates) (for a review see (Kiryushko et al., 2004)). These molecules provide the framework or “roadways” by which neurites can adhere and grow along. (2) Chemotropic guidance cues, on the other hand, act as the “road signs”, instructing the growth
cone on which way to go. Like adhesion molecules, these cues can be located on the cell membrane or extracellular matrix. In addition, these cues can also be diffusible molecules that are released from neighboring or distant cells to bind to specific receptors on growth cones, affecting the trajectory of the advancing neurite.

Netrins, semaphorins, ephrins, and slits are the most commonly studied guidance cues; although, a wide range of molecules have been identified to act as guidance signals including neurotransmitters (Mattson et al., 1988, van Kesteren and Spencer, 2003), neuropeptides (Hokfelt et al., 2008), neurotrophic factors (Sanford et al., 2008), and transcription factors (Brunet et al., 2005, Butler and Tear, 2007). Upon binding to the growth cone, guidance cues trigger signaling cascades that affect the stability of actin and microtubule filaments. Depending on whether this stabilization occurs on the proximal or distal side of the growth cone, in respect to the guidance cue, determines whether a guidance cue is a chemoattractant or a chemorepellant; in other words, a growth cone will move towards an attractive cue and move away from a cue that acts as a repellent. While it was once thought that guidance cues were either strictly an attractant or repellant, a number of studies have shown that the turning response induced by a particular cue is dependent on (1) the presence of the receptor for the guidance cue and (2) the internal state of the growth cone. For example, during the development of commissural interneurons, axons will cross over the midline, unresponsive to Slit, which typically acts as a repulsive cue. Upon crossing the midline, these neurons upregulate Robo, the receptor for Slit, and repel axons, preventing them from crossing back over the midline (Kaprielian et al., 2001). In mammals, the particular type of Robo also determines the effect of Slit; in which, the expression of Robo3 prevents Slit repulsion (Sabatier et al., 2004). Adding to the complexity of the expression of a particular receptor subtype, the internal components/molecular makeup of the growth cone
can also determine whether a cue is attractive or repulsive. In Xenopus spinal neurons, low levels of cyclic adenosine monophosphate (cAMP) switch netrin-1 mediated growth cone attraction to repulsion (Ming et al., 1997). Cyclic nucleotide levels also affect whether nerve growth factor (NGF) induces attractive or repulsive growth cone turning in superior cervical ganglia neurons of the rat (Thompson et al., 2011). Collectively, these findings illustrate the complex interplay between receptors and their signaling pathways in the determination of a growth cone response to a guidance signal. Moreover, these findings demonstrate the importance of identifying the targets of guidance cues and their underlying mechanisms to gain insight how the nervous system develops and regenerates.

1.2.3 Calcium signaling

Calcium is a common downstream signaling target of many guidance factors and has been well documented for its role in growth cone motility, affecting the rate and direction of neurite outgrowth (Kater and Mills, 1991, Gomez and Spitzer, 2000, Sutherland et al., 2014). The specific effects that calcium has on growth cone motility are determined by the overall intracellular calcium concentration ([Ca^{2+}]_i) and the localization of calcium gradients within the growth cone. For example, extremely low and high [Ca^{2+}]_i elicited throughout the growth cone result in the suppression of neurite outgrowth; whereas, moderate [Ca^{2+}]_i (200-300 nM) promote neurite outgrowth (Lankford and Letourneau, 1991). Alternatively, localized elevations of calcium on one side of the growth cone induces growth cone turning; the direction of which is also determined by the [Ca^{2+}]_i (low and high calcium levels result in repulsion and moderate calcium levels result in attraction) (Zheng, 2000). These findings point to an optimal calcium range or “set
point” for the production of specific growth cone behaviors; deviations from this range can change the behavior elicited by the growth cone.

Changes in growth cone $[\text{Ca}^{2+}]_i$ occur through two main pathways: (1) the release of calcium from intracellular calcium stores and (2) the influx of calcium through ion channels embedded in the plasma membrane. Calcium release from intracellular calcium stores involves the activation of either inositol triphosphate receptors (IP$_3$R) or ryanodine receptors (RyR); both of which are located on the membrane of the endoplasmic reticulum (ER), a major calcium store. IP$_3$Rs are activated by inositol triphosphate (IP$_3$), which is a byproduct of the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) by phospholipase C (PLC). Guidance cues Netrin-1 and NGF utilize the PLC/IP$_3$R pathway to modulate neurite outgrowth and growth cone turning, respectively (Xie et al., 2006, Akiyama et al., 2009), demonstrating the importance of calcium release in growth cone motility. In the case of RyR, calcium functions as the primary ligand for the opening of RyR, a process termed calcium-induced calcium release (CICR). While CICR can amplify calcium signals, a number of other signaling molecules have also been identified to modulate RyR activity, such as NO, ryanodine, protein kinase A (PKA), caffeine, etc. (for a review see (Van Petegem, 2012)). Like IP$_3$Rs, the activation of RyRs plays an important role in growth cone motility. For example, CAMs, L1 and N-cadherin, elicit attractive growth cone turning in the presence of functional RyRs; knockout of these receptors switches the growth cone turning response to repulsion (Ooashi et al., 2005). This finding indicates that calcium release via RyR is necessary to amplify CAM-mediated calcium signaling to levels necessary to evoke an attractive growth cone turning response. Moreover, it also indicates that the activation of RyRs in general can play an important role in the determination of growth cone behaviors.
In addition to the release of calcium from stores, calcium influx through membrane bound channels affects the [Ca^{2+}]_{i}. There are 5 main channel types that gate calcium entry into the cell: (1) voltage-gated calcium channels (VGCC), (2) store operated calcium (SOC) channels, (3) transient receptor potential (TRP) channels, (4) receptor-operated channels (ROC), and (5) arachidonic acid regulated calcium (ARC) channels. Each channel has specific gating properties and kinetics to allow for the influx of calcium and are described briefly below:

1 – VGCCs – Voltage-gated calcium channels are gated through changes in membrane potential and are classified into 2 main groups: low voltage activated (LVA) and high voltage activated (HVA) calcium channels. LVA calcium channels begin to activate at or near hyperpolarized membrane potentials (around -60 mV), and contribute to the depolarization of the resting membrane, playing a role in the production of rhythmic firing activity (Chevalier et al., 2006, Deleuze et al., 2012). The LVA class of calcium channels consist of a single ion channel type, the transient opening or T-type calcium channel, which is named for its rapid inactivation kinetics upon depolarization of the membrane potential (for a review see (Perez-Reyes, 2003)). Unlike LVA calcium channels, HVA calcium channels begin to activate at more depolarized membrane potentials (around -40 mV) and have slower inactivation kinetics, thus allowing for more calcium entry into the cytosol. These channels have a wider diversity of channel subtypes and include the L-type (long-lasting), N-type (neuronal-type), P/Q-type (Purkinje type), and R-type (residual type) calcium channels. HVA calcium channels are involved in the release of neurotransmitters and hormones as well as excitation-contraction coupling (L-type channel) (for a review of all VGCCs see (Neumaier et al., 2015)). In addition to these effects, VGCCs transduce changes in electrical activity of developing and generating neurons into calcium signals that in
turn modulate growth cone behaviors as well as responses to incoming guidance cues (discussed in detail in the section “Electrical activity in development”).

2 – SOC – Store operated calcium channels are triggered through the depletion of calcium from intracellular calcium stores. Stromal interaction molecule-1 (STIM1) is localized at the ER and contains an EF-hand motif that extends inside the ER lumen to detect calcium. Upon depletion of calcium, STIM1 extends towards the plasma membrane to form a complex with the protein Orai1, establishing a calcium release-activated calcium (CRAC) channel and allowing for the influx of calcium into the cytosol (Prakriya, 2009). During development, the influx of calcium through these channels is necessary for semaphorin mediated neurite collapse as well as BDNF induced growth cone turning (Mitchell et al., 2012). In addition, SOC channels are necessary for the transduction of filopodial calcium transient in *Xenopus* growth cones and for netrin-1 induced attractive turning (Shim et al., 2013), demonstrating the importance of SOC channels in the mediation of growth cone behaviors.

3 – TRP – Transient receptor potential channels form a superfamily of non-selective cation channels that are permeable to calcium as well as sodium and magnesium. There are 28 identified mammalian TRP channels that are classified into 6 groups based on their amino acid sequence homology: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucolipin) (for a review see Ramsey et al., 2006). An additional TRP channel type has been identified in non-mammalian cells, TRPN (no mechanoreceptor potential C) (Walker et al., 2000, Li et al., 2006). TRP channels act as signal transduction mechanisms with a diverse range of physiological effects. Within the developing nervous system, the activation of TRPC5 channels modulates growth cone morphology. For example: Over-expression of TRPC5 channels results in thin neurites and growth cones with long filopodia,
which is indicative of high [Ca$^{2+}$], (Greka et al., 2003). In *Xenopus* spinal neurons, the inhibition of TRP channels block chemoattractive turning produced by BDNF and netrin-1 (Henle et al., 2011). These studies reveal an important role for TRP channels in the transduction of guidance signals into changes in growth cone [Ca$^{2+}$], affecting growth cone morphology.

4 – ROC – Receptor-operated calcium channels are voltage-independent, non-selective cation channels that are permeable to calcium and are primarily activated downstream of G-protein coupled receptor activation via a variety of agonists, including some common neurotransmitters and hormones (McFadzean and Gibson, 2002). Some ionotropic receptors, like N-methyl-D-aspartate receptors, have been categorized as ROC channels in neurons (Bertolino and Llinas, 1992); however, they are not commonly classified as ROC channels in the literature, pointing out the need for a clear definition of these channel types. It may be that a clear definition of ROC channels is lacking due to the overlap of ROC channels with TRP and SOC channel subunits and functionality. Regardless of their identity, ROC channels have been identified for their ability to permit calcium entry into cells and thus stand as potential modulators of growth cone motility.

5 – ARC - Arachidonic acid regulated calcium channels are similar to SOC channels in that they utilize Orai subunits to form highly selective calcium channels in the plasma membrane; however, unlike SOC channels, ARC channels open independently of the depletion of intracellular calcium stores via the binding of arachidonic acid (for a review see (Shuttleworth, 2009)). While the functional role for ARC channels in growth cone motility remains to be determined, the production of arachidonic acid downstream of PLC signaling, suggests that the activation of ARC channels may be an important downstream component for the regulation of the [Ca$^{2+}$], mediated by guidance cues that act through PLC signaling.
While the multiple mechanisms for calcium entry into the cytosol could be viewed as redundant, assuring a generalized change in calcium for the reproduction of a generalized growth cone behavior, it is more likely that these multiple mechanisms allow for the precise titration of calcium, as evident by their individual activation properties and kinetics, thus defining neuron-type-specific growth cone responses to a myriad of guidance signals.

In addition to calcium entry, growth cone calcium levels are determined by a number of calcium buffering mechanisms. These mechanisms include pumps and transporters that extrude calcium from the cytosol into the extracellular space (Ex: Sodium/calcium exchanger) and take up calcium into intracellular stores (Ex: the sarco/endoplasmic reticulum calcium ATPase (SERCA) pump) as well as buffering molecules proteins that sequester free calcium in the cytosol (Ex: calretinin). Collectively, these buffering mechanisms help maintain calcium homeostasis within the growth cone and ensure the response of growth cones to incoming signals.

1.2.4 Electrical activity in development

A hallmark of neuronal communication is the production of an action potential (AP), mediating the relay of information from one neuron to the next. In recent years, it has become increasingly more evident that electrical activity also relays developmental information to growth cones. Early evidence for the involvement of electrical activity in the wiring of the nervous system came from the application of field currents to developing growth cones, resulting in changes in neurite outgrowth (Hinkle et al., 1981, Cohan and Kater, 1986, McCaig, 1986) and growth cone turning direction (Patel and Poo, 1982, Patel and Poo, 1984). While a number of studies since these initial findings have shown that electrical stimulation, occurring through either direct current stimulation or induced chemically, affects growth cone motility (Neely and Nicholls,
it was not until more recently that the role of intrinsically produced electrical activity was found to be critical for the guidance of axons to their appropriate synaptic locations. For example, the disruption of spontaneous firing activity in lateral geniculate nucleus (LGN) neurons using tetrodotoxin results in the inappropriate projection of LGN axons to cortical subplate areas instead of to the thalamus, resulting in miswiring of neural connections (Catalano and Shatz, 1998). In developing chick spinal motoneurons, spontaneous electrical activity was necessary for the dorsal/ventral pathfinding decision of axons as they exited the spinal column to innervate muscles; the inhibition of electrical activity in these neurons leads to aberrant neurite projections in a medial/lateral direction, demonstrating axonal pathfinding errors (Hanson and Landmesser, 2004). Collectively, these studies demonstrate that electrical activity plays another important role in the nervous system beyond the communication of information across synapses: the guidance of synaptic connections.

The changes in growth cone motility and axonal pathfinding, mediated by electrical activity (referred to as activity-dependent changes), occur, in large part, through the modulation of growth cone \([\text{Ca}^{2+}]\). During the production of an AP, depolarization of the membrane potential activates voltage-gated calcium channels (described in the section “calcium signaling”) embedded in the plasma membrane, resulting in the influx of calcium into the cytosol of neuronal cell bodies, neurites, and growth cones (Bolsover and Spector, 1986, Cohan et al., 1987, Ross et al., 1987, Torreano and Cohan, 1997, Kuznetsov et al., 2012) and affecting growth cone behaviors (Zheng and Poo, 2007). In the absence of extracellular calcium, depolarization of the membrane potential may still provide some changes to the growth cone via increased activation of adenylyl cyclase (Reddy et al., 1995), which can in turn increase cytosolic cAMP concentrations that have
been shown to affect growth cone turning (Guirland et al., 2003). Given these findings, it is plausible that any extrinsic signal that manipulates electrical activity would also affect growth cone motility; however, this is not always the case. Increases in the firing activity of rat superior cervical ganglia neurons increases growth cone [Ca\(^{2+}\)], but does not change the rate of neurite outgrowth (Garyantes and Regehr, 1992). In the snail *Helisoma trivolvis*, nitric oxide (NO) increases firing activity of two identified neurons, B5 (Artinian et al., 2010) and B19 (Zhong et al., 2013c), but only affects filopodial length in B5 neurons (Van Wagenen and Rehder, 2001). One of the goals of this dissertation is to determine how electrical activity affects one neuron-type and not another, specifically focusing on the neuron-type-specific effects of NO on electrical activity and growth cone motility.

### 1.3 Gaseous neuromodulators

In addition to instructing developing and regenerating neuronal processes on where to go, a number of guidance signals function as neuromodulators, altering neuronal activity and cellular signaling pathways that ultimately affect how other guidance factors are interpreted by the growth cone. For example, NGF modulates the response of developing chick DRG neurons to semaphorin, reducing neurite collapse induced by semaphorin (Dontchev and Letourneau, 2003). By altering growth cone responses to guidance factors, neuromodulators can play an integral role in establishing neuron-specific connections.

This dissertation focuses on a particular set of neuromodulators, nitric oxide (NO) and carbon monoxide (CO). Because these neuromodulators are gases, they are capable of diffusing across cell membranes to “globally” modulate the surrounding cellular environment, including growth cones navigating within the diffusion radius of these signals. It is therefore likely that
these gases act as regulators of growth cone motility and the establishment of neuronal connections.

### 1.3.1 Nitric oxide

NO, like the other gaseous signaling molecules CO and hydrogen sulfide, is a toxic gas, acting as a highly reactive radical molecule. The discovery of its endogenous production through the conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS), has led to the description of NO as a physiological signaling molecule in the circulatory, immune and nervous system (for a review see (Guix et al., 2005)). The physiological effects of NO are attributed to two main modes of action: the binding of NO to the thiol group of a protein (S-nitrosoylation) and the binding of NO to transition metals, such as iron, found in proteins and enzymes. Through these mechanisms NO can regulate the function of ion channels, transcription factors, and enzymes, affecting the activity of a cell.

Studies of NO within the nervous system have led to its description as an important developmental signaling molecule. In the early 90s, NO was first implicated as an effector of neurite outgrowth in rat DRG neurons, causing neurite advance to stop within 2 minutes of NO exposure via S-nitrosylation that prevented fatty acid acylation (Hess et al., 1993). Since then a number of vertebrate studies support the notion that NO acts as a stop signal for neurite outgrowth through a cGMP-independent pathway (Renteria and Constantine-Paton, 1996, Stroissnigg et al., 2007). Interestingly, in a study of *Helisoma* neurons, NO was also found to decrease neurite outgrowth, but this decrease was found to occur through a soluble guanylyl cyclase (sGC)/cGMP dependent pathway (Trimm and Rehder, 2004). While the overall effects of NO on neurite outgrowth are the same in these studies, the difference in signaling pathways may
be due to differences in model systems. Additional growth cone studies conducted on *Helisoma* neurons show that NO modulates growth cone filopodial length through the same sGC/cGMP pathway and that these pathways ultimately culminate in an increase in growth cone \([\text{Ca}^{2+}]_i\) (Van Wagenen and Rehder, 1999, Trimm and Rehder, 2004, Welshhans and Rehder, 2005, Tornieri and Rehder, 2007). This change in calcium may represent a common convergent signaling mechanism for NO-mediated effects on growth cone motility across species; however, more studies will need to be conducted to verify this hypothesis.

While NO produced similar effects on neurite outgrowth across neuron-types, the effects of NO on filopodial length can vary, even within the same species. Two identified *Helisoma* neurons, B5 and B19, show very different morphological responses to NO: NO increases filopodial length in B5 neurons and NO has no effect on filopodial length in B19 neurons (Van Wagenen and Rehder, 2001). Moreover, electrophysiological studies of these neurons show that NO increases firing activity in both neurons (Artinian et al., 2010, Zhong et al., 2013c). In theory increases in firing activity in both neurons should increase growth cone \([\text{Ca}^{2+}]_i\), which can increase filopodial length in both B5 and B19 growth cones (Van Wagenen and Rehder, 2001). The differences in effects produced by NO on two neuron-types represent an interesting paradox; in which, similar electrical responses can produce neuron-specific growth cone behaviors. While this paradox is likely to extend to a number of other signaling molecules, one goal of this dissertation is to understand how neuron-type-specific effects arise from NO stimulation.

1.3.2 *Carbon monoxide*

CO, produced from the incomplete combustion of fossil fuels, is a toxic gas that at high concentrations can lead to hypoxia and death. In 1949, it was first discovered that CO is produced by the
human body (Sjostrand, 1949); the production of which is estimated to be 16.4 µM/hour (Coburn, 1970) and upwards of 500 µM/day (Coburn et al., 1964). Since its initial discovery, the endogenous production of CO has been identified in almost every living organism and cell-type, suggesting that it is a highly conserved signaling molecule. Its production occurs primarily through the enzymatic degradation of heme by the enzyme heme oxygenase (HO), generating, in addition to CO, ferrous iron and biliverdin. Aside from heme oxygenase, the breakdown of heme methylene bridges and the inactivation of cytochrome P450 have been also shown to release CO (Wu and Wang, 2005). Two main isoforms of HO have been described: an inducible form (HO-1; also referred to as heat shock protein 32), which is activated by a number of signaling pathways as well as a diverse range of cellular signals such as hypoxia, cytokines, growth factors, etc., and a constitutive form (HO-2), which can be activated by glucocorticoids as well as opiates, protein kinase C (PKC), estrogen, etc. (for a review see (Wu and Wang, 2005)).

The physiological effects ascribed to CO are diverse, ranging from vasodilation to anti-inflammation, anti-apoptosis, cell proliferation, and neurotransmission. Like NO, CO has an affinity for transition metals, enabling CO to bind to the heme moiety of target proteins, such as sGC, to initiate signaling events. In the nervous system, CO activation of sGC/cGMP has been shown to affect circadian rhythms (Artinian et al., 2001), learning and memory (Cutajar and Edwards, 2007), nociception (Steiner et al., 2001), and olfaction (Zufall and Leinders-Zufall, 1997). In addition to the modulation of sGC/cGMP, CO has also been shown to act on cGMP-independent pathways. In recent years, increasing evidence implicates the direct modulation of ion channels by CO, including Na⁺ channels (Althaus et al., 2009, Dallas et al., 2012), K⁺ channels (Wang and Wu, 1997, Dallas et al., 2011), and VGCCs (Lim et al., 2005) (Scragg et al., 2008) (Boycott et al., 2013). While a majority of these studies have been conducted on non-neuronal cells, they highlight additional mechanisms by which CO
may affect the nervous system. Given that electrical activity not only plays a role in neuronal communication but in development as well, the effects of CO on ion channel activity indicate a possible role for CO as a modulator of neuronal development and regeneration, of which little is known.

What is known about the HO/CO system in development/regeneration? In mammals, HO-2 transcripts are present starting on prenatal day 1 and increase in expression into adulthood, while HO-1, on the other hand, remains at relatively low transcription levels throughout the brain with increases occurring in localized brain regions over the course of development (Sun et al., 1990, Bergeron et al., 1998). Interestingly, following spinal cord injury, HO-1 expression dramatically increases as does HO-2 expression albeit in a more delayed time scale of 16 hours following injury (Panahian and Maines, 2001). This finding supports the notion that the HO/CO system functions as a cytoprotectant and suggests that CO production may aide in the regeneration of injured connections, which remains to be determined. In 2009, the first direct study of CO in neural development was conducted by Knipp and Bicker. They found that CO acts as a slowdown signal for the migration rate of locust enteric neurons (Knipp and Bicker, 2009), indicating that CO can act as a developmental signal. More recently, CO was shown to decrease microglial migration while increasing neurite outgrowth in human NT2 cells (Scheiblich and Bicker, 2014), providing evidence that CO may indeed aide in the development/regeneration of neuronal connections. Using these findings as a springboard, this dissertation aims to look at the mechanisms by which CO can affect developing/regenerating neurons, particularly focusing on how CO can affect ion channel activity, calcium dynamics, and growth cone motility.

1.4 *Helisoma trivolvis* a model for neuronal development

While a wide range of invertebrate and vertebrate model systems have been utilized for the study of neuronal development and regeneration, the molluscan nervous system, in particular,
has been used for the extensive study of the processes that underlie neuronal form and function, including some landmark studies on the ionic mechanisms of APs using squid axons (Hodgkin and Huxley, 1952), the cellular and molecular mechanisms of learning and memory using the sea slug *Aplysia*, (Kandel, 1981, Kandel and Schwartz, 1982), and insights into neuronal development using the fresh water snail *Helisoma trivolvis* (Cohan and Kater, 1986, Cohan et al., 1987). In this dissertation, the *Helisoma trivolvis* model system was used to characterize the effects of NO and CO on neuronal development and regeneration for a number of reasons. (1) The snail nervous system, like other invertebrates, is relatively simple, containing fewer neurons and connections than vertebrate nervous systems. Because of this, *Helisoma* neurons are (2) highly identifiable, i.e. individual neurons are capable of being distinguished and experimentally characterized in one animal preparation to the next. Moreover, the large identifiable neurons of *Helisoma* can be (3) easily dissected and isolated in cell culture, allowing for the optimal control of the extracellular conditions during experiments. Once in cell culture, these neurons are (4) capable of developing/regenerating long neurites tipped with motile growth cones within 2 days of culture. (5) *Helisoma* neurons, have been used for a number of neurodevelopmental studies, providing the background and frame work that future studies can be built upon to give a more comprehensive understanding of how the nervous system develops and regenerates. Collectively, the advantages of the *Helisoma* model system allow for the characterization of both internal and external signaling molecules without the interference of neighboring electrical and chemical signals, providing for the best conditions for investigating how neuromodulators directly impact growth cone motility.
2 THE ROLE OF ACTION POTENTIALS IN DETERMINING NEURON-TYPE-SPECIFIC RESPONSES TO NITRIC OXIDE


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2.2 Abstract

The electrical activity in developing and mature neurons determines the intracellular calcium concentration ([Ca$^{2+}$]$_i$), which in turn is translated into biochemical activities through various signaling cascades. Electrical activity is under control of neuromodulators, which can alter neuronal responses to incoming signals and increase the fidelity of neuronal communication. Conversely, the effects of neuromodulators can depend on the ongoing electrical activity within target neurons; however, these activity-dependent effects of neuromodulators are less well understood. Here, we present evidence that the neuronal firing frequency and intrinsic properties of the action potential (AP) waveform set the [Ca$^{2+}$]$_i$ in growth cones and determine how neurons respond to the neuromodulator nitric oxide (NO). We used two well-characterized neurons from
the freshwater snail *Helisoma trivolvis* that show different growth cone morphological responses to NO: B5 neurons elongate filopodia, while those of B19 neurons do not. Combining whole-cell patch clamp recordings with simultaneous calcium imaging, we show that the duration of an AP contributes to neuron-specific differences in $[\text{Ca}^{2+}]_i$, with shorter APs in B19 neurons yielding lower growth cone $[\text{Ca}^{2+}]_i$. Through the partial inhibition of voltage-gated K$^+$ channels, we increased the B19 AP duration resulting in a significant increase in $[\text{Ca}^{2+}]_i$ that was then sufficient to cause filopodial elongation following NO treatment. Our results demonstrate a neuron-type specific correlation between AP shape, $[\text{Ca}^{2+}]_i$, and growth cone motility, providing an explanation to how growth cone responses to guidance cues depend on intrinsic electrical properties and helping explain the diverse effects of NO across neuronal populations.

**KEYWORDS:** electrical activity, calcium, development, filopodia, growth cone

### 2.3 Introduction

The highly regulated intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) in neurons determines a variety of cellular processes, such as neurotransmitter release (Schneggenburger et al., 2012), axonal guidance (Zheng and Poo, 2007), synaptic plasticity and gene regulation (Cohen and Greenberg, 2008). The spiking activity of a neuron is a major factor determining $[\text{Ca}^{2+}]_i$, and neuromodulators can contribute to changes in $[\text{Ca}^{2+}]_i$ by changing firing frequencies and action potential parameters within cell bodies, neurites and growth cones (Bolsover and Spector, 1986, Cohan et al., 1987, Ross et al., 1987, Torreano and Cohan, 1997, Kuznetsov et al., 2012).

During development, growth cones guide extending axons and dendrites towards their synaptic targets, a process shown to be regulated by $[\text{Ca}^{2+}]_i$ (Zheng and Poo, 2007). Changes in
[Ca^{2+}]_{i} affect the growth cone cytoskeleton, enabling for an array of growth cone behaviors, such as turning (Robles et al., 2003, Henley and Poo, 2004, Wen et al., 2004), neurite outgrowth (Gomez and Spitzer, 2000, Trimm and Rehder, 2004) and filopodial motility (Rehder and Kater, 1992, Van Wagenen et al., 1999, Tornieri and Rehder, 2007, Welshhans and Rehder, 2007, Zhong et al., 2013b). While electrical activity can affect growth cone behaviors (Neely and Nicholls, 1995), the effect is often cell-type specific. Stimulation of rat superior cervical ganglia neurons increases growth cone [Ca^{2+}]_{i}, but does not change the rate of neurite outgrowth (Garyantes and Regehr, 1992). Alternatively, stimulation of developing retinal ganglia neurons increases the rate of outgrowth (Goldberg et al., 2002). Several molecular signals present in the vicinity of navigating growth cones act through changes in [Ca^{2+}]_{i} and can determine the trajectory of advancing neuronal processes (Hong et al., 2000, Li et al., 2005, Gomez and Zheng, 2006, Togashi et al., 2008, Tojima et al., 2011).

Given that many neuromodulators function through [Ca^{2+}]_{i}, and that intrinsic firing activity is a major contributing factor to [Ca^{2+}]_{i}, it is important to investigate how the effects of neuromodulators may depend on the ongoing electrical activity of target neurons to explain activity-dependent effects of neuromodulation. Performing such experiments on isolated, cultured neurons has the advantage that the spiking activity can be manipulated, neuromodulators can be targeted to individual neurons, and effects of neuromodulators on spiking, [Ca^{2+}]_{i}, and growth cone motility can easily be recorded. Here, we used identified B5 and B19 neurons from Helisoma trivolvis grown in single cell culture as model neurons and the gaseous volume transmitter, nitric oxide (NO), as a neuromodulator. NO is known to increase the firing activity in both neurons (Artinian et al., 2010, Zhong et al., 2013c), but only B5 neurons show a calcium-dependent in-
crease in growth cone filopodial length (Van Wagenen and Rehder, 2001). An increase in filopodia length is the earliest morphological growth cone response to an increase in [Ca\(^{2+}\)]\(_i\), and given the importance of filopodia for growth cone steering (Marsh and Letourneau, 1984, Bentley and Toroian-Raymond, 1986, McCaig, 1989, Robles et al., 2003, Geraldo and Gordon-Weeks, 2009), it was used here as an indicator for an effect of spiking activity on growth cone morphology. To determine the reason for the cell type-specific responses to NO, we first studied the relationship between the neuronal firing frequency and the resulting [Ca\(^{2+}\)]\(_i\) in growth cones, and then investigated whether the effect of the neuromodulator NO depended on the ongoing electrical activity in these neurons. Using whole-cell patch clump recordings with simultaneous calcium imaging we found that intrinsic firing frequency, as well as cell-specific AP properties, are major determinants of growth cone [Ca\(^{2+}\)]\(_i\), which in turn determine the morphological response of the growth cone to signaling molecules such as NO.

2.4 Methods

2.4.1 Neuronal culture

Identified B5 and B19 neurons were dissected from the buccal ganglia of the adult freshwater pond snail, *Helisoma trivolvis*, and plated in isolation onto poly-L-lysine (hydrobromide; molecular weight of 70,000–150,000; 0.25 mg/ml; Sigma) coated glass coverslips glued to the bottom of 35 mm cell culture dishes (Falcon 1008), as previously described (Zhong et al., 2013a, Zhong et al., 2013c). Briefly, neurons were cultured in 2 mL of conditioned medium at room temperature and used for experiments 24-48 hours after plating, at which time neurons had extended neurites tipped by growth cones which were at least 140-180 \(\mu m\) (2 cell body diameters for B19 and B5, respectively) from the soma. Conditioned medium was prepared by incubating
2 H. trivolvis cerebral ganglia per 1 mL of Leibowitz L-15 medium (Invitrogen, Carlsbad, CA) for 4 days (Wong et al., 1981). L-15 consisted of the following (mM): 44.6 NaCl, 1.7 KCl, 1.5 MgCl₂, 0.3 MgSO₄, 0.14 KH₂PO₄, 0.4 Na₂HPO₄, 1.6 Na pyruvate, 4.1 CaCl₂, 5 HEPES, 50 μg mL⁻¹ gentamicin, and 0.15 mg mL⁻¹ glutamate in distilled water, pH 7.4.

2.4.2 Electrophysiology

Recordings from cultured H. trivolvis B5 and B19 neurons were performed in whole-cell current-clamp mode, as previously described (Artinian et al., 2012, Zhong et al., 2013b, c), while simultaneously imaging calcium concentrations within the growth cone. In brief, patch electrodes were pulled from borosilicate glass tubes (OD 1.5 mm; ID 0.86 mm; Sutter Instruments) on a Sutter Instruments micropipette puller (P-87) and heat polished (Micro Forge MF-830; Narishige) with a resistance of 5-10MΩ. Petri dishes containing cultured neurons were placed on a fixed stage that was centered below the objectives of an upright microscope (BX51W1F, Olympus, Japan). To allow for movement of the field of view and the imaging of different locations while maintaining whole-cell patch clamp, the upright microscope was mounted on a manual translation stage (Siskiyou, San Francisco, CA). Recordings were conducted using an Axopatch 700B amplifier (Molecular Devices, Union City, CA), an analog-to-digital converter (Digidata 1440), and an Imaging Control Unit (Till Photonics, FEI) used to simultaneously trigger electrical recordings with calcium imaging. Acquisition and analysis were performed using Axon Instrument pClamp software (v10; Molecular Devices). Leibowitz L-15 medium (Gibco, Grand Island, NY, USA) was used as the normal extracellular solution; however, in some experiments, L-15 medium was replaced with a NaCl-free solution that consisted of the following (mM): 51.3 N-methyl-D-glucamine (NMDG), 1.7 KCl, 4.1 CaCl₂, 1.5 MgCl₂, and 5 HEPES, pH
7.3 (127 mOsm). The intracellular recording solution contained the following (mM): 54.4 K-aspartate, 2 MgCl₂, 5 HEPES, 5 Dextrose, 5 ATP, and 0.1 EGTA (127 mOsm). The low concentration of EGTA was chosen because it did not affect spontaneous firing activity (Artinian et al., 2010). Recording and manipulation of membrane potential and firing activity were conducted in current-clamp configuration and signals filtered at 5kHz (-3 dB, four-pole Bessel filters). The membrane potential was not corrected for liquid junction potential, which was calculated using Clampex software (v10; Molecular Devices) to be approximately -15.7 mV for L-15 and -11.0mV for NaCl-free solution at 20ºC.

2.4.3 Calcium imaging

Growth cone calcium measurements were performed as previously described (Welshhans and Rehder, 2005, Zhong et al., 2013b), while simultaneously recording electrical activity from the soma. In short, B5 and B19 neurons were injected with the cell-impermeable calcium indicator dye, Fura-2 pentapotassium salt (10 mM in H₂O; Molecular Probes, Eugene, OR) using a Picospritzer (General Valve Corporation, USA). Care was taken to load cells just enough to yield sufficient fluorescence and avoid overloading with Fura-2, which we observed can affect spontaneous firing activity. We estimated the Fura-2 concentration inside the cell to be between 500 µM and 1 mM. Growth cone calcium images were recorded using the dual system described above equipped with a cooled CCD camera (Andor Technology, Model # DR328GC01SIL9JCI). Acquisition and analysis of calcium images were conducted using Live Acquisition and Offline Analysis software (Till Photonics, FEI, Germany). Fura-2 was excited at 340 and 380 nm with 100 ms exposure time at 20% light intensity every 5-10 seconds. Emission ratios (340/380) taken from the center of each growth cone were background corrected and converted to an estimated
calcium concentration following calibration with the Calcium Calibration Buffer Kit #1 (Molecular Probes, USA) and conversion using the Grynkiewicz formula (Grynkiewicz et al., 1985):

\[[\text{Ca}^{2+}] = K_d [(R - R_{\text{min}})/(R_{\text{max}} - R)] \times (F_o/F_s)\]

with \(R_{\text{min}}: 0.2188, R_{\text{max}}: 8.2592, K_d \beta: 4779\), where \(\beta\) represents \((F_o/F_s)\).

While data acquisition of electrical activity and calcium concentration occurred simultaneously, the exact timing of an AP and calcium measurement varied. To minimize the effects of these fluctuations, we averaged firing frequency and growth cone calcium concentration over 10 seconds when the neurons reached an observed stable level of firing; measurements prior to these stable levels were omitted from the current study. The ratiometric FURA-2 images presented in the figures were taken from these stable firing periods, evenly adjusted for contrast, and cropped using Adobe Photoshop CS3 (v.10.0.1; USA). In order to compare the magnitude of calcium change per firing frequency in experiments that showed different firing frequencies, calcium concentrations were estimated from the linear fit equation for each experiment at defined frequencies: 0, 0.5, 1.0, 1.5, and 2.0 Hz.

2.4.4 Growth cone filopodial dynamics

Growth cones were imaged using a 100X oil-immersion objective on an inverted microscope (Olympus IX70, Japan) with a cooled CCD camera (Photometrics C350, Tucson, AZ, USA), as previously described (Welshhans and Rehder, 2007). Images were acquired using MetaMorph software (Universal Imaging Corporation, Dowington, PA, USA) and stored on a Universal Imaging Corporation PC. Phase contrast images were acquired at defined time points both before (-10, -5, and 0 min) and after (2, 5, 10, 15, 20 and 30 min) treatment with pharmacological agents. Filopodial length, determined by measuring all filopodia on a growth cone from the
tip to the edge of the central domain, was analyzed with Scion Image software (Scion Corporation, Frederick, MD). Filopodial data were normalized to the time point $t = 0$ and expressed as a percentage change, minimizing the individual variability occurring from growth cone size as well as baseline filopodial length between different growth cones.

### 2.4.5 Pharmacological agents

The NO-donor, 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propyamine (NOC-7; Calbiochem) was dissolved in 100 mM NaOH to make a stock solution of 100 mM. 4-aminopyridine (4AP; Sigma) and tetraethylammonium (TEA; Sigma) were dissolved in L-15 the day of an experiment at a stock concentration of 250 mM and 500 mM, respectively. All drug treatments were bath applied unless otherwise stated. Replacement of L-15 with the NaCl-free NMDG containing solution involved the removal of 1 mL of L-15 and replacement with 1 mL of NMDG containing solution. This process was repeated 5 times to complete the replacement of NaCl in the extracellular solution.

### 2.4.6 Statistical analysis

All data were expressed as mean ± SEM, unless otherwise stated. For calcium imaging results, the MWU test was used for testing statistical significance between defined frequencies. For growth cone filopodial analysis, a repeated-measures ANOVA was employed for testing overall statistical significance between conditions (SPSS statistical software, SPSS, Chicago, IL). The Tukey test was used for post hoc analysis of preplanned comparisons. Linear fit between growth cone calcium concentration and firing frequency was determined using ORIGIN DATA ANALYSIS AND GRAPHING software (OriginLab, Northampton, MA). Goodness of fit for
linear correlations were considered weak $R^2 < 0.5$, significant $R^2 = 0.5-0.8$, and strong $R^2 >0.8$.

For electrophysiological data analysis, the significance of effects was evaluated by one-way ANOVA and Tukey's *post hoc* test using ORIGIN DATA ANALYSIS AND GRAPHING software (OriginLab, Northampton, MA). Significant differences are indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

### 2.5 Results

#### 2.5.1 Neuronal firing frequency sets $[Ca^{2+}]_i$ in B5 growth cones

To investigate how neuronal spiking activity affected calcium levels within B5 growth cones, electrical activity and the $[Ca^{2+}]_i$ in growth cones were measured simultaneously in cultured neurons [Fig. 1(A)]. B5 neurons exhibited spontaneous tonic firing frequencies that ranged from 0.75 to 1.24 Hz (n=12) and resulted in $[Ca^{2+}]_i$ between 306 to 615 nM (n=12). To systematically investigate how the neuronal firing frequency determined $[Ca^{2+}]_i$ in growth cones, we employed a range of current injections from -40 pA to +40 pA designed to mimic the range of physiological firing frequencies typically observed in B5 neurons [Fig. 1(Ai and Aii)]. Firing frequency and $[Ca^{2+}]_i$ were decreased with hyperpolarizing current injections in a stimulation-dependent manner, with the strongest hyperpolarizing current injection (-40 pA) leading to a complete silencing of firing activity and an average growth cone $[Ca^{2+}]_i$ of 91 ± 7 nM (n=12). Conversely, depolarizing current injections increased firing frequency and $[Ca^{2+}]_i$ in a stimulation-dependent fashion. The correlation between growth cone calcium levels and neuronal firing frequency could be fitted in first approximation by a linear relationship for the stimulation range tested (Pearson’s $r^2=0.98\pm 0.002$, n=12) [Fig. 1(B)]. Taken together, these results suggested that
the frequency of APs generated at the cell body determines the basal [Ca$^{2+}$]$_i$ within developing growth cones.

2.5.2 Evoked APs lead to a frequency-dependent elevation of [Ca$^{2+}$]$_i$ in growth cones of B19 neurons

We next wanted to test whether the correlation and magnitude of response in B19 neurons, which are known to show an activity-dependent increase in growth cone [Ca$^{2+}$]$_i$ (Torreano and Cohan, 1997), were similar to that of B5 neurons. Unlike B5 neurons, B19 neurons exhibited one of two activity states: they were either silent or spontaneous spiking. A majority of B19 neurons were silent prior to stimulation with an average growth cone [Ca$^{2+}$]$_i$ of 55 ± 4 nM. Spontaneously firing B19 neurons produced an average growth cone [Ca$^{2+}$]$_i$ of 81 ± 14 nM. We then applied current steps from -20 pA to +20 pA to elicit physiologically relevant changes in firing frequency while measuring growth cone [Ca$^{2+}$]$_i$. The increase in the amplitude of injected current led to an increase in firing frequency and an elevation of [Ca$^{2+}$]$_i$ in growth cones [Fig. 2(A)].

When the steady-state frequency was plotted against the [Ca$^{2+}$]$_i$, it showed a strong, positive linear correlation between electrical stimulation-induced firing frequency and growth cone [Ca$^{2+}$]$_i$ (Pearson’s $R^2$=.97 ± 0.005, n=12) [Fig. 2(B)]. Interestingly, while both B5 and B19 neurons showed a positive correlation between their firing frequency and the growth cone [Ca$^{2+}$]$_i$ during electrical stimulation, B5 neurons had a greater rate of increase, indicated by the slope of the linear fit (353 ± 28, n = 12), than B19 neurons (138 ± 9, n = 12), suggesting that B5 neurons exhibit a stronger Ca$^{2+}$ increase per firing frequency than B19 neurons. Because the firing frequency induced by current injection varied between experiments and neuron types, we used the linear fit equation derived from each neuron to determine the [Ca$^{2+}$]$_i$ reached at defined frequencies.
While both B5 and B19 neurons showed similar growth cone $[\text{Ca}^{2+}]_i$ when silent (MWU, $p = 0.386$), B5 neurons exhibited a larger increase in growth cone $[\text{Ca}^{2+}]_i$ at defined firing frequencies than B19 neurons (MWU, $p < 0.001$ in each case) [Fig. 2(C)]. Taken together, these results provide further evidence that the neuronal firing frequency plays an important role in setting growth cone $[\text{Ca}^{2+}]_i$ and that the magnitude of activity-dependent $[\text{Ca}^{2+}]_i$ is neuron-type specific.

### 2.5.3 Longer AP durations increase the activity-dependent growth cone calcium concentration

To explain the activity-dependent differences in $[\text{Ca}^{2+}]_i$ observed in B5 and B19 neurons, we next compared the basic electrical properties of the AP in these neurons. Upon first examination, each neuronal type showed a distinct AP shape [Fig. 3(Ai and Aii)] that significantly differed from one another. Further characterization of each AP [Table 1] revealed B19 neurons have a significantly higher AP amplitude than B5 neurons (MWU, $U = 0, p = 0.0001$) while B5 neurons have a significantly longer-lasting AP duration, measured as AP half-width (MWU, $U = 0, p \leq 0.0001$). With the rationale that both the amplitude and duration of an AP would likely determine the degree to which voltage gated calcium channels (VGCCs) are open and thus contributing to the growth cone $[\text{Ca}^{2+}]_i$, we next compared the total area under a single AP from threshold between the two neuron types. Not surprisingly, the area under a single AP was significantly larger for B5 than for B19 neurons (MWU, $U = 0, p = 0.0001$). These findings, coupled with the larger increase in calcium at defined frequencies in B5 neurons over B19 neurons, suggest that AP shape plays a significant role in determining growth cone $[\text{Ca}^{2+}]_i$.

To further test whether wider APs directly impacted the activity-dependent growth cone $[\text{Ca}^{2+}]_i$, we next pharmacologically broadened the width of APs in B19 neurons and measured
the \([\text{Ca}^{2+}]_i\) in growth cones in response to electrical stimulation [Fig. 3(Aiii)]. While application of a solution containing a mixture of 4AP (250 µM) and TEA (500 µM) to inhibit K\(^+\) channels did not significantly affect AP amplitude of B19 neurons (MWU, U = 16; p = 0.758), it significantly increased AP half-width (MWU, U = 0; p = 0.005) and area (MWU, U = 1; p = 0.009) [Table 1]. Injection of current into the cell bodies of TEA and 4AP treated neurons resulted in a strong temporal relationship between firing frequency and \([\text{Ca}^{2+}]_i\) in growth cones, as in pretreatment controls [Fig. 3(B)]. The plot of \([\text{Ca}^{2+}]_i\) in growth cones against firing frequency showed that the slope of the regression line increased following TEA and 4AP treatment (Pre-treatment slope: 190 ± 9, n = 6; Post-treatment slope: 441 ± 35, n = 6)[Fig. 3(C)]. Further analysis showed that silent B19 neurons had similar growth cone \([\text{Ca}^{2+}]_i\) before and after TEA and 4AP treatment (MWU, U = 14, p = 0.522), suggesting that treatment did not affect basal calcium levels. At defined firing frequencies, B19 neurons with widened APs showed significantly higher \([\text{Ca}^{2+}]_i\) in their growth cones than control neurons (MWU, p ≤ 0.001) [Fig. 3(D)]. Taken together, these results suggested that wider APs were sufficient to increase the amount of \([\text{Ca}^{2+}]_i\) influx in growth cones during evoked electrical activity and supported our hypothesis that AP width can account for the difference in \([\text{Ca}^{2+}]_i\) observed in growth cones of B5 and B19 neurons spiking at the same frequency.

### 2.5.4 NO-donor NOC-7 transiently increases firing frequency and \([\text{Ca}^{2+}]_i\) in growth cones of B5 neurons

After having established the correlation between evoked neuronal firing rate and growth cone \([\text{Ca}^{2+}]_i\), we next investigated if the same correlation would be obtained in neurons under
physiological stimulation conditions. We have shown previously that the gaseous neuromodulator, NO, causes a transient increase in neuronal firing in B5 (Artinian et al., 2010) and B19 (Zhong et al., 2013c) neurons. Application of the NO donor NOC-7 (100 µM) to B5 neurons resulted in an initial average increase in the firing rate from 0.7 ± 0.1 to 1.3 ± 0.1 Hz, which then slowly returned towards baseline firing rates over several minutes and eventually resulted in neuronal silencing [Fig. 4(Ai)], supporting earlier results (Artinian et al., 2010). As it was observed in current injection experiments, the changes in spiking frequency were closely correlated with \([\text{Ca}^{2+}]_i\) [Fig. 4(Aii and Aiii)] and showed a linear relationship \((R^2 = 0.84 ± 0.03, n = 6)\) [Fig. 4(B)]. While silent neurons showed a significant difference in \([\text{Ca}^{2+}]_i\) between current injection experiments and NO treatment \((\text{MWU, } U = 4; \ p = 0.003)\), there was no distinguishable difference in calcium levels at defined firing frequencies \((\text{MWU, } p > 0.10)\) [Fig. 4(C)], providing further evidence that the electrical activity of a neuron defines the growth cone \([\text{Ca}^{2+}]_i\).

**2.5.5 NO acts through an increase in spiking activity to elevate \([\text{Ca}^{2+}]_i\) and elongate filopodial**

We have previously shown that increases in growth cone \([\text{Ca}^{2+}]_i\), mediated by NO lead to a transient increase in filopodial length in B5 neurons (Van Wagenen and Rehder, 1999). Transient increases in filopodial length, which would start as early as 2 minutes after an increase in \([\text{Ca}^{2+}]_i\), were shown to result in significant increases in the area that could be surveyed by advancing growth cones (Van Wagenen et al., 1999), and were reminiscent of filopodial changes observed in growth cones encountering locations along their migratory path where pathfinding decisions had to be made (Taghert et al., 1982, Tosney and Landmesser, 1985, Holt, 1989). Here we wanted to test whether the effects of NO on \([\text{Ca}^{2+}]_i\) and filopodial length were mediated
through the transient increase in spiking activity observed following NO treatment. To do this, we first tested how NO affected $[\text{Ca}^{2+}]_i$ in the absence of spiking activity. Because voltage-gated sodium channels in these neurons are insensitive to TTX, to block these channels we replaced extracellular sodium with the impermeable cation N-methyl-D-glucamine (NMDG) as described previously (Artinian et al., 2010). In the presence of the NMDG solution, spontaneous firing activity was silenced [Fig. 5(Ai)]. The subsequent addition of NOC-7 failed to elicit APs. It did, however, result in a small depolarization of the membrane potential from $-31.8 \pm 1.5$ to $-27.0 \pm 1.5$ mV ($n = 7$) [Fig. 5(Aii)] coupled with a small increase in $[\text{Ca}^{2+}]_i$ from $118 \pm 5$ nM to $176 \pm 6$ nM ($n = 7$) [Fig. 5(Aiii)]. To assess how the AP contributed to the overall magnitude of $[\text{Ca}^{2+}]_i$ mediated by NO, we next compared the maximum increase in $[\text{Ca}^{2+}]_i$ between normal and NaCl-free conditions. While the increase of growth cone $[\text{Ca}^{2+}]_i$ in response to NO in spiking neurons was $210 \pm 36$ nM ($n = 5$), this value was significantly lower in the presence of NMDG ($58 \pm 6$ nM; MWU, $U = 0$, $p = 0.003$, $n = 7$), suggesting that the presence of APs is critical for generating robust changes in growth cone $[\text{Ca}^{2+}]_i$ by NO. We then wanted to determine whether the absence of APs and smaller changes in $[\text{Ca}^{2+}]_i$ had an impact on the ability of NO to affect filopodial length. While NOC-7 caused a robust increase in filopodial length as early as two minutes after addition (Repeated-measures ANOVA, Tukey’s post hoc, $p \leq 0.001$), it failed to increase filopodial length significantly in the presence of NMDG (Repeated-measures ANOVA, Tukey’s post hoc, $p = 0.544$) [Fig. 5(B and C)]. Taken together, these findings suggest that the presence of action potentials plays a critical role in determining the neuronal response to NO.
2.5.6 **NO elevates the spiking frequency and growth cone [Ca^{2+}] in B19 neurons**

We have previously shown that the application of NO donor, SIN-1, failed to elongate filopodia in B19 neurons (Van Wagenen and Rehder, 2001), but that NO donors NOC-7 and DEA/NO depolarized their membrane potential and resulted in an increase in firing frequency in B19 neurons (Zhong et al., 2013c). To understand why B19 neurons showed a typical electrical response to NO but failed to produce a morphological response, we first determined the effect of firing frequency induced by NO on B19 growth cone [Ca^{2+}]. Bath application of NOC-7 (100 µM) resulted in an initial increase in firing activity from either a silent (n = 3) or spontaneously firing state (average frequency: 0.4 ± 0.1; n = 3) to 1.7 ± 0.3 Hz (n = 6) followed by a sustained firing period [Fig. 6(Ai)]. As observed in B5 neurons, the increase in firing frequency in B19 neurons [Fig. 6(Aii)] corresponded with a similar increase in growth cone [Ca^{2+}], over time [Fig. 6(Aiii)].

The temporal relationship between firing frequency and growth cone [Ca^{2+}], mediated by NO showed a strong positive linear relationship (R²=0.85 ± 0.02, n=13) [Fig. 6(B)] that was similar to the growth cone [Ca^{2+}], responses to electrical stimulation (MWU, p > 0.10) [Fig.6 (C)], suggesting further that the spiking activity of a neuron sets growth cone [Ca^{2+}], regardless of the stimulus that caused the neuron to fire at this frequency.

2.5.7 **Widening of action potentials increases sensitivity to NO and results in filopodial elongation in B19 neurons**

Intriguingly, both B5 and B19 neurons showed a strong positive correlation between firing frequency and growth cone [Ca^{2+}], following NO treatment; however, only B5 neurons showed a reliable increase in filopodial length. Similar to what we had observed with electrical
stimulation and $[\text{Ca}^{2+}]_i$ responses between B5 and B19 neurons [Fig. 2(C)], the slope of the linear correlation following NO exposure was steeper in B5 neurons than B19 neurons (B5: 362.21 ± 48.49, n = 6 vs. B19: 135.67 ± 9.36, n = 13). Additionally, the NO-mediated increase in growth cone $[\text{Ca}^{2+}]_i$ at defined frequencies was higher in B5 neurons (MWU, $U \leq 10$, $p \leq 0.01$) [Fig. 4(B) and Fig. 6(B)]. While the larger increase in $[\text{Ca}^{2+}]_i$ observed in B5 neurons may account for the NO-mediated increase in filopodial length, it is possible that the smaller increase in $[\text{Ca}^{2+}]_i$ observed in NO-treated B19 neurons was not sufficient or robust enough to have an effect on filopodial length. Having shown above that AP width serves as an important determinant in setting growth cone $[\text{Ca}^{2+}]_i$, we next tested whether the width of the B19 AP was determining both its calcium dynamics and filopodial response to NO.

We first tested whether pharmacological broadening of B19 APs with TEA and 4AP would increase the $[\text{Ca}^{2+}]_i$ within the growth cone following NO treatment. We observed a strong positive linear relationship between firing frequency and growth cone $[\text{Ca}^{2+}]_i$ in B19 neurons pretreated with 500 µM TEA and 250 µM 4-AP and exposed to 100 µM NOC-7 ($R^2 = 0.85 \pm 0.04$, $n = 4$)[Fig. 7(A)]. As hypothesized, wider B19 APs resulted in an increase in the slope of the linear correlation (NOC-7 only: 135.67 ± 9.36, $n = 13$; NOC-7 after TEA and 4AP: 409.71 ± 53.58, $n = 4$)[Fig. 7(A)] and a significant increase in $[\text{Ca}^{2+}]_i$ when firing action potentials at defined firing frequencies compared with NO treatment only (MWU, $U = 0$, $p \leq 0.001$)[Fig. 7(A and B)]. It should be noted that there was no significant difference in growth cone $[\text{Ca}^{2+}]_i$ between the two conditions when the neurons were silent (MWU, $U = 21$, $p = 0.571$).

We next tested whether this increase in $[\text{Ca}^{2+}]_i$ would result in filopodial elongation in B19 neurons. While treatment with NOC-7 alone did not affect filopodial length [Fig. 7(C)], addition of NOC-7 (100 µM) to B19 neurons treated with 4AP and TEA resulted in a significant
increase in filopodial length (Repeated Measures ANOVA, Tukey’s post hoc, p = 0.001) [Fig.7(D)], suggesting that AP shape contributed significantly to whether NO affected growth cone filopodia. Taken together, these data demonstrated that NOC-7 treatment alone did not elevate [Ca$^{2+}$]$_i$ within B19 growth cones high enough to result in filopodial elongation, whereas the widened APs elevated [Ca$^{2+}$]$_i$ beyond a presumed threshold to trigger filopodial elongation. Therefore, the shape of APs in a neuron is an important determinant of whether NO will affect growth cone filopodia during development or not.

2.6 Discussion

Waves of spontaneous and patterned electrical activity propagate throughout the developing nervous system, playing an important role in the establishment of neural connections (Spitzer, 2006, Yamamoto and Lopez-Bendito, 2012). Disruption of this electrical activity can lead to errors in pathfinding (Hanson and Landmesser, 2004) and the formation of synaptic connections with inappropriate targets (Catalano and Shatz, 1998). These activity-dependent changes in pathfinding likely involve alterations in [Ca$^{2+}$]$_i$ (Neely and Nicholls, 1995, Spitzer, 2006, Zheng and Poo, 2007), and [Ca$^{2+}$]$_i$ in growth cones plays important roles in the organization of the growth cone cytoskeleton, enabling for an array of growth cone behaviors, such as turning (Robles et al., 2003, Henley and Poo, 2004, Wen et al., 2004), neurite outgrowth (Gomez and Spitzer, 2000, Trimm and Rehder, 2004) and changes in the sensory radius of the growth cone via changes in filopodial length (Rehder and Kater, 1992, Van Wagenen et al., 1999, Tornieri and Rehder, 2007, Welshhans and Rehder, 2007, Zhong et al., 2013b).
Neuromodulators can affect the electrical activity of developing neurons, and in order to understand their effects on neuronal activity and developmental processes, it is critical to also investigate how the effects of neuromodulators depend on the ongoing electrical activity within target neurons. Here, we provide evidence for neuron-specific growth cone responses to electrical activity, as well as the first direct investigation of how AP shape, and its resulting change in $[\text{Ca}^{2+}]_i$, determine the responsiveness of developing neurons to the volume transmitter, NO.

In the current study, we combined whole-cell patch-clamp recording with ratiometric calcium imaging and found that firing frequency and growth cone $[\text{Ca}^{2+}]_i$ have a strong positive correlation in both B5 and B19 neurons. Due to a longer AP duration, B5 neurons produced larger calcium responses at defined firing frequencies than B19 neurons. These cell-specific differences in AP were found to be critical for shaping the responses of growth cone filopodia to NO treatment. After widening B19 APs with $\text{K}^+$ channel blockers, NO was then capable of increasing filopodial length. Taken together, we conclude that the composition of ionic currents that shape the AP and its frequency define growth cone $[\text{Ca}^{2+}]_i$ and ultimately the response of a growth cone to a given guidance signal.

2.6.1 Electrical activity sets growth cone calcium

With the aim of obtaining a quantitative description of how spontaneous firing activity affects the calcium dynamics within the growth cone, we manipulated the spontaneous firing activity with hyperpolarizing and depolarizing current injections into the soma and simultaneously investigated the effects of propagating AP on $[\text{Ca}^{2+}]_i$ in growth cones. Growth cones of *Helisoma* B5 and B19 neurons produce regenerative APs, which are indistinguishable from those generated in their somata, suggesting the presence of the same ion channels in both cellular compartments
(Guthrie et al., 1989). Measurements of calcium showed a tight temporal relationship between firing frequency and growth cone $[Ca^{2+}]_i$, which has been described previously in studies of both invertebrate (Cohan et al., 1987, Ross et al., 1987, Torreano and Cohan, 1997) and vertebrate (Bolsover and Spector, 1986, Fields et al., 1990) neurons. Here we further develop these findings by showing that deviations from spontaneous activity during depolarizing or hyperpolarizing stimuli can increase and decrease $[Ca^{2+}]_i$, respectively, suggesting for a wider range of fluctuating $[Ca^{2+}]_i$ in developing B5 and B19 neurons than previously thought. Furthermore, these data provided the foundation needed to next compare the effects of electrically-induced APs on $[Ca^{2+}]_i$ with those elicited by NO.

The presence of VGCCs in growth cones (Vigers and Pfenninger, 1991, Spafford et al., 2004, Hui and Feng, 2008) coupled with findings that depolarization-induced increases in $[Ca^{2+}]_i$ require extracellular calcium (Rehder and Kater, 1992) suggests that VGCCs are major contributors determining the activity-dependent $[Ca^{2+}]_i$ in growth cones. The fast activating and slow inactivating current of the L-type calcium channel may play a particularly important role in establishing growth cone calcium levels and has been shown to affect the rate of neurite outgrowth (Robson and Burgoyne, 1989, Schindelholz and Reber, 2000, Kulbatski et al., 2004). A lack of specific pharmacological inhibitors for invertebrates prevented the immediate characterization of the VGCCs involved in our system, but future studies aimed at identifying the molecular identity of VGCCs will aid in our understanding of how individual calcium channel kinetics affect growth cone $[Ca^{2+}]_i$.

While firing frequency sets the $[Ca^{2+}]_i$ in both B5 and B19 growth cones, it does not explain the neuron-specific differences in $[Ca^{2+}]_i$ achieved at similar spiking frequencies. Bolsover et al. (1986) first showed that the duration of an AP can shape the magnitude of the resulting
[Ca\textsuperscript{2+}]_i. Since then a number of studies have supported the notion that AP duration dictates the amount of calcium brought in by an AP (Ross et al., 1987, Torreano and Cohan, 1997, Kuznetsov et al., 2012). We showed that B5 neurons reach a higher [Ca\textsuperscript{2+}]_i per defined firing frequency than B19 neurons, which can be attributed to wider APs in B5 neurons. By widening the B19 AP using the K\textsuperscript{+} channel blockers TEA and 4AP, we increased the growth cone [Ca\textsuperscript{2+}]_i per AP, similar to what was found previously in Helisoma using long term 45 minute electrical stimulations (Torreano and Cohan, 1997). Therefore, intracellular signaling events that change the AP waveform are expected to strongly influence cell-specific calcium dynamics, a finding we needed below to test the relationship between AP parameters, [Ca\textsuperscript{2+}]_i, and filopodial elongation.

2.6.2 Intrinsic electrical properties determine effects of NO on growth cone morphology

NO is a physiological neuromodulator that plays critical roles in neuronal development, including growth cone motility (Hess et al., 1993, Van Wagenen et al., 1999), axonal pathfinding (Tojima et al., 2009), and synaptogenesis (Ogilvie et al., 1995, Nikonenko et al., 2008). Modulation of growth cone dynamics by NO is largely mediated through calcium and electrical activity (Seidel and Bicker, 2000, Tojima et al., 2009); less is known about how intrinsic neuronal firing activity defines the response of a growth cone to NO. Here, we found that the increase in spiking activity elicited by NO strongly correlated with changes in growth cone [Ca\textsuperscript{2+}]_i, and more importantly, intrinsic AP properties were critical for mediating NO-induced growth cone responses. Cell-specific responses to NO have been shown to be regulated by the presence of NO receptor proteins, such as soluble guanylyl cyclase (Garthwaite, 2008), as well as a past history of NO exposure (Halvey et al., 2009). To our knowledge, our findings are the first to demonstrate that the
neuronal firing activity can affect cellular responses to NO. In addition, our findings lend further explanation to how the history of prior exposure to NO can affect future NO responses. For example, the endogenous production of NO has the capability of regulating intrinsic neuronal activity (Artinian et al., 2012) thus affecting the $[\text{Ca}^{2+}]_i$. Activity-dependent changes in $[\text{Ca}^{2+}]_i$ can affect endogenous NO production (Bredt, 1999, Vincent, 2010), which, through NO neuromodulation, can affect neuronal activity and subsequent responses to NO. It is likely that a combination of cell intrinsic activity coupled with NO-activated signaling pathways will generate cell-specific responses to NO as well as to other guidance cues.

2.6.3 Modulating neuronal responses through intrinsic AP properties: the role of $K^+$ channels

The composition of ion channels is important for determining the electrical activity of a neuron and is controlled by the developmental state and stimulation history of a neuron. The up- and down-regulation of ion channels over the course of development can shift AP parameters, affecting both firing frequency and the AP waveform itself (Moody and Bosma, 2005, Suwabe et al., 2011). Such changes can be critical for generating cell-specific responses to signaling molecules during axonal guidance. $K^+$ channels, which play an important role in defining the repolarization phase of the AP, are particularly important in setting neuronal firing frequency (Kuznetsov et al., 2012) and have been shown to affect neuronal pathfinding (Mizuno et al., 2007, Wang et al., 2007). Through the modulation of activity-dependent calcium dynamics, as demonstrated here, $K^+$ channels can regulate neuronal responses to NO. Through the same mechanism, changes in $K^+$ channel activity can affect other neuromodulatory signals functioning via calcium as well as affect other calcium-mediated cellular processes. For example in *Aplysia*, the
closure of K+ channels increases AP duration and neurotransmitter release regulating the sensitization of the gill withdrawal reflex (Abrams et al., 1984). In Xenopus, the type of neurotransmitter produced by a neuronal population can be affected by the level of K+ channel expression (Borodinsky et al., 2004). Interestingly, recent findings show the activation of VGCCs can also determine the type of neurotransmitter produced by a neuron (Lewis et al., 2014), suggesting that the modulation of AP duration by K+ channels could affect neurotransmitter phenotype through their effect on VGCCs.

2.6.4 Implications for the role intrinsic AP properties play in development

With the composition of ion channels changing over development, the spontaneous electrical activity generated by a developing neuron is likely to change. Changes in electrical activity can produce an array of growth cone behaviors that are often cell-type specific (Neely and Nicholls, 1995). For example, electrical stimulation-induced increases in [Ca2+]i can lead to the slowdown and cessation of neurite outgrowth in both invertebrate (Cohan and Kater, 1986, Cohan et al., 1987) and vertebrate (Fields et al., 1990) neurons. Alternatively, electrical stimulation increases neurite outgrowth in developing retinal ganglion neurons (Goldberg et al., 2002), and it has been shown to have no effect on the rate of outgrowth in developing rat sympathetic neurons, although an increase in [Ca2+]i was observed (Garyantes and Regehr, 1992). These cell-specific effects may be attributed to the intrinsic properties of the APs, producing a set point of [Ca2+]i in the growth cone to elicit distinct behaviors. Our findings as well as those of others (Bolsover and Spector, 1986, Ross et al., 1987, Torreano and Cohan, 1997, Kuznetsov et al., 2012), suggest that the width of an AP may be important in how the electrical activity affects the calcium dynamics and thus the resulting behavior produced by the growth cone.
In addition to the variable effects that electrical activity can have on growth cone behaviors, the electrical activity can also serve as a gateway for how the growth cone responds to incoming guidance cues. For instance, electrical stimulation has been shown to change the turning direction of *Xenopus* spinal neuron growth cones induced by myelin-associated glycoprotein from repulsive to attractive following electrical stimulation greater than 0.2 Hz (Ming et al., 2001). In *Helisoma* B5 and B19 neurons, a similar increase in electrical activity occurs when stimulated by NO (Artinian et al., 2010, Zhong et al., 2013c), but the neurons differ in their growth cone filopodial responses to this induced electrical activity. With our findings that filopodia responses to NO can be changed based on the duration of the AP and the resulting change in 

\[ [\text{Ca}^{2+}] \], it is likely that the intrinsic properties that constitute a given AP waveform establish a cell-specific program of growth cone behaviors and responses to guidance signals. Future studies will have to determine which roles these neuron type-specific differences in response to electrical activity and NO may play during neuronal pathfinding.
2.7 Figures and tables

Figure 2.1 Correlation of firing frequencies and growth cone calcium concentrations in B5 neurons.

(A) Current injections were applied in 10 pA steps from -40 pA to +40 pA into the cell body of developing B5 neurons for 30 seconds while simultaneously recording electrical activity and
growth cone $[\text{Ca}^{2+}]_i$. (Ai) Examples taken from the step protocol show a change in firing frequency and growth cone $[\text{Ca}^{2+}]_i$. An overlay of firing activity with growth cone $[\text{Ca}^{2+}]_i$ showed a tight temporal relationship between firing frequency and resulting $[\text{Ca}^{2+}]_i$. (Aii) Pseudocolored ratiometric Fura-2 images of growth cone $[\text{Ca}^{2+}]_i$ illustrate increases and decreases in $[\text{Ca}^{2+}]_i$ as firing frequency increased and decreased. Measurements of growth cone $[\text{Ca}^{2+}]_i$ were taken in the center of each growth cone (white circle). Note that growth cones from the same neuron behave similarly, showing comparable $[\text{Ca}^{2+}]_i$. (B) Averages of firing frequency and growth cone $[\text{Ca}^{2+}]_i$ showed a strong linear correlation ($R^2 = .98$).
Figure 2.2 Correlation of firing frequencies and growth cone [Ca^{2+}] in B19 neurons.

(A) Current injections were given in 5 pA steps from -20 pA to +20 pA. Examples taken from the step protocol show (Ai) changes in firing frequency and growth cone [Ca^{2+}]. Note that some
B19 neurons showed sporadic firing of action potentials and that a majority of recordings showed no firing activity prior to current injection. (Aii) Ratiometric Fura-2 images illustrate an increase in growth cone [Ca\(^{2+}\)]\(_i\) as firing activity increased. (B) Averages of firing frequency and [Ca\(^{2+}\)]\(_i\) showed a strong linear correlation between firing frequency and growth cone [Ca\(^{2+}\)]\(_i\) (R\(^2\) = .99). (C) Comparisons of [Ca\(^{2+}\)]\(_i\) between B5 and B19 neurons at defined firing frequencies show that B5 neurons (black) experienced a larger increase in [Ca\(^{2+}\)]\(_i\) per firing frequency than B19 neurons (grey; p < 0.001).
Figure 2.3 Action potential duration affects the magnitude of activity-dependent growth cone [Ca^{2+}].

(A) APs of B5 neurons (Ai) have a longer half-width (12.5 ms) than those of B19 neurons (Aii; half-width: 2.5 ms). Treatment with a combination of TEA (500 μM) and 4AP (250 μM) lengthened the AP duration of B19s to approximately 5 ms (Aiii). (B) Examples taken from the step protocol show similar firing frequencies of approximately 0.8 Hz before (Bi) and after combined treatment with TEA and 4AP (Bii). (Right) Ratiometric Fura-2 images of a growth cone match-
ing the recordings on the left show higher [Ca\textsuperscript{2+}]\textsubscript{i} in TEA and 4AP treated neurons at similar firing frequencies. (C) Current injections showed a simultaneous increase in firing frequency and [Ca\textsuperscript{2+}]\textsubscript{i} as previously observed. While the linear correlation between growth cone [Ca\textsuperscript{2+}]\textsubscript{i} and firing frequency was strong before (R\textsuperscript{2} = .97) and after treatment (R\textsuperscript{2} = .97), the widened APs following TEA and 4AP treatment resulted in an increased slope. (D) Comparison of [Ca\textsuperscript{2+}]\textsubscript{i} at defined frequencies shows that the longer AP duration significantly increased the [Ca\textsuperscript{2+}]\textsubscript{i} in the growth cone (p < 0.001).
Figure 2.4 Nitric oxide increases the firing frequency and growth cone $[\text{Ca}^{2+}]_i$ in B5 neurons.

(Ai) Bath application of NO-donor, NOC7, (treatment indicated by black bar) transiently increased the firing activity of B5 neurons and later resulted in neuronal silencing. Simultaneous recordings of electrical activity and growth cone calcium show a tight temporal relationship between firing frequency (Aii) and growth cone $[\text{Ca}^{2+}]_i$ (Aiii). (B) Further analysis of this relationship revealed a strong linear correlation between firing frequency and growth cone $[\text{Ca}^{2+}]_i$ ($R^2 = .88$). (C) Both electrically evoked firing frequencies (black bars) and NO induced firing frequencies (grey bars) resulted in similar increases in growth cone $[\text{Ca}^{2+}]_i$ at defined frequencies.
Figure 2.5 APs are necessary for NO to modulate growth cone filopodia.

(A) Na replacement with N-methyl-D-glucamine (NMDG) silenced spontaneous firing activity in B5 neurons. The addition of NO (indicated by black bar) did not elicit APs (Ai); however, it depolarized the membrane potential transiently by about 6mV (Aii). Growth cone [Ca^{2+}]i closely followed the change in membrane potential (Aiii), even in the absence of AP generation. (B) Phase contrast images of B5 growth cones show filopodial elongation 10 min after treatment with NOC7 in normal medium (Bi), whereas no elongation was visible in the presence of NMDG (Bii). (C) Quantification of the change in filopodial length shows that Na replacement inhibited the NO-mediated increase in filopodial length. Note that treatment with NMDG alone had no significant effect on filopodia.
Figure 2.6 Nitric oxide increases firing frequency and growth cone calcium in B19 neurons.

(Ai) Bath application of NO-donor, NOC7, (treatment indicated by black bar) increased the firing frequency of B19 neurons. Simultaneous recordings of electrical activity and growth cone [Ca\(^{2+}\)]\(_i\) showed that firing frequency (Aii) and growth cone [Ca\(^{2+}\)]\(_i\) (Aiii) shared a tight temporal relationship. (B) Further analysis of this relationship revealed a strong linear correlation between firing frequency and growth [Ca\(^{2+}\)]\(_i\) (R\(^2\) = .80). (C) Summary of experiments showing that growth cone [Ca\(^{2+}\)]\(_i\) reached at defined firing frequencies in response to NO (grey) were not significantly different from those obtained after evoked current injection (black).
Figure 2.7 Nitric oxide causes filopodial elongation in B19 neurons after broadening of their APs.

(A) A strong linear correlation between firing frequency and growth cone $[Ca^{2+}]_{i}$ was observed in TEA and 4AP treated neurons following NO bath application. Note the increase in slope compared to NO treatment alone (dotted line). (B) Broadening the AP of B19 neurons significantly increased the growth cone $[Ca^{2+}]_{i}$ at defined firing frequencies ($p < 0.001$). (C) Phase contrast images taken before and 10 minutes after treatment with NOC7 show that B19 growth cones experience little change in filopodial length (Ci) whereas filopodial length in neurons pretreated with TEA and 4AP, followed by NO bath application increased (Cii). (D) Quantification of changes in filopodial length from experiments such as shown in (C). Compared to the control
groups, a significant increase in filopodial length is seen in the TEA and 4AP treatment group following NO treatment ($p < 0.001$).
Table 2.1 Neuron-type specific action potential characteristics.

<table>
<thead>
<tr>
<th>Cell Type/Treatment</th>
<th>Peak Amplitude (mV)</th>
<th>Half-width (ms)</th>
<th>Total Area (mV * ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B5 control, n = 12</td>
<td>60.9 ± 0.9 a</td>
<td>17.3 ± 1.0 a</td>
<td>1322.0 ± 71.3 a</td>
</tr>
<tr>
<td>B19 control, n = 9</td>
<td>75.0 ± 1.2</td>
<td>2.0 ± 0.2 b</td>
<td>250.9 ± 16.3 b</td>
</tr>
<tr>
<td>B19 TEA + 4AP, n = 4</td>
<td>74.2 ± 1.3</td>
<td>4.3 ± 0.3</td>
<td>440.8 ± 39.0</td>
</tr>
</tbody>
</table>

Statistical comparisons between Cell Type/Treatment groups were conducted for each characteristic using MWU. Significant differences for each group are indicated as follows: a for control B5 vs control B19 and b for B19 control vs B19 TEA + 4AP.
3 REGULATION OF ELECTRICAL ACTIVITY AND NEURONAL EXCITABILITY IN HELISOMA TRIVOLVIS BY CARBON MONOXIDE

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3.2 Abstract

Carbon monoxide (CO), like other gaseous neuromodulators, has a dual nature as both a toxic gas and a physiologically relevant signaling molecule. In the nervous system, high concentrations of CO can lead to neuronal injury while lower concentrations are found to be neuroprotective. The number of cellular targets affected by physiological concentrations of CO are rapidly growing and include ion channels in various cell types. The modulation of ion channels by CO in neurons, however, and the effect that this may have on neural activity are incompletely understood. Here, the well-characterized buccal neurons, B5 and B19, of the freshwater snail, Helisoma trivolvis, were used to investigate the role that CO plays in regulating spontaneous firing activity and neuronal excitability. Neurons were studied in single cell culture, thereby removing other signals normally present in the intact nervous system and allowing for the optimal characterization of the physiological effects of CO. We found that the CO donor molecule, carbon monoxide releasing molecule-2 (CORM-2), hyperpolarized the resting membrane potential of B5 neurons and silenced their spontaneous firing activity. These effects were mediated through the inhibition of a persistent sodium current. CORM-2 also inhibited neuronal excitability, defined as the ability of B5 neurons to produce action potentials upon stimulation. This effect was mediated by the inhibition of voltage gated calcium channels by CO. The general findings of CO acting as a hyperpolarizing signal and
an inhibitor of neuronal excitability extended to B19 neurons. Taken together, these findings suggest that CO is a potent modulator of ion channels with broad implications for the modulation of neural activity in a wide range of neuron-types.

**Keywords:** CO, Neuron, Voltage-gated calcium channel, Persistent sodium current, CORM-2

### 3.3 Introduction

Carbon monoxide (CO) is typically perceived as a toxic gas; however, the identification of its endogenous production in numerous species (Sjostrand, 1949, Maines, 1997, Wu and Wang, 2005) has led to its description as an important physiological signaling molecule. Within the nervous system, CO has been shown to affect circadian rhythms (Artinian et al., 2001), learning and memory (Cutajar and Edwards, 2007), nociception (Steiner et al., 2001, Carvalho et al., 2011), olfaction (Zufall and Leinders-Zufall, 1997), and neuronal migration (Knipp and Bicker, 2009). In addition, CO has been found to function as a neuroprotectant through anti-inflammatory and anti-apoptotic mechanisms (Vieira et al., 2008, Biermann et al., 2010, Queiroga et al., 2012, Schallner et al., 2012). In recent years, CO has gained increasing importance as a regulator of ion channel activity in a variety of cell-types (Wilkinson and Kemp, 2011b, Peers et al., 2014).

Spontaneously firing neurons utilize a combination of ion channels to bring the resting membrane potential (RMP) to threshold and initiate intrinsically produced action potentials (APs) in the absence of presynaptic input. This spontaneous firing activity is important for the pacemaking activity of the heart (DiFrancesco, 2010), as well as rhythmic, burst and sporadic firing activity in the brain (Sadaghiani and Kleinschmidt, 2013). It also plays an important role in
the establishment of neuronal connections during development (Yamamoto and Lopez-Bendito, 2012). The ionic mechanisms underlying spontaneous firing activity involve three main conductances: a hyperpolarization-activated cationic current, a persistent sodium current (I_{NaP}), and a low voltage-gated calcium channel (VGCC) current. Inhibition of these mechanisms has been reported to lead to hyperpolarization of the RMP and silencing of spontaneous firing activity (Ghamari-Langroudi and Bourque, 2000, Chevalier et al., 2006, Chu and Zhen, 2010, Xie et al., 2011, Artinian et al., 2012). Conversely, activation of these currents can increase spontaneous firing activity (Nakashima et al., 2013).

Another gaseous signaling molecule, nitric oxide (NO), has been shown to regulate neuronal firing activity through the modulation of three ionic conductances: I_{NaP}, VGCC current and small-conductance calcium-activated potassium (SK) channel current (Artinian et al., 2010, Artinian et al., 2012). Given that NO and CO share similar cellular targets, we wanted to determine whether CO affected spontaneous firing activity, and if so, by which mechanism(s). To do this, experiments were performed using well characterized B5 neurons from the buccal ganglia of the freshwater snail, Helisoma trivolvis, which were grown in single cell culture. Single cell culture provides conditions in which the direct effects of CO can be studied while excluding potential confounds of signaling molecules produced by other cells. Using whole-cell patch clamp techniques, we identified CO as a potent modulator of both spontaneous firing activity and neuronal excitability. Examination of the ion channels responsible for these effects revealed that CO hyperpolarized the membrane potential through the inhibition of I_{NaP} and that CO decreased neuronal excitability through the inhibition of VGCC. The effects of CO on multiple ionic conductances suggest that CO can serve as a potent modulator of electrical activity. Using another well characterized neuron from the buccal ganglia, the B19 neuron, we found that the general effects
of CO on RMP and excitability extended to this neuron-type as well. Collectively, this study provides further insight into the role of CO as a modulator of ion channels and a regulator of firing activity and implicate CO as a potentially important effector of neuronal function and developmental events that are dependent on neuronal electrical activity.

3.4 Methods

3.4.1 Neuronal culture

Identified neurons, B5 and B19, were dissected from the buccal ganglia of the freshwater snail, *Helisoma trivolvis*, and plated onto poly-L-lysine (hydrobromide; molecular weight of 70,000–150,000; 0.25 mg/ml; Sigma) coated glass coverslips glued to the bottom of 35 mm cell culture dishes (Falcon 1008), as previously described (Estes et al., 2014). In brief, neurons were cultured for 24-48 hours in 2 mL of conditioned medium at room temperature. Neurons displaying neurites that were at least 2 cell body lengths long (approximately 180 µm) from the soma were used for experiments. Conditioned medium was generated by incubating 2 *H. trivolvis* cerebral ganglia per 1 mL of Leibowitz L-15 medium (Invitrogen, Carlsbad, CA) for 4 days (Wong et al., 1981). L-15 consisted of the following (mM): 44.6 NaCl, 1.7 KCl, 1.5 MgCl₂, 0.3 MgSO₄, 0.14 KH₂PO₄, 0.4 Na₂HPO₄, 1.6 Na pyruvate, 4.1 CaCl₂, 5 HEPES, 50 µg mL⁻¹ gentamicin, and 0.15 mg mL⁻¹ glutamate in distilled water, pH 7.4.

3.4.2 Electrophysiology

Electrical activity of cultured *H. trivolvis* B5 and B19 neurons was recorded in whole-cell current-clamp configuration, as previously described (Artinian et al., 2012, Zhong et al., 2013b, c). Briefly, patch electrodes were pulled from borosilicate glass tubes (OD 1.5 mm; ID 0.86 mm;
Sutter Instruments) using a Sutter Instruments micropipette puller (P-87) and heat polished (Micro Forge MF-830; Narishige) with a resistance of 5-10MΩ. To obtain whole-cell patch, petri dishes containing cultured neurons were placed on a fixed stage that was centered below the objectives of an upright microscope (BX51W1F, Olympus, Japan). Neurons were recorded using an Axopatch 700B amplifier (Molecular Devices, Union City, CA) and analog-to-digital converter (Digidata 1440). Acquisition and analysis of data were conducted using pClamp software version 10 (Molecular Devices). Current-clamp mode was used to evaluate membrane potential, spontaneous and evoked firing properties. Voltage-clamp mode (holding voltage = -60 mV) was used to evaluate the whole-cell configuration parameters as well as to generate voltage-current protocols for measuring $I_{\text{NaP}}$ and calcium currents. Leibowitz L-15 medium was generally used as the extracellular recording solution. When recording calcium channel activity L-15 was replaced with an extracellular calcium solution (Hui and Feng, 2008) consisting of (mM): 10 CaCl₂, 45.7 TEA-Cl, 1 MgCl₂, 10 HEPES, and 2 4-AP, pH adjusted to 7.4 using TEA-OH. The intracellular solution in the recording pipette generally consisted of (mM): 54.4 K-aspartate, 2 MgCl₂, 5 HEPES, 5 Dextrose, 5 ATP, and 0.1 EGTA (127 mOsm). In experiments recording calcium channel activity, the intracellular solution consisted of (mM): 29 CsCl, 2.3 CaCl₂, 2 MgATP, 0.1 GTP-Tris, 11 EGTA, 10 HEPES, pH adjusted to 7.4 by CsOH. In current clamp only, the membrane potential was corrected for liquid junction potential, which was calculated using Clampex software (v10; Molecular Devices) to be approximately -15.7 mV.

3.4.2.1 Measurement of excitability and afterhyperpolarization (AHP)

Excitability was measured as the number of spikes produced after injecting depolarizing current (0.1, 0.5, and 1.0 nA) for 1 s to stimulate the production of evoked AP. The afterhyperpolarization (AHP) was measured in the mentioned above protocol as the region of voltage trace in
which the membrane potential fell below the resting membrane potential. The amplitude of the AHP was measured as an antipeak of the membrane potential occurring 100-200 ms after the end of stimulation. The duration of the AHP was measured as the time it took for the membrane potential to return to baseline resting levels immediately following removal of current injection.

3.4.2.2 Measurement of persistent sodium channel current ($I_{NaP}$)

Characterization of the $I_{NaP}$ was conducted as previously described (Harvey et al., 2006, Artinian et al., 2012). In brief, $I_{NaP}$ was measured in voltage-clamp mode by applying a slow voltage ramp from -80 to – 30 mV with an increment of 5 mV/s. Using the linear current from the passive leak conductance, measured between -80 and -70 mV, [dashed line, Fig. 2 (A)], we extrapolated the fit to obtain the peak current amplitude [vertical dotted line, Fig. 2 (A)]. Currents were analyzed by normalizing the peak inward current for each cell to the cell capacitance (pA/pF).

3.4.2.3 Measurement of voltage-gated calcium currents

To study total calcium currents, membrane potential was held at -60 mV and stepped from -60 mV to + 60 mV for 500 ms with increments of +10 mV, as previously shown (Artinian et al., 2012, Zhong et al., 2013a). To characterize high VGCC currents, the membrane potential was first stepped to -20 mV for 200 ms to fully inactivate the low VGCC currents, followed by stepping membrane potential from -60 to +60 mV for 500 ms. Low VGCC currents were calculated by subtraction of high VGCC currents from total calcium currents (Haydon and Man-Son-Hing, 1988). Currents were analyzed by normalizing the peak inward current for each cell to the cell capacitance (pA/pF).
3.4.3 Pharmacological agents

The carbon monoxide donor, carbon monoxide releasing molecule-2 (CORM-2; Tricarbonyldichlororuthenium(II) dimer; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to make a stock of 50 mM. CORM-2 was mixed with L-15 10 minutes following the generation of stock solution allowing for saturation of CO at final concentrations of 5, 50, and 100 µM. Riluzole (Sigma-Aldrich) was dissolved in DMSO at a stock concentration of 40mM and diluted in L-15 to a final concentration of 20µM. All drugs were mixed with L-15 and bath applied to obtain final concentrations.

3.4.4 Statistics

All values described were expressed as mean ± SEM, unless otherwise stated. Data plots were designed using ORIGIN DATA ANALYSIS AND GRAPHING software (version 9.1, OriginLab, Northampton, MA). The significance of effects between control and treatment was evaluated using the Mann-Whitney U test and the Kruskal-Wallis test for comparison of effects over time (SPSS statistical software, SPSS, Chicago, IL). Comparison of the effects before and after treatment were evaluated with the Wilcoxon-Signed-Ranks Test. Significant differences are indicated as *p < 0.05, **p < 0.01, and ***p < 0.001.

3.5 Results

3.5.1 Carbon monoxide hyperpolarizes $V_m$ and silences spontaneous firing activity

We showed previously that the gaseous signaling molecule, nitric oxide (NO), is capable of increasing the spontaneous firing activity and excitability of developing B5 neurons (Artinian et al., 2010). Given that CO, like NO, is produced in the nervous system (Wu and Wang, 2005)
and is present during development (Raju et al., 1997, Bergeron et al., 1998, Li Volti et al., 2004), we wanted to test whether CO affected neuronal firing activity. To characterize the effects of CO on electrical activity, we used the CO-donor, carbon monoxide releasing molecule-2 (CORM-2), which has been used and shown to have no adverse effects at concentrations ranging from 20 to 210 µM (Motterlini et al., 2002, Knipp and Bicker, 2009). CORM-2 was bath applied to isolated B5 neurons while their electrical activity was recorded using a whole-cell current clamp configuration. Prior to CO treatment, B5 neurons had resting membrane potentials (RMP) of -45.1 ± 0.8 mV (n = 25) and were spontaneously active producing a range of firing frequencies from 0.4 to 1.6 Hz, similar to our previous findings (Artinian et al., 2010, Zhong et al., 2013a). Following treatment with CORM-2 (5, 50, or 100 µM), all neurons showed hyperpolarization of the RMP and a decrease in firing frequency [Fig. 3.1 (A)]. The maximum hyperpolarization of the RMP induced by 5 µM CORM-2 was -9.2 ± 0.6 mV (n = 5) [Fig. 3.1 (B)] taking an average of 35 ± 7 min (n = 5) to reach its maximal effect [Fig. 3.1 (C)]. The hyperpolarization of the RMP by 5 µM CORM-2 was statistically significant when compared with the solvent control DMSO (-1.9 ± 2.5, n = 4, MWU, U = 0, p = 0.014). In addition to the CO-induced hyperpolarization of the RMP, the spontaneous firing activity in 2 out of the 5 cells was silenced. Increasing the CORM-2 concentration to 50µM [Fig. 3.1(Aii)] and 100µM [Fig. 3.1(Aiii)] also led to a significant hyperpolarization of the RMP compared to the solvent control (50 µM: -9.1 ± 1.4 mV, n = 10, MWU, U = 1, p = 0.007; 100 µM: -11.0 ± 3.4 mV, n = 5, MWU, U = 2, p = 0.05) [Fig. 1 (B)] but did not change the RMP beyond what was observed with 5µM CORM-2. Moreover, the average time to reach maximum hyperpolarization of the RMP was decreased at the higher concentrations of CORM-2 (50 µM: 9 ± 2 min, n = 10; 100 µM: 8 ± 2 min, n = 5) [Fig. 3.1 (C)]. We also observed that higher CORM-2 concentrations completely silenced spontaneous firing activity of
all cells within 4 ± 1 min (n = 10) and 4 ± 1 min (n = 5) at 50 and 100 µM, respectively. Given that 50 µM CORM-2 produced a significant hyperpolarization of the RMP within a few minutes and resulted in a consistent silencing of spontaneous activity, we utilized this concentration from here on to further characterize the effects of CO on electrical activity. We next wanted to identify which ionic conductance was modulated by CO leading to the hyperpolarization of the RMP.

3.5.2 CO inhibits a persistent sodium current to hyperpolarize the membrane potential

In previous studies, we found that B5 neurons contain a persistent sodium current (I_{NaP}) that is active at hyperpolarized membrane potentials and contributes to the RMP as well as to spontaneous firing activity (Artinian et al., 2012); therefore, we tested first whether the CO-induced hyperpolarization of the RMP involved modulation of I_{NaP}. To determine the effects of CO on the I_{NaP}, we used a slow-voltage ramp protocol from -80 to -30 mV, which inactivates fast-inactivating voltage-gated ion channels allowing for the characterization of the I_{NaP} (Harvey et al., 2006). We observed an inward current that developed around -60 mV and peaked at -30 mV [Fig. 3.2 (A)], which was similar to our previous findings (Artinian et al., 2012). Bath application of CORM-2 caused significant inhibition of I_{NaP} within 5 minutes by -35.0 ± 7.0 % of baseline (Wilcoxon Signed Ranks test, p = 0.028, n = 6) and that inhibition was sustained over time [Fig. 3.2 (B)], mirroring the time course described for the effect of CO on RMP above (Fig. 3.1). If the CO-induced hyperpolarization of the RMP was due to the inhibition of I_{NaP}, pretreatment of B5 neurons with the I_{NaP} inhibitor, riluzole (20 µM), prior to CO treatment should prevent the effect of CO. Riluzole treatment alone significantly hyperpolarized the membrane potential from baseline by an average of 25.7 ± 4.8 % from -44.2 ± 0.6 mV to -55.7 ± 2.6 mV (Wilcoxon
Signed Ranks Test, p = 0.012, n = 8) and silenced spontaneous firing activity. The combination of riluzole treatment with the solvent control, DMSO, or CORM-2 did not hyperpolarize the membrane potential further compared to riluzole treatment alone (MWU, DMSO: U = 10, p = 0.683, n = 3; CORM-2: U = 27.5, p = 0.636, n = 8) [Fig. 3.2(C and D)], suggesting that CO primarily affects the RMP through the inhibition of the $I_{\text{NaP}}$.

### 3.5.3 CO inhibits neuronal excitability

To test whether CO merely caused a hyperpolarization of RMP or also affected the ability of B5 neurons to generate action potentials (APs), we next injected depolarizing current into the neuron to evaluate evoked APs for potential effects of CO on neuronal excitability. Depolarization of the membrane potential through the injection of current resulted in evoked spiking that increased with the amount of injected current (0.1, 0.5, or 1.0 nA). CORM-2 (50µM) had no significant effect on spiking 5 minutes after addition [Fig. 3.3(A)], which was the time point when the RMP had been maximally hyperpolarized by CO. Instead, significant changes in evoked APs were not observed until 10 to 15 minutes post CO treatment when compared with solvent control DMSO (Kruskal-Wallis, p ≤ 0.05) [Fig. 3.3 (B)], indicating that the underlying mechanism may have a slower time course than observed above for the hyperpolarization. In summary, CORM-2 (50µM) resulted in a significant decrease in the number of evoked APs over time, from an average of 4.2 ± 0.2 to 2.0 ± 0.6 at 0.1 nA, from 8.4 ± 0.4 to 5.8 ± 1.0 at 0.5 nA, and from 10.4 ± 0.4 to 5.5 ± 1.5 at 1.0 nA.

An investigation of the afterhyperpolarization potential (AHP) before and after treatment with CORM-2 showed a delay in the return of the membrane potential to rest after current injections [Fig. 3.3(C)], indicating an increase in the duration of the AHP. In experiments with a 1 nA
current injection, which produced a robust AHP, CO increased the duration of the AHP, defined as the time taken for the membrane potential to return to resting potential immediately following removal of the current injection, from $2.1 \pm 0.4 \text{ s}$ to a maximum of $3.5 \pm 0.4 \text{ s}$ [Fig. 3.3(D)]. The significant increase in the AHP duration was transient occurring 5 to 10 minutes post CO treatment compared with DMSO (Kruskal-Wallis, 5 min: $p = 0.05$, 10 min: $p = 0.027$, 15 min: $p = 0.142$, 20 min: $p = 0.086$ and 30 min: $p = 0.248$). Changes in the duration of the AHP may be due to either an increase in outward calcium-activated K currents or the inhibition of inward currents that are active at hyperpolarized voltages. We have previously shown that SK channels are major contributors to the AHP amplitude in B5 neurons (Artinian et al., 2010), suggesting that CO may increase the AHP duration through the activation of SK channels. We did not, however, observe any significant changes in AHP amplitude while the AHP duration was increased (Kruskal-Wallis, 5 min: $p = 0.462$ and 10 min $p = 0.142$), arguing against the involvement of SK channels. This finding provided additional evidence that CO likely modulated depolarizing currents necessary for bringing the membrane potential towards AP threshold. Taken together with our findings that the hyperpolarization of the RMP occurred within 5 minutes of CO treatment, the slower time course for the inhibition of evoked APs suggested that CO may be inhibiting inward conductances.

### 3.5.4 CO inhibits neuronal excitability through the inhibition of VGCCs

Previously, we reported that calcium currents are a major determinant of the AP waveform in B5 neurons and that by inhibiting VGCC the RMP depolarizes and neuronal excitability decreases (Artinian et al., 2010). We therefore hypothesized that the decrease in neuronal excita-
bility mediated by CO involved the modulation of VGCC activity. Using voltage clamp, we applied voltage steps from -60 to +60 mV to characterize the total calcium current in B5 neurons [Fig. 3.4(A)]. We observed two inward currents upon stimulation: a low voltage current produced at hyperpolarized voltages with a peak conductance around -20 to -10 mV and a high voltage current produced at relatively more depolarized voltages with a peak conductance around 0 to +10 mV [Fig. 3.4(B)]. To determine the effects of CO on each current, we used a second voltage step protocol that stepped the voltage to -20 mV immediately before voltage steps from -60 to +60 mV, thereby isolating the high VGCC current. We extrapolated the low VGCC current by subtracting the high VGCC from the total current. Over a 30 minute time course, CO reduced both low and high VGCC currents by 76.5 ± 3.8 % and 72.0 ± 16.2 % (n = 4), respectively [Fig. 3.5 (C)]. The inhibition of these currents was significant when compared to solvent controls (Kruskal-Wallis, p = 0.034) [Fig. 3.5 (C)], suggesting that CO is a potent inhibitor of VGCCs. Previously, we showed that the inhibition of VGCCs with LaCl$_3$ and CdCl$_2$ fully blocks evoked APs (Artinian et al., 2010). The progressive inhibition of evoked APs after 10 minutes of CO treatment coupled with the progressive inhibition of VGCCs, suggests that the CO regulates neuronal excitability through the inhibition of VGCCs.

### 3.5.5 CO similarly modulates neuronal firing activity of B19 neurons

With CO decreasing spontaneous firing activity and neuronal excitability of B5 neurons, we wanted to test whether these effects extended to another neuron type. Previously, we have characterized the firing activity of another buccal neuron, B19, showing that these neurons are often silent, not producing spontaneous action potentials, and that NO increases firing activity and neuronal excitability of these neurons (Zhong et al., 2013c). Using 2 day old B19 neurons in
culture, bath application of CORM-2 (50 µM) was found to hyperpolarize the RMP of B19 neurons within 8.9 ± 2.5 minutes from an average of -63.2 ± 5.5 to -69.4 ± 3.0 mV (Wilcoxon Signed Ranks Test, p = 0.043, n = 5) [Fig. 3.5(A and B)]. It should be noted that B19 neurons which were already silent prior to CO addition (4 out of 5) showed a smaller hyperpolarization of the RMP (-68.3 ± 2.6 to -71.6 ± 2.7 mV) and that the one spontaneously firing B19 showed a stronger hyperpolarization of the RMP from -42.7 to -60.7 mV, as well as silencing of spontaneous firing activity. We next wanted to determine whether the effects of CO on the RMP affected the ability of B19s to produce evoked APs. B19 neurons produce robust spiking activity following 1 nA current injection, generating on average 33 ± 2 evoked APs [Fig. 3.5(C)]. 30 minute treatment with CO showed an inhibition of evoked activity by 53.0 ± 19.4 % to 16 ± 7 evoked APs (Wilcoxon Signed Ranks Test, p = 0.043, n = 5) [Fig. 3.5(C and D)]. Taken together, these data suggest that CO may act as a general inhibitory signal that hyperpolarizes the RMP of a neuron and decreases neuronal excitability.

### 3.6 Discussion

Here, we assessed the role of CO in the modulation of neuronal firing activity using the CO-donor, CORM-2, and found that CO affected firing activity in two ways: 1) hyperpolarization of the RMP, which led to the silencing of spontaneous firing activity and 2) a decrease in neuronal excitability [Fig. 3.6]. These effects appeared to be time dependent and were primarily mediated through the inhibition of two types of conductances, $I_{NaP}$ and VGCC currents, respectively. We also found that the general effects of CO on the RMP and excitability of B5 neurons extended to another neuron-type, B19. Collectively, our findings suggest that CO can serve as an important modulator of electrical activity in neurons.
3.6.1 $I_{\text{NaP}}$ regulation of the resting membrane potential

The ability of a neuron to fire spontaneous APs requires depolarizing current(s) to be active at hyperpolarized voltages bringing the membrane potential to threshold and thus inducing an AP without the need for external stimulation. The $I_{\text{NaP}}$ has been shown to be an essential depolarizing current for producing spontaneous and rhythmic firing activity in a number of neurons, including hypoglossal neurons (Lamanauskas and Nistri, 2008), spinal neurons (Harvey et al., 2006, Kuo et al., 2006), suprachiasmatic nucleus neurons (Kononenko et al., 2004)) and inspiratory neurons (Del Negro et al., 2005). Previously we showed that the replacement of extracellular sodium ($\text{Na}^+$) with N-methyl-d-glucamine (NMDG) hyperpolarized the RMP and silenced spontaneous firing activity in B5 neurons (Artinian et al., 2010), suggesting $\text{Na}^+$ regulates the RMP in B5 neurons. In a subsequent study, we inhibited the $I_{\text{NaP}}$ with riluzole, and observed similar effects on the RMP and firing activity as with NMDG treatment (Artinian et al., 2012), suggesting that the persistent sodium channel is responsible for the $\text{Na}^+$-driven regulation of the RMP. Here, we found that CO inhibited $I_{\text{NaP}}$ within 5 minutes of exposure, which was similar to the timing for CO to hyperpolarize the RMP and silence spontaneous firing activity. The inhibition of $I_{\text{NaP}}$ prior to stimulation with CO prevented additional hyperpolarization of the RMP, suggesting that the effects of CO on the RMP and thus the spontaneous firing activity were mediated through $I_{\text{NaP}}$. While we show that CO inhibits $I_{\text{NaP}}$ in neurons, it should be noted that CO shows both inhibition and activation of an epithelial $\text{Na}^+$ current (Althaus et al., 2009, Wang et al., 2009) as well as activation of a sustained sodium current in cardio myocytes (Dallas et al., 2012), suggesting that the actions of CO on $\text{Na}^+$ currents is cell-specific. It is likely that the underlying mechanisms that control the gating activity of these channels differ, and while the aim of this study was not to determine the exact mechanism for how CO regulates $I_{\text{NaP}}$, the variable effects
of CO on Na\textsuperscript{+} currents stresses the importance of understanding the molecular identity of these channels and their gating kinetics in future studies.

3.6.2 VGCC regulation of neuronal excitability

In addition to the silencing of spontaneous firing activity, CO was found to decrease neuronal excitability. In the simplest of cases, both effects of CO would have been generated through the inhibition of I\textsubscript{NaP}; however, the timing for these two effects differed. In our previous experiments characterizing I\textsubscript{NaP} using riluzole, there was no discernable effect of I\textsubscript{NaP} inhibition on evoked firing activity (Artinian et al., 2012). In addition, the replacement of extracellular Na\textsuperscript{+} with NMDG showed little impact on the evoked activity of B5 neurons as well (Artinian et al., 2010). Instead, the inhibition of VGCCs with LaCl\textsubscript{3} and CdCl\textsubscript{2} completely blocked evoked APs (Artinian et al., 2010). From this we concluded that the primary conductance for the generation of APs in B5 neurons was through VGCCs. Not surprisingly then, CO was found to inhibit VGCCs by 50\% when excitability began to decrease, suggesting that CO inhibited neuronal excitability through the inhibition of VGCCs. In contrast, B19 neurons are more dependent on Na\textsuperscript{+} for AP generation (Zhong et al., 2015) and showed smaller decreases in evoked APs following CO exposure [Fig. 3.5 (C & D)]. While both neuron-types showed an overall inhibition of evoked AP by CO, individual differences in ion channel expression may account for cell-specific modification by CO. We are currently working to uncover the exact molecular identity of ion channels in B5 and B19 neurons to determine how these cell-specific differences may arise.
3.6.3 Implications of CO in CNS function and development

The endogenous production of CO is primarily mediated by the enzyme heme oxygenase (HO), which is thought to be ubiquitously expressed across phyla and tissue types (Wilks, 2002, Li and Stocker, 2009); however, the amount and location of CO varies based on whether the inducible (HO-1) or constitutive form (HO-2) of the enzyme is present (Maines, 1997). The increased expression of HO-1 following cellular stress/injury has led to the description of CO as an important cytoprotectant (Bilban et al., 2008). Injury to the CNS can result in the hyperexcitability of neurons (Reeves et al., 1995, Kobayashi and Mori, 1998, Gwak and Hulsebosch, 2011), which can lead to aberrant firing activity and neuronal cell death. CO treatment before and up to 3 hours after ischemic injury has been shown to be neuroprotective by modulating apoptotic and inflammatory signaling pathways (Queiroga et al., 2012, Schallner et al., 2012). Additional reports show that CO can function as a neuroprotectant for a range of CNS pathologies including stroke, pain, and neurodegenerative diseases (Queiroga et al., 2014). With a number of ion channels playing a role in the excitability of a neuron (Armstrong and Hille, 1998), the inhibition of ion channel activity by CO may be another way in which CO can provide neuroprotection. Moreover, the predominance of HO-2 throughout the CNS (Maines, 1997) coupled with growing evidence for CO as an ion channel regulator suggests that CO can provide tonic modulation of neural activity in normal physiological conditions. The exact role that CO plays in modulating neural activity will likely be varied and depend on the synaptic connectivity of the neuron that CO is modulating. For example, while the localized silencing of an excitatory neuron by CO can lead to a decrease in neuronal activity in the downstream synaptic partners of that neuron, the silencing of inhibitory neurons will have the opposite effect and could result in increased spiking.
Therefore, the physiological production of CO in the brain is likely well controlled to prevent adverse effects on neuronal electrical properties effecting overall brain activity.

The presence of HO-1 and HO-2 during development (Maines, 1997) suggests that in addition to modulating the activity of a neural circuit, CO can modulate the electrical activity of developing neurons and affect how a neural circuit is formed. Spontaneous and patterned electrical activity generated during neuronal development are vital for the guidance of an axon to its target as well as refining established neuronal connections (Neely and Nicholls, 1995, Yamamoto and Lopez-Bendito, 2012). Disruption of this activity can result in the misguidance of neuronal projections and the formation of inappropriate synapses (Catalano and Shatz, 1998, Hanson and Landmesser, 2004). Electrical activity varies over the course of development for any given neuron and is likely important for establishing distinct neural networks. The growing list of ion channels as targets of CO modulation coupled with the presence of CO during development (Bergeron et al., 1998, Li Volti et al., 2004), suggests that it may play an important role in the establishment of synaptic connections. For early stages of development, particularly the guidance of axons to their synaptic targets, we have shown that the electrical activity in B5 neurons regulates the calcium dynamics of growth cones, the guidance structure of developing axons (Estes et al., 2014). The concentration of calcium within a growth cone is critical for determining an array of guidance behaviors important for the navigation of an axon (Zheng and Poo, 2007). With CO inhibiting firing activity and decreasing excitability, particularly through the inhibition of VGCCs, it is likely that CO regulates growth cone calcium and motility. With evidence that other gaseous neuromodulators, such as NO, affect growth cone behaviors through the regulation
of calcium (Trimm and Rehder, 2004, Welshhans and Rehder, 2005), future studies of CO on development are likely to reveal a modulatory role of CO on growth cone calcium dynamics and motility.
3.7 Figures

Figure 3.1 CO donor, CORM-2, silences spontaneous firing activity of B5 neurons.

(A) Representative examples of CORM-2 at (i) 5, (ii) 50, and (iii) 100 µM show hyperpolarization of the resting membrane potential (RMP) from baseline values (indicated by the black
dashed line) and silencing of spontaneous firing. Note the differences in time scale for each concentration. (B) Quantification of the maximum change in RMP across experiments. Increasing CORM-2 concentrations above 5 µM did not further increase the overall maximum hyperpolarization of the RMP, but (C) it did shorten the time taken to reach maximum hyperpolarization.
Figure 3.2 CO inhibition of $I_{\text{NaP}}$ results in the hyperpolarization of the RMP and silencing of spontaneous firing activity.

(A) Recording of the $I_{\text{NaP}}$ in B5 neurons using a slow-voltage ramp protocol showed a maximal inward current occurring around -30 mV. CO treatment almost completely inhibited the $I_{\text{NaP}}$ current. (B) A time course of the normalized $I_{\text{NaP}}$ showed that CO inhibited $I_{\text{NaP}}$ within 5 minutes when compared with baseline $I_{\text{NaP}}$. (C) The treatment with the inhibitor of $I_{\text{NaP}}$, riluzole, silenced spontaneous firing activity and hyperpolarized the RMP (indicated by the black arrowhead). The
addition of CO only slightly hyperpolarized the RMP further (indicated by the grey arrowhead).

(D) Quantification of the changes in membrane potential showed that riluzole treatment alone hyperpolarized the RMP and that the addition of either the solvent control (DMSO) or CORM-2 did not hyperpolarize the RMP further.
Figure 3.3 CO decreases neuronal excitability.

The injection of depolarizing currents (0.1, 0.5, and 1.0 nA) for 1 second evoked APs in B5 neurons. (A) Representative example from the 1 nA current injection shows that while there was no significant change in evoked firing activity within 5 minutes of CO exposure, evoked activity was inhibited over time. (B) The time course of the change in evoked APs following CO exposure showed that across experiments evoked activity decreased within 10 minutes for 0.5 and 1.0
nA current injections (Kruskal-Wallis, p ≤ .028). (C) Analysis of the afterhyperpolarization (AHP) following 1 nA current injection showed a delay in the late phase of the AHP in which the membrane potential returns to RMP levels. This led to an increase in the duration of the AHP, which is defined as the time from end of stimulation to the return to RMP (indicated by black arrow for control). (D) Analysis of the change in AHP duration in response to a 1 nA current injection showed a significant increase 5 to 10 minutes following CO exposure.
Figure 3.4 CO inhibits voltage gated calcium channels.

(A) A representative example of calcium currents in response to voltage steps from -60 to +60 mV show the partial inhibition of total calcium currents by CO (CORM-2, 50 µM) (B) Measure-
ments of the time course of CO effects on the total calcium current identified two distinct currents. A low VGCC current opening at around -20 mV and a high VGCC current with maximal activation at +10 mV. CO exposure inhibited both calcium currents over time. (C) Quantification of the change in both low and high VGCC currents showed that each current became progressively inhibited over time. High and low voltage components of the total calcium currents were determined as described in the Methods section.
Figure 3.5 CO hyperpolarizes B19 RMP and decreases neuronal excitability.

To test whether the effects of CO extended to other neuron types, we examined the effect of CO on B19 neurons. (A) A representative example of B19 neuron electrical activity shows that the neuron is silent prior to treatment and that the RMP hyperpolarized slightly following CO exposure. Maximum hyperpolarization was reached by approximately 10 minutes. The break in the recording marks the transition between two recording files lasting 5 s. (B) Individual changes in RMP following CO exposure showed that RMP decreased in all neurons recorded. Note that out of the 5 neurons, 1 was spontaneously firing and showed the largest hyperpolarization of the RMP. The average change in RMP from baseline was found to be significant following CO exposure (black line). (C) A representative example showing that the number of APs evoked by the injection of 1 nA current before (Ci) and 30 minutes following CO treatment (Cii). (D) Quantification of the change in the number of evoked APs shows variable but overall significant inhibition of evoked activity following CO treatment.
Figure 3.6 Summary for CO modulation of electrical activity

Proposed model for the modulation of electrical activity by CO in *Helisoma trivolvis* B5 neurons. (Left) Under control conditions, B5 neurons produce spontaneous action potentials utilizing the persistent sodium current ($I_{\text{NaP}}$) to depolarize the resting membrane potential to threshold. As the membrane potential approaches threshold calcium currents ($I_{\text{Ca}}$) from the activation of voltage-gated calcium channels (VGCCs) provide the primary driving force for the generation of an action potential. Depolarizing stimuli produce sustained evoked firing activity. (Right) Upon CO exposure, $I_{\text{NaP}}$ is inhibited leading to hyperpolarization of the resting membrane potential and silencing of spontaneous firing activity. Over time, the inhibition of VGCCs by CO leads to a decrease in neuronal excitability.
THE MODULATION OF GROWTH CONE CALCIUM AND FILOPODIA BY CARBON MONOXIDE

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4.2 Abstract

Carbon monoxide (CO) has been identified as an important physiological signaling molecule; however, the role it plays in neuronal development and regeneration is poorly understood. During these events, axons extend and navigate through a rich cellular environment to locate target cells and establish synaptic connections. Molecular signals encountered en route are integrated by growth cones, which are located at the tip of neurites, to affect neurite guidance. Previously, we have shown that another gaseous signaling molecule, nitric oxide (NO), has potent effects on growth cone motility. With NO and CO sharing similar cellular targets, we wanted to determine whether CO affected growth cone motility as well. As an initial step in understanding the role of CO in growth cone motility, we assessed how CO modulated growth cone filopodial length and the pathway by which these effects were mediated. Using two well-characterized neurons from the freshwater snail, Helisoma trivolvis, we found that the CO donor, carbon monoxide releasing molecule-2 (CORM-2), increased filopodial length. CO utilized a signaling pathway that involved the activation of soluble guanylyl cyclase, protein kinase G, and ryanodine receptor mediated calcium release from intracellular stores. While increases in filopodial length often occur from robust increases in intracellular calcium levels, the timing in which CO in-
creased filopodial length corresponded with low basal calcium levels in growth cones. Taken together, these findings provide evidence for CO as a modulator of growth cone motility and implicate CO as a neuromodulatory signal during neuronal development and/or regeneration.

4.3 Introduction

The identification of carbon monoxide (CO) as an endogenously produced gas has transformed the notion that CO is just a toxic gas and has led to the description of CO as an important physiological signaling molecule (Kim et al., 2006). Its endogenous production primarily occurs through the degradation of heme by the enzyme heme oxygenase (HO), which has been identified to have two main isoforms: (1) an inducible form (HO-1) found throughout the body that is activated by cellular stress and injury and (2) a constitutively active form (HO-2) found primarily in the nervous system and testes. While studies of CO within the mature nervous system have led to its description as an effector of circadian rhythms (Artinian et al., 2001), nociception (Steiner et al., 2001, Carvalho et al., 2011), learning and memory (Cutajar and Edwards, 2007), and olfaction (Zufall and Leinders-Zufall, 1997), the presence of both HO isoforms throughout development in both vertebrates and invertebrates (Maines, 1997, Shi et al., 2008, Knipp and Bicker, 2009, Christie et al., 2014) suggests that CO may also play an important role as a developmental signaling molecule; however, unlike another endogenously produced gas, nitric oxide (NO), little is known about the role CO plays in neuronal development and regeneration.

In the developing and regenerating nervous system, neurons extend axons and dendrites, which are tipped by growth cones that integrate environmental signals into appropriate guidance behaviors (Gomez and Letourneau, 2014). Growth cone filopodia protrude from the leading edge of the growth cone and sample the surrounding environment for guidance cues by elongating and
shortening continuously. Given their extension ahead of the growth cone proper, filopodia can act as the initial contact for guidance signals affecting how the growth cone responds to guidance cues (Davenport et al., 1993, Zheng et al., 1996, Mattila and Lappalainen, 2008) and where the growth cone advances (Hammarback and Letourneau, 1986). In addition, filopodia make the first contact with target cells and can determine whether the developing neurite forms a synaptic connection with that cell (Shen and Cowan, 2010). Without filopodia, growth cones show abnormal steering (Bentley and Toroian-Raymond, 1986, Zheng et al., 1996) and deficits in the rate of neurite outgrowth and terminal axonal branching (Dwivedy et al., 2007), indicating that the regulation of filopodial dynamics are important for neurite guidance. While a direct role of CO in growth cone morphology has not been assessed, it has been shown that CO affects neuronal migration in enteric neurons of the locust (Knipp and Bicker, 2009) and increases the rate of neurite outgrowth in human NT2 neurons (Scheiblich and Bicker, 2014), suggesting that CO is an effector of neuronal development and regeneration.

In this study, we employed a well-established developmental model system: the freshwater snail *Helisoma trivolvis*, to study the effects of CO on filopodia length as a first step in understanding its role as a signaling molecule in growth cone dynamics. We have previously identified that neurons in the buccal ganglia of *Helisoma* contain a heme oxygenase-like protein (unpublished data), suggesting for the production of CO within this nervous system; however, the role it plays in the snail is relatively unknown. A single study shows that CO affects the oscillations of olfactory neurons in the slug, *Limax maximus* (Gelperin et al., 2000), indicating that CO may serve as a signaling molecule in mollusks. Given that developing *Helisoma* neurons endogenously produce another gaseous signal NO (Van Wagenen and Rehder, 1999) and that NO has been shown to modulate neurite outgrowth (Trimm and Rehder, 2004) and filopodial length via
soluble guanylyl cyclase (sGC) (Van Wagenen and Rehder, 1999, Tornieri and Rehder, 2007), a common cellular target of CO, it is possible that CO functions as a physiological modulator of growth cone dynamics. Therefore, we isolated Helisoma neurons B5 and B19 from the buccal ganglia and plated them in single cell culture, where they can be individually exposed to CO, thus allowing for the characterization of CO while eliminating electrical or chemical signals produced by other cells in the intact nervous system. Using phase contrast microscopy, we found that exposure with the CO donor, carbon monoxide releasing molecule-2 (CORM-2) caused a rapid and sustained increase in filopodial length in two neuron-types, B5 and B19. We further determined that the CO-mediated increase in filopodial length occurred through the activation of a soluble guanylyl cyclase (sGC), protein kinase G (PKG), and a ryanodine receptor (RyR) mediated pathway. Analysis of growth cone calcium levels showed that neuronal exposure to CO resulted in a rapid decrease in growth cone calcium in spontaneously firing neurons and that the initial increase in filopodial length occurred when intracellular calcium concentrations were at basal levels. Collectively, these findings suggest CO can act as a regulator of filopodial dynamics and implicate it as a modulator of growth cone motility and effector of neurite pathfinding.

4.4 Methods

4.4.1 Neuronal culture

Primary cell cultures were established by dissecting buccal neurons, B5 and B19, from the adult fresh water snail, Helisoma trivolvis, as described previously (Estes et al., 2014). In short, neurons were plated in 35 mm cell culture dishes (Falcon 1008) with poly-L-lysine (hydrobromide; molecular weight of 70,000–150,000; 0.25 mg/ml; Sigma) coated glass coverslips glued to the bottom of the dish. Cells were cultured in 2 mL of conditioned medium until long
neurites tipped with growth cones extended at least 2 cell body diameter lengths from the soma (24-48 hours in culture). Conditioned medium was prepared by incubating 2 Helisoma trivolvis cerebral ganglia per 1 mL of Leibowitz L-15 medium (Invitrogen, Carlsbad, CA) for 4 days (Wong et al., 1981). L-15 consisted of the following (mM): 44.6 NaCl, 1.7 KCl, 1.5 MgCl₂, 0.3 MgSO₄, 0.14 KH₂PO₄, 0.4 Na₂HPO₄, 1.6 Na pyruvate, 4.1 CaCl₂, 5 HEPES, 50 μg mL⁻¹ gentamicin, and 0.15 mg mL⁻¹ glutamate in distilled water, pH 7.4.

4.4.2 Electrophysiology

Whole-cell current-clamp recordings were performed on cultured H. trivolvis B5 and B19 neurons as previously described (Zhong et al., 2013c, Estes et al., 2014), while simultaneously imaging calcium concentrations within the growth cone. In summary, patch electrodes were pulled from borosilicate glass tubes (OD 1.5 mm; ID 0.86 mm; Sutter Instruments) using a Sutter Instruments micropipette puller (P-87). The electrodes were then heat polished (Micro Forge MF-830; Narishige) and used with a resistance ranging from 5-10MΩ. Cultured neurons were placed below the objectives of an upright microscope (BX51W1F, Olympus, Japan) on a fixed stage. The microscope was placed on top of a translation stage (Siskiyou, San Francisco, CA), allowing for the free movement of the microscope around the stationary platform and culture dish without interfering with the whole-cell patch clamp. Electrical recordings were conducted using an Axopatch 700B amplifier (Molecular Devices, Union City, CA) and an analog-to-digital converter (Digidata 1440, Molecular Devices). An Imaging Control Unit (Till Photonics, FEI) was used to trigger calcium imaging in conjunction with electrical recordings. Acquisition and analysis of recordings were performed using Axon Instrument pClamp software (v10; Molecular Devices). The intracellular recording solution contained the following (mM): 54.4 K-aspartate, 2
MgCl₂, 5 HEPES, 5 Dextrose, 5 ATP, and 0.1 EGTA (127 mOsm). The extracellular solution used for recording consisted of Leibowitz L-15 medium (Gibco, Grand Island, NY, USA), as described above. Recordings were conducted in current-clamp configuration and signals were filtered at 5kHz (-3 dB, four-pole Bessel filters). The liquid junction potential was calculated using Clampex software (v10; Molecular Devices) and found to be approximately -15.7 mV; all membrane potentials were adjusted accordingly.

4.4.3 Calcium imaging

Growth cone calcium levels and electrical activity were simultaneously measured as (Estes et al., 2014). Briefly, the cell-impermeable calcium indicator dye, Fura-2 pentapotassium salt (10 mM in H₂O; Molecular Probes, Eugene, OR) was injected into B5 and B19 neurons using a Picospritzer (General Valve Corporation, USA). Cells were loaded with Fura-2 to yield sufficient fluorescence while avoiding saturation, which has been observed to affect the spontaneous firing activity of a H. trivolvis neurons (Estes et al., 2014). Using a cooled CCD camera (Andor Technology, Model # DR328GC01SIL9JCI), images of growth cones were captured simultaneously with recordings of electrical activity, described above. Images were taken with a 100 ms exposure time at 20% light intensity every minute. Acquisition and analysis were performed using Live Acquisition and Offline Analysis software (Till Photonics, FEI, Germany). 340/380nm emission ratios were taken from the center of each growth cone and background corrected.

4.4.4 Growth cone filopodia dynamics

Phase contrast images of growth cones were acquired using a 100X oil-immersion objective on an inverted microscope (Olympus IX70, Japan) with a cooled CCD camera (Photometrics
C350, Tucson, AZ, USA), as previously described (Welshhans and Rehder, 2007, Estes et al., 2014). Images were captured using MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA) and stored on a Universal Imaging Corporation PC. Images were acquired at defined time points both before (-10, -5, and 0 min) and after (2, 5, 10, 15, 20 and 30 min) treatment with pharmacological agents. Filopodial length, measured from the tip of a filopodium to the edge of the central domain, was analyzed with Scion Image software (Scion Corporation, Frederick, MD) for all filopodia on a growth cone. Filopodial data were normalized to the time point t = 0 and expressed as a percentage change.

### 4.4.5 Pharmacological agents

All drugs were purchased from Sigma, dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at -20°C unless otherwise indicated. All drugs were bath applied and used at biologically active concentrations that had been determined by others and through our own studies.

**Carbon monoxide donor**

The CO-donor, carbon monoxide releasing molecule-2 (CORM-2; Tricarbonyl-dichlororuthenium (II) dimer; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to make a stock concentration of 50 mM. Fresh aliquots of CORM-2 were generated for each experiment 10 minutes prior to application to ensure reproducibility of CO concentration. To control for potential effects of the stable byproducts of CORM-2 once CO was liberated, we generated an inactive form of CORM-2 (iCORM-2), described by (Sun et al., 2008). Fresh CORM-2 stock was degassed overnight at 37°C. To remove residual CO left in the solution, nitrogen was bubbled through the solution for 1 hour and then degassed for an additional 10 minutes. The final solution
was filtered to remove any potential contamination, separated into aliquots, and stored at -20°C. To determine whether the effects of CORM-2 were similar to what could be observed physiologically in B5 and B19 neurons, hemin (Sigma-Aldrich), a substrate for heme oxygenase, was prepared fresh prior to each experiment. Hemin was dissolved in DMSO to make a stock concentration of 1 mM and then bath applied to the culture dish yielding a final hemin concentration of 1 µM.

**Pharmacological inhibitors**

The soluble guanylyl cyclase (sGC) inhibitors ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one) and NS2028 ([4H-8-bromo-1,2,4-oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-one]; Calbiochem) were aliquoted at respective stock concentrations of 20 mM and 10 mM. The protein kinase G (PKG) inhibitor, KT5823, was aliquoted at 2 mM. Ryanodine and dantrolene, inhibitors of calcium release from intracellular calcium stores, were dissolved at stock concentrations of 100 mM and 40 mM. All pharmacological agents were tested for their individual effects on filopodial length and for inhibition of CO effects and were preincubated for 30 minutes prior to CORM-2 addition.

**4.4.6 Statistical analysis**

All data were expressed as mean ± SEM, unless otherwise stated. For growth cone calcium and filopodial analysis, a repeated-measures ANOVA was employed for testing overall statistical significance between conditions (SPSS statistical software, SPSS, Chicago, IL). The Tukey test was used for *post hoc* analysis of preplanned comparisons. To test for statistical significant between individual time points, a paired Student’s *t* test or unpaired *t* test was used
where appropriate. Significant differences are indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

4.5 Results

4.5.1 CO increase filopodia length in B5 and B19 neurons

In light of recent findings that CO can affect neurite outgrowth (Scheiblich and Bicker, 2014), we hypothesized that CO might function as a modulator of growth cone dynamics, specifically filopodial length. To test for the direct effects of CO on filopodia and exclude the effects of its endogenously produced co-byproducts biliverdin and ferrous iron, we bath applied the CO-donor carbon monoxide releasing molecule-2 (CORM-2; 50 µM) to developing B5 and B19 neurons in vitro. It should be noted that while there is no measurement for the amount of CO produced within the snail nervous system, the concentration used here has been shown to produce physiological effects similar to the induction of the HO pathway in other species (Knipp and Bicker, 2009, Scheiblich and Bicker, 2014) and is known to affect the electrical activity of developing B5 and B19 neurons (our unpublished data). Global application of CORM-2 resulted in a significant increase in filopodial length in both B5 and B19 neurons over time when compared with the solvent control, DMSO (repeated-measures ANOVA, Tukey’s HSD B5: $p < 0.001$; B19: $p < 0.001$) [Fig. 4.1(A)]. When comparing with controls, we observed an increase in filopodial length within 2 minutes of CO treatment for both neuron-types (unpaired t-test, B5: $t = -2.279, p = .03$; B19: $t = -3.076, p = 0.005$). B5 neurons reached a maximum filopodial increase of 22 ± 2% 20 minutes following CO treatment, and B19 neurons reached a maximum increase of 35 ± 6% for B19 neurons [Fig. 4.1(B and C)].
While CORM-2 has been established as a potent CO-donor, it has also been reported that the effects of CO can differ from the effects of CO-releasing molecules (Motterlini et al., 2002, Dong et al., 2008, Wilkinson and Kemp, 2011a, Decaluwe et al., 2012), thus requiring appropriate controls for potential biological activity of the end products of CORM decomposition. To account for the possibility that the ruthenium complex contained in CORM-2 itself could affect filopodial length following the release of CO, we degassed CORM-2 to generate inactive CORM-2 (iCORM-2). iCORM-2 was found to have no significant effect on filopodial length compared with solvent controls (repeated-measures ANOVA, Tukey’s HSD B5: $p = 0.821$; B19: $p = 0.996$), suggesting that CO was indeed responsible for the CORM-2 mediated increase in filopodial length. Given that the amount of CO affecting these neurons in vivo is relatively unknown, we next wanted to determine whether the effects of CORM-2 were similar to what could be observed endogenously. Using hemin as a substrate for HO, we found that bath application of hemin (1 µM) resulted in a significant increase in both B5 and B19 filopodial length compared with the solvent control (repeated-measures ANOVA, Tukey’s HSD B5: $p < 0.001$; B19: $p < 0.001$). Moreover, these increases were similar to what we observed with 50 µM CORM-2 application (repeated-measures ANOVA, Tukey’s HSD B5: $p = 0.497$; B19: $p = 0.619$), suggesting that the concentration of CORM-2 utilized here may be within physiological concentrations.

Previously, we observed that increases in filopodial length often correspond with a decrease in the overall number of filopodia (Rehder and Kater, 1992); therefore, we quantified the change in filopodial number following CO treatment as well. Interestingly, there was no significant change in either B5 or B19 filopodial number following CORM-2 treatment (repeated-measures ANOVA, Tukey’s HSD B5: $p = 0.915$; B19: $p = 0.541$) [Fig. 4.1(D and E)]. Application of iCORM-2 also showed no significant change in filopodial number (repeated measures
ANOVA, Tukey’s HSD B5: $p = 0.895$; B19: $p = 0.502$), indicating that neither CO nor the by-products of CORM-2 affected filopodial number. We next tested whether stimulation of endogenous CO production affected filopodial number and found that application of hemin led to a delayed increase in the number of filopodia in B19 neurons only (repeated-measures ANOVA, Tukey’s HSD, $p = 0.046$). This increase occurred 20 minutes after hemin treatment (unpaired t-test, $t = -2.640$, $p = 0.015$) and persisted throughout the remainder of the experiment, suggesting that the endogenous production of CO may have some late effects on B19 growth cones that differ from extrinsic CO treatment. It should also be noted that while B5 neurons did not show a significant change in filopodial number across treatments (repeated-measures ANOVA, $p = 0.758$), we observed a general increase in filopodial number within the last ten minutes of hemin treatment, indicating that filopodial number may also increase in B5 neurons over an extended time period. Taken together, these results identify CO as a general effector of filopodial length during neurite outgrowth.

**4.5.2 CO increases filopodia length through a soluble guanylyl cyclase pathway**

Next we wanted to determine the signaling pathway in which the effects of CO on filopodial length were mediated. Previously, we have shown that activation of soluble guanylyl cyclase (sGC) and an increase in cyclic guanosine monophosphate (cGMP) yield an increase in filopodial length (Van Wagenen and Rehder, 2001). sGC is a major physiological target for CO (Kim et al., 2006), and its activation has been shown to result in a 4 fold increase in sGC activity to produce cGMP (Martin et al., 2006). To test whether CO modulated filopodial length through the activation of sGC, we utilized the sGC specific inhibitor, NS2028. While NS2028 showed no significant change in filopodial length on its own compared with solvent control (repeated
measures ANOVA, Tukey’s HSD, B5: $p = 0.882$; B19: $p = 0.719$) [Fig. 4.2], preincubation of NS2028 for 30 minutes prevented the CO-mediated filopodial elongation in B5 and B19 neurons (Repeats measures ANOVA, Tukey’s HSD B5: $p < 0.001$; B19: $p < 0.001$) [Fig. 4.2(A and B)]. To test independently whether CO mediated filopodial elongation through sGC, another sGC inhibitor, ODQ, was employed. Similar to NS2028, ODQ alone did not have any effect on filopodial length compared with solvent control (repeated measures ANOVA, Tukey’s HSD, B5: $p = 0.883$; B19: $p = 0.998$) but when preincubated for 30 minutes it blocked the increase in filopodial length mediated by CO (Repeats measures ANOVA, Tukey’s HSD B5: $p < 0.001$; B19: $p < 0.001$). Taken together, these findings suggest that CO affects changes in growth cone filopodial length through the activation of sGC.

### 4.5.3 CO increases filopodia length through activation of protein kinase G

With CO changing filopodial length through sGC, we next determined how the modulation of sGC by CO increased filopodial length. Previously, we have shown that injection of the cGMP analog, 8-bromo-cGMP, can lead to an increase in filopodial length of both B5 and B19 neurons (Van Wagenen and Rehder, 2001). Increases in this activity were found to occur through the activation of protein kinase (PKG); therefore, we used the PKG inhibitor, KT5823 to investigate whether PKG was a downstream signaling target of CO. KT5823 treatment by itself did not significantly affect the filopodia of B5 or B19 neurons when compared with the solvent control (repeated measures ANOVA, Tukey’s HSD, B5: $p = 0.223$; B19: $p = 0.924$). In both B5 and B19 neurons, the preincubation of KT5823 for 30 minutes blocked the increase in filopodial length mediated by CO alone (repeated measures ANOVA, Tukey’s HSD B5: $p < 0.001$; B19 $p <$
suggesting that CO increases filopodial length through an increase in PKG activity.

4.5.4 CO increases filopodia length through activation of ryanodine receptors

PKG is involved in a number of cellular signaling pathways that include the activation of cyclic adenosine diphosphoribose (cADPR), which acts as an intracellular messenger affecting the release of calcium from intracellular calcium stores (Lee, 2001). Changes in growth cone calcium levels are known to alter the growth cone cytoskeleton leading to different pathfinding behaviors elicited by the growth cone (Zheng and Poo, 2007). Injection of cADPR into B5 neurons triggers an increase in calcium as well as an increase in filopodial length (Welshhans and Rehder, 2007), suggesting that the release of calcium from internal stores is a mechanism involved in filopodial elongation. Given that CO modulates filopodial length through a sGC/PKG pathway, we wanted to directly test whether calcium release from intracellular stores was involved in the CO-mediated filopodial elongation. To do this, we used pharmacological inhibitors, dantrolene and ryanodine, to block ryanodine receptors, which mediate the release of calcium from intracellular stores. The application of dantrolene alone had no effect on filopodial length of B5 and B19 neurons (repeated measures ANOVA, Tukey’s HSD, dantrolene: B5: \( p = 0.610 \); B19: \( p = 0.999 \)) [Fig. 4.4 (A and B)]. The pretreatment with dantrolene for 30 minutes completely inhibited the increase in filopodial length induced by CO (repeated measures ANOVA, Tukey’s HSD, B5: repeated measures ANOVA, Tukey’s HSD B5: \( p < 0.001 \); B19: \( p < 0.001 \)). These findings suggest that the elongation of filopodia mediated by CO involved the release of calcium from intracellular stores. We wanted to verify these findings using another RyR inhibitor, ryanodine (100 µM). Treatment of B5 and B19 neurons with ryanodine alone did not
affect filopodial length compared with the solvent control (repeated measures ANOVA, Tukey’s HSD, B5: \( p = 0.776 \); B19: \( p = 1.000 \)). The pretreatment of B5 and B19 neurons with ryanodine for 30 minutes followed by CO treatment blocked the CO-mediated increase in filopodial length (repeated measures ANOVA, Tukey’s HSD, B5: repeated measures ANOVA, Tukey’s HSD, B5: \( p < 0.001 \); B19: \( p < 0.001 \)) [Fig. 4.4 (A and B)]. Taken together, these findings strongly suggest that CO affects filopodial length through the release of calcium from intracellular calcium stores.

4.5.5 CO-induced changes in filopodia occur through undetectable changes in calcium

With CO modulating filopodial length through RyRs, we next wanted to determine how CO affected cytoplasmic growth cone calcium levels. Neurons were loaded with the calcium indicator dye, Fura-2, and fluorescence emission ratios of growth cones at 340 and 380nm excitation wavelengths were captured every minute. In B5 neurons, CO led to a significant decrease in calcium from 0.72 ± 0.03 to 0.33 ± 0.01 within 5 minutes of treatment (paired t-test, \( t = 12.885, p < 0.001, n = 14 \)) [Fig. 4.5 (A and B)]. B19 neurons on the contrary, showed little change in calcium following CO treatment (0.40 ± 0.01 to 0.39 ± 0.01; paired t-test, \( t = 0.952, p = 0.353, n = 20 \)). With electrical activity dictating the calcium concentration in B5 and B19 neurons, the differences in the effect of CO on calcium levels were likely due to differences in electrical activity (Estes et al., 2014). To test this hypothesis, we combined calcium imaging with electrophysiology in a subset of these neurons. B5 neurons firing at an average frequency of 0.59 ± 0.03 Hz (n = 3) had an average growth cone calcium ratio of 0.67 ± 0.01 (n = 10). CO addition led to a rapid decrease in calcium to 0.34 ± 0.01 (paired t-test, \( t = 21.381, p < 0.001, n = 10 \)) that corresponded
with the silencing of firing activity [Fig. 4.5 (C)]. In B19 neurons, a majority of neurons were silent (3 out of 4) with an average calcium ratio of 0.39 ± 0.02 (n = 11). The addition of CO did not significantly change the growth cone calcium level (average ratio 0.37 ± 0.01; paired t-test, t = 1.164, p = 0.271) [Fig. 4.5 (D)], supporting our previous findings that growth cone calcium concentrations reach basal levels when neurons are silent (Estes et al., 2014). It should be noted that the one B19 neuron that showed spontaneous firing activity was silenced following CO treatment and that the one growth cone imaged had a decrease in calcium ratio from 0.55 to 0.39. Taken together these findings suggest that the robust decrease in calcium is due to the silencing of firing activity in response to treatment with CO.

Interestingly, changes in filopodial length occurred within 2 minutes of CO treatment when growth cone calcium levels were at or close to basal levels. It was not until approximately 9 and 20 minutes post CO treatment (B5 and B19 neurons, respectively) that calcium began to increase, suggesting that if an increase in intracellular calcium was required for filopodial length change it would have been relatively small and undetectable under these experimental conditions. To determine whether the delayed increase in calcium was due to the release of calcium from intracellular stores, we pretreated neurons with ryanodine for 30 minutes prior to the addition of CO. We observed no significant differences in growth cone calcium levels following ryanodine and CO treatment in either B5 or B19 neurons when compared with CO treatment alone (repeated measures ANOVA, B5: p = 0.516, B19: p = 0.999) [Fig. 4.5 (E and F)], suggesting that calcium release from intracellular stores did not have a detectable effect on the overall calcium concentration.
4.6 Discussion

In this study, we assessed the role of CO in the regulation of growth cone filopodia using a CO-releasing molecule and found that CO increased filopodial length of two neuron-types, B5 and B19, while having no effect on filopodial number. The changes in filopodia were mediated through a sGC/PKG/RyR pathway occurring at low growth cone calcium levels [Fig. 4.6]. Collectively, these findings demonstrate that CO functions as a modulator of filopodial dynamics and provide further evidence implicating it as an effector of growth cone motility.

4.6.1 CO modulates growth cone filopodia

During neuronal development, the presence of both HO isoforms suggests that CO is being produced both constitutively and in response to environmental and cellular stimuli, suggesting that CO may play a role in developmental processes. Here, we investigated the effects of CO on growth cone motility, a critical cellular activity observed during neuronal development and regeneration, when neurites undergo pathfinding in search of their appropriate synaptic target. As a first step in our understanding of the role that CO may play in growth cone motility, we found that both CORM-2 and hemin treatment elongated growth cone filopodia of B5 and B19 neurons. Given the sensory-motor nature of filopodia (Davenport et al., 1993), the increases in filopodial length mediated by CO may be important for increasing the exploratory radius of the growth cone, which can enable growth cones to traverse obstacles encountered within the cellular environment that may impede neurite advancement (Hammarback, 1986 #185).

Surprisingly, we found that the CO-mediated increases in filopodial length in both neuron types were not accompanied by decreases in filopodial number, which is known to occur
Even more unexpectedly, we observed an increase in filopodial number in B19 neurons 20 minutes after hemin treatment. By increasing filopodial length and maintaining or in some case increasing filopodial number, CO effectively increases the sampling radius and the sampling density of the growth cone. While these findings suggest that CO may increase the ability of growth cones to detect guidance cues at a distance, it is clear that these findings are only the beginning of our understanding for the role that CO plays in development and that more experiments need to be conducted to determine the functional implications of CO modulation of filopodial length. In addition, discrepancies in the modulation of filopodial number between CORM-2 and endogenous CO production induced by hemin suggest that some differences may exist between endogenous CO production and CO-donor molecules. It may be that differences between these two treatments is due to the fact that CO production also results in the production of two co-byproducts, ferrous iron or biliverdin, which have been shown to have their own physiological effects on tissues (Wu and Wang, 2005). Given that little is known about the role CO plays in development and that even less is known about the role its co-byproducts play, it will be important to conduct future studies on the role that ferrous iron and biliverdin can play in neuronal development as well in order to fully understand how the HO/CO pathway affects the wiring of the nervous system.

4.6.2 CO signaling: a sGC/PKG/RyR mediated pathway that modulates filopodial length

Soluble guanylyl cyclase is a major cellular target of CO. CO binds to the heme moiety of sGC leading to a 4-fold increase in its enzymatic activity converting GTP to cGMP (Kim et al., 2006) and affecting a number of physiological processes including: the relaxation of smooth
muscles in the ileum (Utz and Ullrich, 1991), platelet aggregation (Brune and Ullrich, 1987, Chlopicki et al., 2012), and the protection of neurons following ischemic brain injury (Schallner et al., 2013). While the CO/sGC pathway has been proposed to play a role in neuronal migration (Knipp and Bicker, 2009), there is very little direct evidence for the involvement of CO/sGC signaling in neuronal development. Here we showed that the effect of CO on filopodial length was mediated via sGC in both B5 and B19 neurons, because the inhibition of sGC with NS2028 and ODQ resulted in the full inhibition of CORM-2 mediated filopodial elongation. Interestingly, the effects of CO seen in this study differed from those obtained with an activator of sGC, YC-1, which increased filopodial length in B5 neurons and not in B19 neurons (Van Wagenen and Rehder, 2001). Moreover, the other gaseous neuromodulator known to stimulate sGC, NO, was shown to cause filopodia elongation in B5, but not in B19 neurons. These differences in response to activation of sGC may be due to differences in the binding properties of these sGC activators. CO, like NO, binds to the prosthetic heme group of sGC to increase its catalytic activity (Ma et al., 2007). YC-1, on the other hand, is an allosteric activator of sGC showing both a heme-dependent and a heme-independent mechanism of activation (Martin et al., 2001). Furthermore, the activation of sGC by YC-1 requires sGC to be in a reduced state in order to affect its catalytic activity (Evgenov et al., 2006), an effect that has not been reported with CO treatment. We have observed qualitative differences in the redox states of B5 and B19 neurons in preliminary experiments, in which B19 neurons appeared to be in a more oxidized state than B5 neurons (data not shown). Therefore, it is possible that B19 neurons were insensitive to YC-1 treatment due to their oxidative state, whereas CO might be functioning as a more general modulator of sGC because it is capable of activating sGC independent of oxidative state. Future studies are aimed at elucidating how the oxidative state of a neuron determines its cellular response to CO.
The production of cGMP by sGC plays an important role in neuronal development affecting neurite outgrowth (Trimm and Rehder, 2004, Yamazaki et al., 2004, Bau et al., 2005, Koriyama et al., 2009) and growth cone turning (Gundersen and Barrett, 1980, Murray et al., 2009). In developing B5 and B19 neurons, treatment with the cGMP analog 8-Br-cGMP increases filopodial length (Van Wagenen and Rehder, 2001). cGMP activates a number of cellular targets including: phosphodiesterases, cyclic nucleotide gated ion channels, and PKG. During NO-signaling, the activation of PKG is necessary for the modulation of filopodial dynamics and neurite outgrowth (Yamazaki et al., 2004, Welshhans and Rehder, 2005, Koriyama et al., 2009). Here, the inhibition of PKG with KT5823 blocked the elongation of filopodia in both B5 and B19 neurons, demonstrating that PKG activity is necessary for the modulation of filopodial dynamics by CO, as well.

Further downstream of PKG activation, the modulation of RyR results in an efflux of calcium from intracellular stores and thus an increase in cytosolic calcium levels (Takasago et al., 1991, Lee, 2001, Francis et al., 2010). Similar to effects reported for stimulation with NO, the inhibition of RyRs using dantrolene or ryanodine also prevented the CO-mediated change in filopodial length; however, unlike NO, we did not detect an increase in growth cone calcium that matched the timing of filopodial elongation in either neuron. The emptying of growth cone calcium stores using the sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase inhibitor, thapsigargin, does not show a detectable increase in growth cone calcium levels (Welshhans and Rehder, 2005), suggesting that the release of calcium from these stores may be undetectable with our current methods.

Prior to the addition of CO, we observed large differences in cytoplasmic calcium levels between B5 and B19 neurons, as previously described (Estes et al., 2014). B5 neurons, which
had higher baseline calcium levels than B19 neurons, showed a rapid decrease in growth cone calcium following CO treatment to levels that were similar to B19 neurons. This drop in cytoplasmic calcium corresponded to the time when spontaneous firing activity in B5 neurons stopped, supporting earlier findings that electrical activity of a neuron is the major determinant of growth cone calcium levels (Bolsover and Spector, 1986, Ross et al., 1987, Torreano and Cohan, 1997, Kuznetsov et al., 2012, Estes et al., 2014). CO has been shown to inhibit as well as activate voltage-gated calcium channels (Lim et al., 2005, Scragg et al., 2008), which implicate it as both a negative and positive effector of intracellular calcium concentrations. Surprisingly, little is known about the direct role CO plays in the regulation of intracellular calcium concentrations except that in guinea pig mast cells, CO increases cGMP production while decreasing cytosolic calcium levels (Ndisang et al., 1999) and that in pancreatic acinar cells CO increases intracellular calcium concentrations through a phospholipase C mediated pathway (Moustafa and Habara, 2014). While it is likely that the differences in calcium regulation by CO are due to cell-specific differences, it is clear that further studies are needed to determine how these cell-specific differences in calcium homeostasis may arise and what impact this can have on the physiology of a cell.

4.6.3 CO and NO: implications on growth cone motility

Filopodia protruding from the leading edge of the growth cone are continually extending and retracting, sampling the surrounding cellular environment for adhesive molecules and guidance cues. With longer filopodia, growth cones can detect guidance cues at a distance (Davenport et al., 1993) and allow developing neurites to traverse areas of low adhesion.
Conversely, growth cones that lack filopodia show aberrant growth cone steering (Bentley and Toroian-Raymond, 1986, Zheng et al., 1996) as well as decreases in neurite outgrowth (Dwivedy et al., 2007). Alterations in filopodial length and number may play an important role in a growth cone’s ability to navigate and establish proper neuronal connections.

NO increases filopodial length, decreases filopodial number, and slows the rate of neurite outgrowth (Trimm and Rehder, 2004), resulting in a “slow-down and search” behavior that is thought to enable a neurite to slow its advance while assessing the surrounding environment for signals that instruct it on its next pathfinding step. CO, on the other hand, resulted in what could be termed “search” behavior. CO increased filopodial length while having no effect on filopodial number, thereby effectively increasing the sampling radius of the growth cone while maintaining its sampling resolution. Interestingly, in human NT2 neurons CO increases neurite outgrowth and decreases migration of BV-2 microglia (Scheiblich and Bicker, 2014), suggesting that CO does not elicit a “slow-down” mechanism while exploring the environment. Unfortunately, the effects of CO on filopodial length in these neurons is unknown; however, it did lead to an increase in F-actin containing microspikes in BV-2 microglia, suggesting that CO modulates F-actin dynamics. While NO and CO share many cellular targets and pathways, subtle differences in the magnitude of their effects on signaling pathways and calcium dynamics may yield different growth cone responses and outgrowth trajectories. How these subtle differences affect the overall pathfinding of a neurite remains to be explored.
4.7 Figures

**Figure 4.1 CO effects on filopodia.**

CO increased filopodial length of B5 and B19 neurons. (A) Phase contrast images showed bath application of the CO donor, CORM-2 (50µM) increased growth cone filopodial length in B5 (top) and B19 (bottom) neurons over time. Note that filopodial elongation began as early as 2 minutes following CO treatment. (B) Quantification of the change in filopodial length over time showed that CO significantly increased filopodial length in B5 neurons when compared to the
solvent control, DMSO. Treatment with the inactivated CORM-2 (iCORM-2) had no effect on filopodial length, while the application of hemin, the substrate for heme oxygenase, showed a significant increase in filopodial length that was similar to the application of CORM-2. (C) Quantification of the change in B19 neurons showed that CO significantly increased filopodial length when compared to the solvent control. Application of iCORM-2 had no effect on filopodial length. As with B5 neurons, hemin led to a significant increase in filopodial length that was similar to the addition of CORM-2. While increases in filopodial length often correspond with decreases in filopodial number, CORM-2 treatment did not affect the number of filopodia in (D) B5 or (E) B19 neurons. It should be noted though that while hemin did not significantly affect filopodial number in B5 neurons, there was a significant increase in filopodial number in B19 neurons within 20 minutes of hemin treatment that persisted for the remainder of the recording.
Figure 4.2 CO acts through sGC

Inhibition of sGC blocked the CO mediated increase in filopodial length. (A) Treatment of B5 neurons with sGC inhibitors, NS2028 (10 µM) and ODQ (20 µM), did not have any effect on filopodial length when compared to the DMSO control treatment. 30 minute pretreatment with each inhibitor blocked the CO-mediated increase in filopodial length. (B) Treatment of B19 neurons with NS2028 and ODQ did not affect filopodial length. Pretreatment with either NS2028 or ODQ blocked the CO mediated increase in filopodial length.
Figure 4.3 CO acts through PKG

Inhibition of PKG blocked the CO-induced increase in filopodial length. (A) B5 neurons treated with KT5823 (2 µM) alone did not have a significant effect on filopodial length. 30 minute pretreatment with KT5823 prevented the CO mediated increase in filopodial length. (B) Treatment of B19 neurons with KT5823 did not affect filopodial length. The pretreatment of B19 neurons with KT5823 prevented the increase in filopodial length mediated by CO.
Figure 4.4 CO acts through RyR

Inhibition of the RyRs blocked the CO mediated increase in filopodial length. (A) Treatment of B5 neurons with RyR inhibitors, dantrolene (40 µM) and ryanodine (100 µM), did not have any effect on filopodial length when compared to the DMSO control treatment. 30 minute pretreatment with each inhibitor blocked the CO-mediated increase in filopodial length. (B) Treatment of B19 neurons with dantrolene and ryanodine did not affect filopodial length. Pretreatment with either dantrolene or ryanodine blocked the CO mediated increase in filopodial length.
Figure 4.5 CO decreases calcium

Low calcium levels corresponded with CO-mediated increases in filopodial length. (A) Ratio-metric images of (top) B5 and (bottom) B19 neurons before and 2 minutes after CORM-2 treatment (50µM) showed that CO rapidly decreased the calcium concentration in B5 neurons. (B)
Quantification of the calcium ratio showed that the decrease in calcium occurred for all B5 neurons and that B19 neurons showed little change in calcium over time. (C) Combined electrical recording with calcium imaging showed that (i) CO silenced spontaneous firing activity leading to the rapid decrease in growth cone calcium. (ii) Average growth cone calcium ratios for each neuron recorded showed that calcium levels decreased with CO exposure. These changes all coincided with the silencing of spontaneous firing activity observed during baseline recordings. Note that increases in filopodial length occurred within 2 minutes of CO treatment and that this timing corresponded with basal calcium ratios. (Di) Combined recording of B19 electrical activity with calcium imaging showed basal levels in silent B19 neurons. (Dii) The average growth cone calcium ratio for individual neurons (grey lines) as well as the average growth cone calcium ratio for all neurons (black line) showed little change in growth cone calcium when filopodial elongation occurred (2 minutes post CO treatment). Note that 1 out of 4 B19 neurons (Neuron 3) generated spontaneous firing activity that produced a higher calcium ratio typical for spiking neurons. CO treatment of this neuron led to a rapid decrease in calcium that corresponded with the silencing of spontaneous firing activity. (E) B5 and (F) B19 neurons were pretreated with ryanodine (100 µM) for 30 minutes prior to CO treatment and calcium imaging. The inhibition of ryanodine receptor-mediated calcium release from intracellular stores did not affect the changes in calcium observed by CO treatment alone.
Figure 4.6 CO proposed model.

This schematic model summarizes the results of CO on the modulation of filopodial length. CO increased filopodial length through the activation of sGC leading to an increase in cGMP production. cGMP activatedPKG which led to the opening of RyRs on intracellular calcium stores. Low levels of calcium release from intracellular stores were required for an increase in filopodial length through a currently unknown mechanism.
5 GENERAL DISCUSSION AND CONCLUSIONS

During neuronal development and regeneration, guidance signals converge at the growth cone to influence the navigation of axons and dendrites to their synaptic targets. The specific guidance behavior elicited by the growth cone depends on how the growth cone integrates all of these signals. With calcium functioning as the common downstream signaling target of many guidance signals, it is therefore important to understand the mechanisms by which various signals affect growth cone calcium dynamics in order to understand how the nervous system becomes wired. This dissertation focused, in large part, on how electrical activity of a neuron affects growth cone calcium levels, and more specifically, on how two gaseous neuromodulators, nitric oxide (NO) and carbon monoxide (CO), affect growth cone calcium levels and the impact this has on growth cone filopodia.

The current studies showed that electrical activity is a major determinant of growth cone calcium levels in two identified *Helisoma* neurons, B5 and B19. Upon bath application of NO, both B5 and B19 neurons showed marked increases in firing frequency as well as growth cone intracellular calcium concentration ([Ca\(^{2+}\)]\(_{i}\)). Interestingly, B5 neurons showed larger increases in growth cone [Ca\(^{2+}\)]\(_{i}\) than B19 neurons at specific firing frequencies due to their longer action potential (AP) duration. By increasing the duration of B19 APs through the inhibition of K\(^{+}\) channels, the amount of calcium influx driven by specific firing frequencies in these neurons increased. In addition, the increase in B19 AP duration switched the filopodial response of B19 neurons from being unresponsive to NO stimulation to responsive, showing filopodial elongation.

In addition to NO affecting growth cone [Ca\(^{2+}\)], CO also affected neuronal firing activity and growth cone calcium levels. CO treatment hyperpolarized the membrane potential of both
B5 and B19 neurons resulting in the silencing of spontaneous firing activity and the simultaneous drop in growth cone calcium. The changes in spontaneous firing activity mediated by CO occurred through the inhibition of a persistent sodium current (I\textsubscript{NaP}). In addition to affecting spontaneous firing activity, CO was found to decrease neuronal excitation through the inhibition of voltage-gated calcium channels (VGCCs). Given the effects of CO on electrical activity, it was hypothesized that CO would not affect filopodial length; however, CO treatment increased filopodial length of both B5 and B19 neuronal growth cones. This increase occurred through the activation of a soluble guanylyl cyclase (sGC), protein kinase G (PKG), ryanodine receptor (RyR) mediated pathway. Taken together, these studies suggest that NO and CO are potent modulators of electrical activity and growth cone calcium levels during neuronal development and regeneration. Moreover, while each gas elicits opposite effects on growth cone calcium levels (increases for NO and decreases for CO), they both increase the exploratory radius of the growth cone via filopodial elongation, allowing for the reception of guidance signals in the surrounding environment.

### 5.1 Nitric oxide: a gaseous effector molecule of neuronal development

While NO is not viewed as a conventional guidance signal, its gaseous properties make it an intriguing effector molecule of developing and regenerating neuronal processes. NO, which is capable of diffusing across cell membranes, has the potential to affect all nearby neurons within its diffusion radius, which was originally modeled to be as far as 200 µm from the source of NO production (Wood and Garthwaite, 1994). More recently, NO diffusion from a single neuron was shown to increase filopodial length of growth cones that were within 100 µm of its production.
(Tornieri and Rehder, 2007), supporting the notion that NO acts as a volume signaling molecule during development.

NO signaling affects many aspects of neuronal development including neuronal migration (Bicker, 2005), growth cone motility (Hess et al., 1993, Renteria and Constantine-Paton, 1996, Van Wagenen et al., 1999, Yamada et al., 2006, Stroissnigg et al., 2007, Tojima et al., 2009), and synaptogenesis (Ogilvie et al., 1995, Nikonenko et al., 2008). The effects of NO on these processes have been well characterized, involving two primary mechanisms: modulation of metalloproteins, like sGC, and through S-nitrosylation (also referred to as S-nitrosation) of protein cysteines. Interestingly, while developing neurons may contain these cellular targets, cell-specific effects of NO on growth cone motility have been observed (Ball and Truman, 1998, Van Wagenen and Rehder, 2001). How individual effects of NO may arise is not well understood. In locusts, NO selectivity occurs through the expression levels of sGC (Ball and Truman, 1998). In developing Helisoma neurons, B19 neurons do not exhibit changes in filopodial length following NO treatment and show little to no immunoreactivity for sGC, while B5 neurons show significant filopodial elongation following NO treatment and show strong immunoreactivity for sGC (Van Wagenen and Rehder, 2001). These findings indicate that cell-specific responses to NO in Helisoma neurons may also be due to differences in the expression of sGC. More recently, however, single-cell RT-PCR of B19 neurons showed that these neurons do express sGC (Zhong et al., 2015). Moreover, the administration of the cGMP analog, 8-Bromo-cGMP, to B19 neurons resulted in an increase in filopodial length (Van Wagenen and Rehder, 2001). Collectively, these findings suggest that B19 neurons contain the receptor for NO, sGC, and that the downstream effectors of sGC activation are also present in B19 neurons and capable of modulating filopodial dynamics.
In Chapter 2 of this dissertation, the electrical properties of a neuron were found to determine the effects of NO on growth cone filopodial length, providing an alternative explanation for how neuron-specific effects of NO may arise. In both B5 and B19 neurons, NO increases neuronal firing activity through the inhibition of small conductance potassium (SK) channels and the subsequent depolarization of the membrane potential (Artinian et al., 2010, Zhong et al., 2013c). While electrical stimulation of developing neurons correlates with increases in growth cone calcium in both invertebrate (Cohan et al., 1987, Ross et al., 1987, Torreano and Cohan, 1997) and vertebrate (Bolsover and Spector, 1986, Fields et al., 1990) neurons, a direct correlation between the NO-induced firing activity and growth cone [Ca\(^{2+}\)]\(_i\) remains to be determined. In Chapter 2 of this dissertation, I found that electrical stimulation of developing Helisoma B5 and B19 neurons increased the firing frequency of both neuron types, correlating with a linear increase in growth cone [Ca\(^{2+}\)]\(_i\). Moreover, these effects were replicated through NO-mediated increases in firing frequency, suggesting that firing activity is a major determinant of growth cone [Ca\(^{2+}\)]\(_i\). By inhibiting spontaneous firing activity in B5 neurons through the replacement of extracellular sodium with N-methyl-D-glucamine (NMDG), NO was no longer capable of producing robust increases in growth cone [Ca\(^{2+}\)]\(_i\). The inability of NO to affect firing activity and thus calcium in B5 neurons was also found to prevent NO-mediated increases in B5 filopodial length, providing evidence that firing activity, growth cone calcium levels, and growth cone motility are causally linked.

Interestingly, we observed cell-specific differences in calcium influx that corresponded to the duration of an AP. With VGCCs activating at depolarized membrane potentials, I hypothesized that longer AP durations would result in greater activation of VGCCs and thus an increase in growth cone [Ca\(^{2+}\)]\(_i\). While I did not directly measure the calcium current to verify that longer
AP duration result in an increase in VGCC activation, I did measure the resulting change in \([\text{Ca}^{2+}]_i\) before and after increasing the AP duration of B19 neurons, finding that longer AP duration of B19 neurons increased growth cone \([\text{Ca}^{2+}]_i\). While these findings support previous studies that AP duration effects growth cone \([\text{Ca}^{2+}]_i\) (Ross et al., 1987, Torreano and Cohan, 1997, Kuznetsov et al., 2012), this study showed, for the first time, that NO mediated growth cone \([\text{Ca}^{2+}]_i\) was determined by the presence of APs, and more specifically, by their duration. Furthermore, it was found that the duration of the AP determined the neuron-specific effects of NO on growth cone filopodial responses. Given that the composition of ion channels located in the plasma membrane can shift over the course of development, affecting both firing frequency and the AP waveform itself (Moody and Bosma, 2005, Suwabe et al., 2011), the mechanisms for the neuron-specific effects of NO, described here, may extend beyond neuron-type specific effects and include developmentally specific effects of NO on an individual neuron. With observations that B5 neurons do not respond to NO early in culture (≤ 1 day), future studies assessing developmental or regenerative changes in electrical properties of these neurons may provide insight into the developmental/regenerative responses of an individual neuron over time.

5.2 Generating specific growth cone behaviors: electrical activity

The findings of this study highlight the details of a more generalized mechanism involved in the regulation of neuronal responses to guidance factors: electrical activity. Throughout development neurons show patterns of spontaneous firing activity that are thought to be necessary for the wiring of the nervous system (Yamamoto and Lopez-Bendito, 2012). Disruption of this activity can lead to miswiring of neuronal projections and the formation of inappropriate synapses.
In Chapter 2, the disruption of spontaneous firing activity in B5 neurons, via the replacement of extracellular sodium with NMDG, inhibited increases in filopodial length mediated by NO. With neuronal firing activity driving growth cone calcium levels, it is likely that the minor increase in growth cone \([Ca^{2+}]_i\) following NO treatment of silent B5 neurons was insufficient for inducing filopodial elongation, providing evidence that the generation of specific growth cone behaviors require a specific threshold or “set point” of calcium. This may also explain why B19 neurons were unresponsive to NO treatment alone; the increases in B19 firing activity induced by NO and the simultaneous increase in growth cone \([Ca^{2+}]_i\) were likely unable to reach a calcium threshold necessary to initiate filopodial elongation.

The necessity for growth cone calcium levels to reach a particular threshold to initiate a growth cone behavior, as demonstrated in Chapter 2 with NO and filopodial elongation, is not an uncommon phenomenon. A number of studies have shown that there are optimal growth cone \([Ca^{2+}]_i\) for the production of specific growth cone behaviors. For example, moderate levels of growth cone \([Ca^{2+}]_i\) (200-300nM) elicit neurite elongation in chick DRG neurons, while deviations from this “moderate” \([Ca^{2+}]_i\) result in the suppression neurite outgrowth (Lankford and Letourneau, 1991). In Xenopus neurons, the elevation of growth cone \([Ca^{2+}]_i\) on one side of the growth cone results in attractive turning towards the side of calcium elevation; alternatively, low levels of calcium localized to one side of the growth cone result in growth cone repulsion (Zheng, 2000). While these findings illustrate how calcium levels directly affect growth cone behaviors, effectors of growth cone calcium levels can also determine how incoming signals are integrated by the growth cone to produce a specific behavior. For example, in Xenopus spinal neurons that are only 6-10 hours in culture, electrical stimulation switches netrin-1 mediated
growth cone repulsion to attraction (Ming et al., 2001). Moreover, Ming and colleagues were able to show that changes in electrical stimulation altered growth cone calcium levels, providing additional evidence that electrical activity acts as a major determinant of growth cone [Ca\(^{2+}\)]i. In another study by Nishiyama and colleagues, it was shown that the depolarization of the membrane potential in developing *Xenopus* neurons shifts Sema3A-mediated growth cone repulsion to attraction (Nishiyama et al., 2008). Furthermore, they demonstrated that changes in the membrane potential affected the magnitude of calcium entry into the growth cone and the turning direction elicited by that growth cone (high calcium led to attractive turning and low calcium led to repulsive turning). Taken together, both of these studies demonstrate that electrical activity can regulate the behaviors elicited by a growth cone to a given guidance cue.

While many of the effects of electrical activity described in this dissertation involve the direct modulation of calcium dynamics to affect growth cone motility, it is also possible that shifts in electrical activity can have downstream effects on growth cone motility. For example, activity-dependent changes in calcium can affect the transcription of guidance cue receptors. During the navigation of thalamocortical axons, the upregulation of Robo1, occurring through the silencing of spontaneous firing activity and subsequent decrease in calcium, is necessary for slowing axonal outgrowth; inhibition of Robo1 transcription via Robo1 knockout results in faster outgrowth (Mire et al., 2012). Moreover, Mire and colleagues, were also able to show that inhibition of calcium entry with the calcium channel blocker, nifedipine, decreased the rate of outgrowth in Robo1 knockouts, providing additional evidence that calcium thresholds determine specific growth cone behaviors. Through the regulation of receptor and ion channel transcription, activity-dependent changes in calcium are likely to play important roles in the timing of receptor
expression that enables neuronal processes to navigate to their target locations. Therefore, activity-dependent changes in calcium mediated by NO, as described in this dissertation, may also affect transcription of guidance receptors; although the transcriptional regulation of growth cone guidance receptors by NO remains to be directly determined. Collectively, the studies described in this dissertation provide evidence for the importance of electrical activity during neuronal development and how intrinsic electrical activity of a neuron can generate specific growth cone behaviors.

5.3 Carbon monoxide: a novel regulator of neuronal electrical activity

Like NO, CO is an unconventional signaling molecule that diffuses across cell membranes to affect nearby cells; however, unlike NO, CO is not a radical, suggesting that its diffusion radius may be larger than that of NO. While there is a need for cellular CO sensors to establish the exact diffusion radius of CO, it is known that the main pathway for clearance of CO in the body involves the sequestration of CO by nearby blood cells for the transport of CO to the lungs where it is exhaled (Wu and Wang, 2005, Levitt and Levitt, 2015), suggesting that CO does diffuse across extracellular space to affect nearby cells. The production of CO throughout the nervous system by the enzyme heme oxygenase (HO) has led to the description of CO as an important signaling molecule in the nervous system, affecting learning and memory (Cutajar and Edwards, 2007), nociception (Steiner et al., 2001), circadian rhythms (Artinian et al., 2001), and olfaction (Zufall and Leinders-Zufall, 1997). While many of the effects of CO are attributed to its activation of sGC (Kim et al., 2006), emerging evidence from a variety of cell-types (mostly non-neuronal cells) suggest that CO is a regulator of ion channel activity (Wilkinson and Kemp, 2011b). Therefore, with ion channel activity affecting the electrical activity of a neuron and in
turn electrical activity affecting growth cone motility, as described above, this dissertation set out
to understand how CO affects electrical activity in developing neurons, which has seen little at-
tention to date.

Spontaneous firing activity, which is critical for the proper wiring of the nervous system,
requires the activation of ion channels that depolarize the membrane potential to threshold, in-
ducing an AP without external stimulation. The activation of a persistent sodium current (I_{NaP}),
which is active at hyperpolarized membrane potentials, can be critical for the generation of spont-
aneous firing activity. In B5 neurons, the inhibition of I_{NaP} leads to hyperpolarization of the
membrane potential and the rapid silencing of spontaneous firing activity (Artinian et al., 2012).
It has been shown in a number of other neuron types that I_{NaP} regulates their spontaneous and
rhythmic firing activity, including hypoglossal neurons (Lamanauskas and Nistri, 2008), spinal
neurons (Harvey et al., 2006, Kuo et al., 2006), suprachiasmatic nucleus neurons (Kononenko et
al., 2004) and inspiratory neurons (Del Negro et al., 2005). Given that the molecular identity of
the persistent sodium channel is unknown, little is known about the endogenous regulation of
these channels and their currents. What is known is that both exogenous application of NO as
well as the induction of hypoxia can increase the I_{NaP} (Hammarstrom and Gage, 1998, 1999).
Furthermore, in Helisoma neurons, the endogenous production of NO by nNOS attenuates the
I_{NaP} (Artinian et al., 2012), providing evidence for the endogenous regulation of I_{NaP} by NO. In
Chapter 3 of this dissertation, I show for the first time that CO can act as an inhibitory regulator
of the I_{NaP} in developing neurons. Within 5 minutes of CO treatment, using the CO-donor carbon
monoxide releasing molecule-2 (CORM-2), the inward current that is characteristic of the I_{NaP}
was inhibited. The timing of this inhibition was similar to the time frame for the CO-induced hy-
perpolarization of the resting membrane potential (RMP) and subsequent silencing of spontaneous firing activity in B5 neurons, supporting previous evidence that the I_{NaP} is critical for the generation of spontaneous firing activity in B5 neurons (Artinian et al., 2012). While these findings reveal that CO can function as a novel inhibitory mechanism for the I_{NaP}, they also identify the mechanism through which CO can regulate neuronal firing activity in developing neurons.

In addition to modulating spontaneous firing activity, CO was also found to decrease neuronal excitability, i.e. the electrical response of a neuron to incoming stimulation. This evoked firing activity, like spontaneous firing activity, has been shown to affect growth cone behaviors (Cohan et al., 1987, Fields et al., 1990, Spitzer, 2006). Therefore, the modulation of evoked firing activity by CO may be an important mechanism by which CO regulates neuronal development. Interestingly, while CO decreased evoked firing activity in both B5 and B19 neurons, the decrease in B19 evoked APs was not as large as the decrease in B5 evoked APs, suggesting for cell-specific responses to CO. These differences are likely due to cell-specific differences in AP characteristics, primarily the driving force for AP generation in each neuron. In B5 neurons, APs are primarily driven by calcium, while in B19 neurons, APs are primarily driven by sodium (Zhong et al., 2015). Given that CO effects on evoked firing activity was found to be through inhibition of VGCCs, it is not surprising that CO led to a greater decrease in evoked firing activity of B5 neurons. These findings revealed a potential mechanism by which cell-specific responses to CO may be derived; although further work is needed to determine the impact of these differences.
5.4 Carbon monoxide: a novel modulator of growth cone motility

Filopodia, which extend from the leading edge of the growth cone, act as sensory-motor antennae (Davenport et al., 1993, Mattila and Lappalainen, 2008). They are the first responders to guidance signals in the surrounding environment and their dynamics can be indicative of the behaviors elicited by the growth cone as a whole. For example, in *Xenopus* neurons changes in filopodial calcium transients can be indicative of changes in neurite outgrowth and turning direction (Robles et al., 2003). The elongation of filopodia and the reduction in filopodial number following NO treatment in *Helisoma* neurons precedes the slow-down of neurite outgrowth, a collective phenomenon referred to as “slow down and search” behavior (Trimm and Rehder, 2004). Developing mouse hippocampal neurons also show a decrease in filopodial number that correlates with the suppression of neurite elongation (Kim et al., 2011). In addition to affecting growth cone behaviors directly, filopodia length can determine the path by which neurites can grow by enabling growth cones to traverse areas of low substrate adhesivity to continue pathfinding (Hammarback and Letourneau, 1986). Collectively, these findings demonstrate that filopodial dynamics can serve as an indicator for changes in growth cone motility.

Given that the \([\text{Ca}^{2+}]_i\) is a major determinant of filopodial dynamics, setting filopodial length and thus the exploratory radius of the growth cone for guidance signals (Rehder and Kater, 1992), and that the modulation of spontaneous and evoked firing activity affects growth cone \([\text{Ca}^{2+}]_i\), as shown in this dissertation, CO stands as a potential modulator of growth cone filopodia. Like NO, CO treatment elicited a significant increase in growth cone filopodial length; however, unlike NO, it did not show neuron-type-specific effects on filopodial elongation, i.e.
both B5 and B19 neurons showed filopodial elongation in response to CO. These findings suggest that CO is a general modulator of filopodial dynamics and moreover, implicate it as a novel modulator of growth cone motility as a whole.

Interestingly, the effects of CO on filopodial length were not mediated through detectable increases in growth cone \([\text{Ca}^{2+}]\). Moreover, in spontaneously firing B5 and B19 neurons CO decreased growth cone calcium to basal levels that corresponded with the silencing of spontaneously firing activity, which supported evidence from Chapter 2 that electrical activity regulates growth cone \([\text{Ca}^{2+}]\). Given that increases in growth cone calcium levels are involved in filopodial elongation (Rehder and Kater, 1992, Cheng et al., 2002), the decreases in growth cone calcium levels and subsequent increase in filopodial length mediated by CO suggests for a novel modulatory pathway for filopodial dynamics.

The effects of CO on growth cone filopodia were found to involve the activation of a sGC/PKG/RyR mediated pathway, which is the same pathway described for NO regulation of filopodial dynamics (Van Wagenen and Rehder, 1999, 2001, Welshhans and Rehder, 2005, Tornieri and Rehder, 2007, Welshhans and Rehder, 2007). These findings suggest that the activation of the sGC/PKG/RyR pathway is a common signaling pathway for the modulation of filopodial dynamics. Given that sGC is a major cellular target for both NO and CO (Kim et al., 2006), it is not surprising that these gases both act through sGC to modulate filopodia. The finding that, in addition to sGC activation, NO requires electrical activity and robust increases in calcium to increase filopodia length while CO does not suggests that there is a relationship between sGC, electrical activity and growth cone filopodial dynamics that is currently unknown, raising an interesting paradox for the modulation of filopodial length by these two gases.
While it may be that NO elongation of growth cone filopodia requires both sGC activation and activity-dependent increase in growth cone \([\text{Ca}^{2+}]_i\); and that CO only requires sGC activation [Fig. 5.1], an alternative explanation for this paradox is that both gases do not share the same signaling pathway to affect filopodial length. Based on the following series of evidence from other studies as well as the work from this dissertation, NO may function primarily through activity-dependent changes in growth cone calcium levels to affect filopodial length: (1) B19 neurons contain sGC but do not elicit changes to filopodial length by NO (Van Wagenen and Rehder, 2001, Zhong et al., 2015) except upon the increase of its AP duration, as observed in Chapter 2 of this dissertation, (2) the silencing of spontaneous firing activity in B5 neurons via the replacement of extracellular sodium with NMDG inhibits NO mediated increases in filopodia length, as shown in Chapter 2, and (3) the inhibitors of sGC (ODQ and NS2028) and PKG (KT5823) inhibit spontaneous firing activity in B5 neurons (Artinian et al., 2012). Taken together, these findings suggest that the effects of NO on growth cone filopodial length may be primarily mediated through the modulation of electrical activity, which is known to serve as an important effector of multiple physiological processes determined by the intracellular calcium concentration.
Figure 5.1 Schematic of NO and CO filopodial signaling pathways

(Left) Filopodial elongation mediated by NO involves the activation of a sGC pathway that results in the release of calcium from intracellular calcium stores and the elevation of intracellular calcium concentration. In addition, NO inhibits SK channels leading to the depolarization of the membrane potential and an increase in firing activity. Increases in firing activity activate voltage gated calcium channels on the plasma membrane allowing for an influx of calcium into the cytosol and an increase in intracellular calcium concentration. The increase in calcium concentration in turn results in filopodial elongation. (Right) CO utilizes a similar sGC pathway as NO to affect filopodial length. Alternatively to NO, CO inhibits firing activity through the inhibition of a I_{NaP}. Decreases in firing activity lead to decreases in calcium influx through voltage gated calcium channels and a general decrease in cytosolic calcium levels. The release of calcium from intracellular stores provides the calcium necessary for CO to elongate filopodia.
Regardless of the underlying mechanism behind the NO and CO modulation of filopodial dynamics, it is clear that both gases elongate filopodia, increasing the exploratory radius of the growth cone for guidance signals. Furthermore, growth cones affected by NO show elevated growth cone \( [Ca^{2+}]_i \), while growth cones affected by CO show diminished growth cone calcium levels. It is through these differences in growth cone calcium regulation that CO stands out as a novel modulator of growth cone motility that is separate from NO.

As mentioned previously, guidance signals converging on the growth cone affect the overall growth cone \( [Ca^{2+}]_i \), to elicit a specific growth cone behavior. The modulation of calcium dynamics within a growth cone may be an important mechanism for switching growth cone responses to guidance cues and for the establishment of specific neuronal projections. For example, the attractive turning response of a growth cone to netrin1 involves the localized elevation of calcium. Inhibition of calcium influx switches the attractive turning response induced by netrin1 to repulsive (Hong et al., 2000). The inhibition of VGCCs and the decrease in growth cone calcium levels mediated by CO, as shown in this dissertation, stand as a possible physiological mechanism for the switching of netrin turning responses from attractive to repulsive as well as other guidance signals that act through elevations in calcium.

In addition to affecting growth cone turning responses, CO may also modulate neurite outgrowth directly and through the modulation of other signaling targets. In a recent study by Scheiblich and colleagues, CO was shown to increase neurite outgrowth in human NT2 neurons (Scheiblich and Bicker, 2014). It may be possible that CO acts a modulator of guidance signals found in the environment to affect neurite outgrowth. For example, Sema3A induces neurite collapse in developing mouse DRG neurons via the elevation of growth cone calcium levels, specif-
ically through the activation of VGCCs; the inhibition of these channels blocks the Sema3A mediated collapse (Treinys et al., 2014), suggesting that physiological inhibitors of VGCCs, such as CO, can affect neurite outgrowth. Taken together these studies show CO can function as both a signaling molecule to affect growth cone motility as well as a modulator of incoming guidance signals, affecting how these signals are interpreted by the growth cone.

5.5 Special considerations for neural regeneration: CO as a therapeutic

A major goal for the study of neuronal development and regeneration is to understand the underlying mechanisms for the establishment of neural connections. In understanding these mechanisms, it becomes possible to develop therapeutics to aid in the recovery of neuronal injury to the central nervous system, which fails to regenerate on its own due to a non-permissive environment. Both traumatic brain injury and spinal cord injury are characterized by an initial injury event that directly crushes or severs neurons and/or their axons. Following the initial injury event is an ongoing deleterious cascade of effects, including: ischemia, excitotoxicity, vascular dysfunction, oxidative stress, and inflammation that generate secondary collateral damage. The physiological effects of CO described here and by others (Kim et al., 2006) suggests that CO may be a potential therapeutic for the treatment of each of these secondary effects and for the promotion of regeneration. For example, excitotoxicity following neuronal injury is characterized by elevated glutamate concentrations and activation of sodium channels, generating spontaneous firing activity. It is currently being proposed that Riluzole, a I_{NaP} inhibitor, be used for the treatment of this excitotoxicity (Wilson and Fehlings, 2014). Given the findings of this dissertation that CO acts as a potent inhibitor of I_{NaP}, CO may be a potential therapeutic for the treatment
of injury-induced excitotoxicity, as well. Furthermore, the finding that CO inhibited VGCCs suggested that it can also provide therapeutic action by minimizing calcium influx that triggers glutamate release into the surrounding environment, effectively stopping the positive glutamate feedback loop. CO has also been shown to act as an anti-inflammatory signal (Otterbein et al., 2000) and a suppressant of microglial migration (Scheiblich and Bicker, 2014), suggesting it can minimize the inflammation at the site of injury and prevent microglia translocation to the injury site, which contribute to the formation of the glial scar. Lastly, the effects of CO on growth cone motility, described in this dissertation, and the increase in neurite outgrowth of human NT2 (Scheiblich and Bicker, 2014) suggest that CO may also aide in the regeneration of axons back to their target locations. Collectively, the diverse effects of CO make it a unique therapeutic for the treatment of traumatic brain and spinal cord injury.

5.6 Conclusions

This dissertation focused on three specific aims to understand the physiological roles of NO and CO in neuronal development, specifically on how they affect growth cone motility. In aim 1, the electrical activity of developing neurons was shown to be a major determinant of growth cone calcium levels as well as the morphological response of a growth cone to NO. Furthermore, it was shown that the intrinsic characteristics of an AP, specifically its duration, can determine how guidance signals, like NO, affect growth cone motility. In aim 2, CO was identified as an inhibitory signal, hyperpolarizing the membrane potential, silencing spontaneous firing activity, and decreasing neuronal excitability. Moreover, CO was shown to be an effector of multiple ionic conductances to regulate this activity. Finally, in aim 3, CO was shown to increase filopodial length through the activation of a sGC/PKG/RyR mediated pathway. Additionally, CO
treatment did not show any significant increases in calcium, but rather, it showed a decrease in growth cone calcium levels, which corresponded to the silencing of spontaneous firing activity. Collectively, these aims reveal novel mechanisms for the modulation of growth cone motility.

While NO and CO are both potent modulators of filopodial length, the primary mechanisms used by each gaseous signal for this modulation differs: NO effects are primarily mediated through electrical activity and CO effects are primarily mediated through a sGC pathway. Moreover, the calcium dynamics regulated by these gases are different with NO increasing calcium and CO decreasing it. It is through these differences that NO and CO stand as separate unique modulators of growth cone motility, capable of differentially affecting growth cone responses to incoming guidance signals. These studies provide possible explanations for how specific growth cone behaviors can be generated and provide insight into how specific pathfinding trajectories can be generated in vivo. Overall, this dissertation provides a better understanding of the cellular mechanisms involved in the development and regeneration of the nervous system.
REFERENCES


