Activation of 5-HT1A Receptors in the Nucleus Tractus Solitarius Facilitate Ventilatory Responses to Hypoxic Hypercapnia and Promote Sympathetic Recovery Following Hypotensive Hemorrhage

Jaime Vantrease
Loyola University Chicago

Follow this and additional works at: https://ecommons.luc.edu/luc_diss

Recommended Citation
https://ecommons.luc.edu/luc_diss/1310

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 2014 Jaime Vantrease
LOYOLA UNIVERSITY CHICAGO

ACTIVATION OF 5-HT1A RECEPTORS IN THE NUCLEUS TRACTUS SOLITARIUS FACILITATE VENTILATORY RESPONSES TO HYPOXIC HYPERCAPNIA AND PROMOTE SYMPATHETIC RECOVERY FOLLOWING HYPOTENSIVE HEMORRHAGE

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MOLECULAR PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

BY

JAIME ERIN VANTREASE

CHICAGO, ILLINOIS

DECEMBER 2014
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Professor Karie Scrogin, for her tremendous guidance, support and encouragement throughout my graduate studies. She not only taught me essential scientific skills and critical reasoning, but also showed me that hard work and dedication will help me attain my most difficult goals in science and in all aspects of life. She has been someone I can count on to go above and beyond to help me succeed and for that, I will be forever grateful.

I’d also like to thank the other members of my committee including Professor Virginia Brooks, Associate Professor Neil Clipstone, Professor Emeritus EJ Neafsey, and Professor Emeritus Charles Webber Jr. for their time and valuable input throughout my dissertation process. I am also grateful for students in the Department of Molecular Pharmacology and Experimental Therapeutics as well as all of the wonderful people in my laboratory that provided support as well as a wonderful scientific environment.

Finally, I would like to thank my friends and family, especially my parents. Without your unconditional love, support, and encouragement, I would not have come this far.
Dedicated to the science teachers I had growing up. I am forever grateful to them for instilling a love of the natural world in me at an early age.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

LIST OF TABLES vii

LIST OF FIGURES ix

LIST OF ABBREVIATIONS xii

CHAPTER I: INTRODUCTION 1
  Significance 1
  Aim I 10
  Aim II 12
  Aim III 13

CHAPTER II: REVIEW OF RELATED LITERATURE 15
  Current treatment for hemorrhagic shock 15
  Autonomic response to hemorrhage 17
  Overview of baro-, chemo- and cardiopulmonary reflexes during hypotensive hemorrhage 20
  Anatomy of the arterial baroreflex 22
  Physiological role of the arterial baroreflex and hypotensive hemorrhage 25
  Anatomy of the peripheral chemoreflex 26
  Physiological role of the peripheral chemoreflex and hypotensive hemorrhage 29
  Peripheral chemoreceptor and baroreceptor interactions 30
  Central chemoreflex 31
  Nucleus tractus solitarius 37
    Anatomical features of the NTS 38
    Neurotransmission in the NTS 41
  Overview of central serotonergic system 49
    Anatomy of serotonergic system 50
    Serotonin receptors 54
    Major function of serotonin 56
    Serotonin and 5HT\textsubscript{1A} receptors in hemorrhage 57
      General functions of serotonin in hemorrhage 57
      5-HT\textsubscript{1A} receptor activation in hemorrhage 60
    Serotonin and central chemoreceptors 64

CHAPTER III: 5-HT\textsubscript{1A} RECEPTORS IN THE NUCLEUS TRACTUS SOLITARIUS FACILITATE SYMPATHETIC RECOVERY FOLLOWING HYPOTENSIVE HEMORRHAGE 69
  Abstract 69
  Introduction 70
CHAPTER IV: 5-HT1A RECEPTORS IN THE NUCLEUS TRACTUS SOLITARIUS ARE RESPONSIBLE, IN PART, FOR THE SYMPATHOEXCITATORY AND PRESSOR EFFECTS OF 8-OH-DPAT ADMINISTRATION DURING HYPOTENSIVE HEMORRHAGE

CHAPTER V: 5-HT1A RECEPTORS IN THE CAUDAL NUCLEUS TRACTUS SOLITARIUS MEDIATE INDICES OF AROUSAL DURING EXPOSURE TO HYPOXIC HYPERCAPNIA

CHAPTER VI: GENERAL DISCUSSION

REFERENCE LIST

VITA
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. shRNA sequences screened in HEK 293 cells</td>
<td>74</td>
</tr>
<tr>
<td>2. Reverse transcriptase PCR primers</td>
<td>77</td>
</tr>
<tr>
<td>3. Primers for qPCR analysis</td>
<td>87</td>
</tr>
<tr>
<td>4. Spontaneous baroreflex sensitivity data from baseline recordings</td>
<td>99</td>
</tr>
<tr>
<td>in 1ARshRNA and ScramshRNA injected rats</td>
<td></td>
</tr>
<tr>
<td>5. Hematocrit and plasma protein concentrations before and after</td>
<td>100</td>
</tr>
<tr>
<td>hypotensive hemorrhage in ScramshRNA and 1ARshRNA rats</td>
<td></td>
</tr>
<tr>
<td>6. Effects of hemorrhage on venous blood gas variables and acid-base</td>
<td>101</td>
</tr>
<tr>
<td>status in ScramshRNA- and 1ARshRNA-injected rats</td>
<td></td>
</tr>
<tr>
<td>7. PCR conditions for 8-OH-DPAT-treated rats</td>
<td>124</td>
</tr>
<tr>
<td>8. Body weight and blood volume indices for 8-OH-DPAT-treated rats</td>
<td>126</td>
</tr>
<tr>
<td>9. Spontaneous baroreflex indices in 8-OH-DPAT-treated rats</td>
<td>127</td>
</tr>
<tr>
<td>10. Venous blood gas and acid-base status for 8-OH-DPAT-treated rats</td>
<td>130</td>
</tr>
<tr>
<td>11. Primers for qPCR analysis of chemoreflex challenged AAV rats</td>
<td>155</td>
</tr>
<tr>
<td>12. The percentage of serotonin-immunoreactive neurons that also</td>
<td>158</td>
</tr>
<tr>
<td>express c-Fos immunoreactivity during chemoreflex challenges</td>
<td></td>
</tr>
</tbody>
</table>
13. Arterial blood gases and acid-base status determined before and after a 60 minute exposure to chemoreflex challenges 160

14. Total number of cells that expressed c-Fos in the commissural and medial NTS during exposure to chemoreflex challenges 166
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Progressive blood loss elicits four distinct phases of autonomic nervous system response</td>
<td>18</td>
</tr>
<tr>
<td>2.</td>
<td>Neural pathways of the arterial baroreceptors</td>
<td>24</td>
</tr>
<tr>
<td>3.</td>
<td>Neural pathways of the peripheral chemoreceptors</td>
<td>28</td>
</tr>
<tr>
<td>4.</td>
<td>Schematic representation of how CO$_2$ and H$^+$ ions in plasma central chemoreceptors</td>
<td>33</td>
</tr>
<tr>
<td>5.</td>
<td>Sagittal view of a rat brain depicting serotonin innervation throughout the CNS</td>
<td>51</td>
</tr>
<tr>
<td>6.</td>
<td><em>In vitro</em> screening of shRNA sequences targeting the rat 5-HT$_{1A}$ receptor</td>
<td>90</td>
</tr>
<tr>
<td>7.</td>
<td>The 5-HT$<em>{1A}$ receptor shRNA #2 reduces 5-HT$</em>{1A}$ receptor mRNA and protein expression <em>in vitro</em></td>
<td>91</td>
</tr>
<tr>
<td>8.</td>
<td>AAV injection sites</td>
<td>93</td>
</tr>
<tr>
<td>9.</td>
<td>Representative coronal rat brain sections after immunohistochemical label of serotonin</td>
<td>94</td>
</tr>
<tr>
<td>10.</td>
<td>5-HT$<em>{1A}$ receptor shRNA selectively and discretely reduces relative 5-HT$</em>{1A}$ receptor gene expression at the target site <em>in vivo</em></td>
<td>95</td>
</tr>
<tr>
<td>11.</td>
<td>Representative sample recordings during hypotensive hemorrhage in ScramshRNA- and 1ARshRNA-injected rats</td>
<td>96</td>
</tr>
<tr>
<td>12.</td>
<td>Mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (RSNA) during hemorrhage and subsequent recovery in ScramshRNA- and 1ARshRNA-injected rats</td>
<td>97</td>
</tr>
<tr>
<td>13.</td>
<td>Lactate levels at baseline, after hemorrhage termination and recovery</td>
<td>102</td>
</tr>
</tbody>
</table>
14. Relationship of 5-HT\textsubscript{1A} receptor mRNA levels in the commissural NTS to sympathetic recovery and and lactate levels 104

15. Diagram of 5-HT\textsubscript{1A} receptor activation during hypotensive hemorrhage 112

16. Effect of ScramshRNA and 1ARshRNA expression in the NTS on cardiovascular responses to 8-OH-DPAT in hemorrhaged rats 128

17. Coronal rat brain sections demonstrating variations in hrGFP expression in AAV-treated rats 131

18. Relationship of 5-HT\textsubscript{1A} receptor mRNA levels and latency to recover 75\% of baseline blood pressure after hypotensive hemorrhage 133

19. Relative 5-HT\textsubscript{7} and \(\alpha\text{AR}_{2\alpha}\) expression in commissural NTS and mNTS of rats injected with ScramshRNA or 1ARshRNA 135

20. Relationship of 5-HT\textsubscript{1A} receptor mRNA levels in the commissural and mNTS to RSNA one minute after 8-OH-DPAT administration 136

21. Effect of ScramshRNA and 1ARshRNA on responses to hemorrhage after exclusion of unsuccessfully transduced rats 137

22. Effect of gas challenges on c-Fos expression in serotonin neurons of the raphe magnus 163

23. Effect of gas challenges on c-Fos expression in serotonin neurons of the raphe obscurus 164

24. Effect of gas challenges on c-Fos expression in the commissural and mNTS 165

25. Ventilatory rate (VR) and minute volume (MV) in rats treated with AAV encoding the scrambled shRNA (ScramshRNA) or the 1ARshRNA 167

26. Ventilatory rate changes of individual rats over the course of exposure to hypoxic hypercapnia 169

27. Effect of ScramshRNA and 1ARshRNA expression in NTS on cumulative minute ventilation (MV) during gas challenges 171

28. Percent of time during each gas challenge spent sniffing 172
29. Relationship of 5-HT$_{1A}$ mRNA expression in commissural and medial NTS to area under the curve of ventilatory rate during exposure to hyperoxic hypercapnia 173

30. Original working model 180

31. Revised working model 187
LIST OF ABBREVIATIONS

5-HT   Serotonin
5-HT-ir Serotonin immunoreactivity
5,7-DHT 5,7-dihydroxytryptamine
8-OH-DPAT 8-hydroxy-2-n-di[propylamino] tetralin
AAV    adeno-associated virus
ATP    adenosine triphosphate
BE     base excess
BRS    baroreflex sensitivity
BP     blood pressure
CMV    cytomegalovirus
cNTS   caudal NTS
CO     cardiac output
CO₂    carbon dioxide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPG</td>
<td>central pattern generator</td>
</tr>
<tr>
<td>CVLM</td>
<td>caudal ventrolateral medulla</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3’-diaminobenzadine tetrahydrochloride</td>
</tr>
<tr>
<td>DBP</td>
<td>diastolic blood pressure</td>
</tr>
<tr>
<td>DIC</td>
<td>disseminated intravascular coagulopathy</td>
</tr>
<tr>
<td>DMX</td>
<td>dorsal motor nucleus of the vagus nerve</td>
</tr>
<tr>
<td>DR</td>
<td>dorsal raphe</td>
</tr>
<tr>
<td>EAA</td>
<td>excitatory amino acid</td>
</tr>
<tr>
<td>EPI</td>
<td>epinephrine</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>HCVR</td>
<td>hypercapnic ventilatory response</td>
</tr>
<tr>
<td>HF</td>
<td>high frequency</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>hrGFP</td>
<td>humanized <em>Renilla reniformis</em> green fluorescent protein</td>
</tr>
<tr>
<td>IBI</td>
<td>interbeat interval</td>
</tr>
<tr>
<td>IML</td>
<td>intermediolateral cell column of the spinal cord</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IR</td>
<td>ischemic reperfusion</td>
</tr>
<tr>
<td>iv.</td>
<td>intravenous</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>KCN</td>
<td>potassium cyanide</td>
</tr>
<tr>
<td>LF</td>
<td>low frequency</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
</tr>
<tr>
<td>MBP</td>
<td>mean blood pressure</td>
</tr>
<tr>
<td>MR</td>
<td>median raphe</td>
</tr>
<tr>
<td>MV</td>
<td>minute volume</td>
</tr>
<tr>
<td>NA</td>
<td>nucleus ambiguus</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>NTS</td>
<td>nucleus tractus solitarius</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>arterial blood CO₂ pressure</td>
</tr>
<tr>
<td>PaO₂</td>
<td>arterial blood O₂ pressure</td>
</tr>
<tr>
<td>p-CPA</td>
<td>p-chlorophenylalanine</td>
</tr>
<tr>
<td>PMN</td>
<td>phrenic motor nucleus</td>
</tr>
<tr>
<td>PPY</td>
<td>parapyramidal region</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real – time PCR</td>
</tr>
<tr>
<td>RM</td>
<td>raphe magnus</td>
</tr>
<tr>
<td>RO</td>
<td>raphe obscurus</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Term</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RP</td>
<td>raphe pallidus</td>
</tr>
<tr>
<td>RR</td>
<td>respiratory rate</td>
</tr>
<tr>
<td>RSNA</td>
<td>renal sympathetic nerve activity</td>
</tr>
<tr>
<td>RTN</td>
<td>retrotrapezoid nucleus</td>
</tr>
<tr>
<td>RVLM</td>
<td>rostroventrolateral medulla</td>
</tr>
<tr>
<td>SBP</td>
<td>systolic blood pressure</td>
</tr>
<tr>
<td>SDNN</td>
<td>standard deviation of blood pressure between normal beats</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>SUB</td>
<td>subependymal region</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>TASK</td>
<td>two-pore domain potassium</td>
</tr>
<tr>
<td>TV</td>
<td>tidal volume</td>
</tr>
<tr>
<td>TPH</td>
<td>tryptophan hydroxylase</td>
</tr>
<tr>
<td>TPR</td>
<td>total peripheral resistance</td>
</tr>
<tr>
<td>VR</td>
<td>ventilatory rate</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

Significance

In the US every year, over 50,000 people die following traumatic injury. It is estimated that 30-40% of these deaths are directly due to extensive blood loss and shock (Centers for Disease Control and Prevention, 2012; Kauvar and Wade, 2005). Current treatment for hemorrhagic shock includes volume resuscitation, the goal of which is to restore perfusion of the vital organs (Perel et al., 2013). However, fluid volume restitution alone is often insufficient to regain perfusion pressure, in which case catecholamines or other vasoconstrictors are given (Hollenberg, 2011). Therefore, such treatment is only administered after sufficient volume resuscitation in order to avoid exacerbation of ischemia and reperfusion injury. During hypoperfusion, the peripheral tissues become hypoxic and undergo anaerobic metabolism. The byproducts of this process can form reactive oxygen species (ROS) upon the reintroduction of blood flow to cause further damage to the microvasculature and induce endothelial injury (Verma et al., 2002). While significant treatment advances have been made in recent years, morbidity and mortality remain high, especially when resuscitation is delayed. Novel methods of resuscitation that can be employed rapidly and easily are required to improve patient survival from traumatic blood loss.
Progressive blood loss produces a complex multiphasic response of the autonomic nervous system. During the initial stage of hemorrhage, arterial pressure is maintained primarily through the unloading of the arterial baroreceptors, which activate sympathetic drive causing vasoconstriction and increased heart rate (Korner et al., 1990; Schadt and Ludbrook, 1991). Once a significant amount of blood has been lost (20-30% total volume in humans and 15% total volume in unanesthetized rats), a reversible decompensatory response is initiated during which there is a decrease in sympathetic outflow and rapid fall in blood pressure (Hasser and Schadt, 1992; Ludbrook and Ventura, 1996). This transient response is hemodynamically similar to vasovagal syncope. This response may be beneficial in that it increases ventricular filling time and cardiac output (Barcroft and Edholm, 1945). The syncopal phase persists until a second set of compensatory responses develop that help regain perfusion pressure to peripheral organs. The second compensatory response is characterized by a slow restoration of sympathetic drive (Haljamae, 1984). Without therapeutic intervention, this secondary compensatory stage eventually plateaus. If blood loss continues beyond this stage, vascular permeability increases, and vascular reactivity declines (Liu et al., 2003). This last stage is known as decompensation and is extremely difficult to treat. Emergency interventions that prolong the secondary compensatory phase and delay decompensation are crucial for patient survival from traumatic blood loss.

Our lab has found that 5-HT1A receptor agonists, including the full agonist, 8-OH-DPAT, accelerate sympathetic recovery and stimulate sympathetic-
dependent increases in venous tone without increasing total peripheral resistance when given after the onset of hypotensive hemorrhage or hemorrhagic shock (Osei-Owusu and Scrogin, 2004; Scrogin, 2003; Scrogin et al., 2000; Tiniakov et al., 2007; Tiniakov and Scrogin, 2006). These effects are prevented by ganglionic blockade or sympathetic denervation of the splanchnic vasculature. Thus, 8-OH-DPAT administration increases perfusion pressure by mobilizing blood from the venous pool in the splanchnic vasculature (Tiniakov et al., 2012; Tiniakov and Scrogin, 2009). This effect persists beyond the duration of drug action, suggesting that an initial increase in sympathetic activation mobilizes blood stores that remain in the effective circulation even after the direct effects of the drug have worn off. Low dose drug injection in the cisterna magna, directly above the floor of the 4th ventricle was found to produce the most robust and rapid rise in sympathetic activity in hemorrhaged rats compared to other routes of administration (Scrogin, 2003). Attempts to better define the site of action of 5HT₁A receptor agonists have been hampered by an inability to observe the sympathoexcitatory effects of 5-HT₁A receptor agonists in anesthetized animals.

In the course of the studies described above, it was found that selective lesion of caudal serotonin neurons attenuated normal ventilatory responses and recovery of sympathetic activity following hypotensive hemorrhage. Destruction of serotonin neurons also exacerbated metabolic acidosis, indicating that lesioned animals had exaggerated peripheral tissue hypoxia (Kung et al., 2010). Excessive tissue hypoxia developed despite high arterial blood O₂ levels. Evidence that tissue hypoxia after hemorrhage is more severe in lesioned
animals despite elevated arterial blood O$_2$ indicates that loss of serotonin cells in the caudal brainstem impairs delivery of blood to peripheral tissue. Nevertheless, blood pressure compensation following hemorrhage was similar in lesioned and sham-lesioned animals, which led us to speculate that lesioned animals maintained blood pressure by increasing peripheral resistance. These data further suggest that serotonin neurons are important for maintenance of cardiac output and peripheral oxygenation during low volume states, a phenomenon that may be dependent upon sympathetic activation. Thus, 8-OH-DPAT and serotonin neurons have similar effects during hemorrhage. Specifically, both increase sympathetic recovery following hypotensive hemorrhage and improve tissue oxygenation, most likely by increasing cardiac output. Thus, we propose that endogenous serotonin may be released during hemorrhage and act on 5-HT$_{1A}$ receptors expressed on non-serotonergic neurons to mediate sympathetic-dependent increases in perfusion. Therefore, 8-OH-DPAT may act by accelerating and further augmenting the normal serotonin-dependent mechanism activated by stimuli associated with hemorrhage.

5-HT$_{1A}$ receptors normally couple to inhibitory G$_{ai}$ proteins (Fargin et al., 1989). Activation of 5-HT$_{1A}$ receptors leads to membrane hyperpolarization (Innis et al., 1988; Penington and Kelly, 1990). Indeed, in euvolemic animals, 8-OH-DPAT produces profound sympathoinhibition, most likely through activation of 5-HT$_{1A}$ receptors expressed on tonically active RVLM cells that excite pre-ganglionic sympathetic nerves (Kubo et al., 1995; Lewis and Coote, 1993). Therefore, the sympathoexcitatory effect of 8-OH-DPAT observed in
hemorrhaged animals may be due to disinhibition of a reflex response that is activated by some stimulus associated with blood loss, but is not active when blood volume is normal. In accord, the sympathoexcitatory effect of 8-OH-DPAT in hemorrhaged animals is attenuated by disruption of afferent input to the CNS from peripheral chemoreceptors and arterial baroreceptors (Osei-Owusu and Scrogin, 2006). These data are consistent with the possibility that chemo and/or baroreceptor afferents convey information that somehow attenuates the full sympathoexcitatory reflex that develops during hemorrhage; and that 5-HT$_{1A}$ receptor activation reverses this effect to enable full expression of compensatory responses.

Afferent nerves that emanate from the carotid body transmit information on arterial blood gases and pH to the central nervous system through the IXth cranial nerve (Berger, 1979). Chemosensory afferents terminate primarily in the commissural subnucleus of the nucleus tractus solitarius (NTS) (Sapru, 1996). Excitation of chemosensory afferents by acute hypoxia promotes increased ventilation, increased sympathetic activity and bradycardia. Injection of excitatory amino acid neurotransmitters into the commissural NTS promotes similar reflex responses (Almado and Machado, 2005).

Blood pressure is sensed by arterial baroreceptor afferents which arise from stretch receptors in the wall of the carotid vessels and the aortic arch. They are activated by vessel stretch, which leads to sympathoinhibition, bradycardia and hypotension. Arterial baroreceptor afferents travel along with the chemosensory afferents in the sinus and IXth cranial nerve in rats (Ciriello,
However, nerve terminals of baroreceptor afferents terminate primarily in more rostral regions of the NTS in the medial and dorsomedial subnuclei. Injection of excitatory amino acids in this region produces responses similar to activation of the arterial baroreflex (Viard and Sapru, 2002).

Disruption of afferent input from both the carotid chemoreceptors and arterial baroreceptors by sinoarotic denervation prevents the increase in sympathetic drive and heart rate that characterizes the initial normotensive phase of hemorrhage (Korner et al., 1990). This leads to a slight acceleration in the onset of the syncopal response to hemorrhage. Sinoaortic denervation also slows the recovery of sympathetic activity, much like serotonin cell lesion (Osei-Owusu and Scrogin, 2006). While it is well known that arterial baroreceptor unloading contributes to the compensatory response to hemorrhage, the role of peripheral chemoreceptors in the sympathetic response to hemorrhage remains controversial. Oxygen pressure within the carotid body declines as blood pressure falls during blood withdrawal in the anesthetized cat (Buerk et al., 1989). The decline parallels increases in sinus nerve discharge, suggesting that the peripheral chemoreceptors are involved in reflex responses to hemorrhage. However, during hemorrhage, arterial blood O₂ levels rise due to an initial increase in ventilation. Rising acidosis also results in bronchodilation which helps to oxygenate blood. Therefore, it is thought that the decline in perfusion of the carotid body is the primary stimulus that increases chemoreflex activation. Nevertheless, studies indicate that the blood pressure and heart rate changes that develop during blood loss and subsequent compensation are not affected by
carotid body artery ligation in the unanesthetized rat, suggesting that chemoreceptors may have little effect on compensation from hemorrhage (Silveira et al., 2005). However, our results have also shown that lesion of caudal raphe serotonin neurons has no effect on blood pressure and heart rate responses to blood loss but does have a significant influence on sympathetic and ventilatory drive during hemorrhage; these factors also have a significant effect on tissue oxygenation. It remains to be determined whether peripheral chemoreceptors contribute to ventilatory and sympathetic responses to recovery from blood loss and whether they modulate the hemodynamic response to compensation.

During hypotensive hemorrhage, loss of tissue perfusion and low pH in the carotid body should activate chemoreceptors, while simultaneous hypotension should unload arterial baroreceptors. Both stimuli normally promote reflex sympathoexcitation and increased ventilation (Brunner et al., 1982; Kara et al., 2003). However, the ventilatory response to hypoxia declines with continued hypoxic exposure (Morrill et al., 1975; Tabata et al., 2001). Sympathetic activity also declines, though the response is somewhat delayed (Hunt et al., 2008). This effect, known as hypoxic ventilatory decline, is dependent upon the integrity of the carotid body; and is associated with increased GABA release in the commissural NTS where chemoreceptor afferents terminate (Tabata et al., 2001). It is not known whether ventilatory decline contributes to the sympathoinhibitory response to severe hemorrhage. However, we have noted that rats subjected to lesion of hindbrain serotonin cells show a significant decline in ventilation that
begins to develop at the onset of blood loss, while intact animals show an initial increase in ventilation that returns to baseline. Thus, it is plausible that endogenous serotonin release normally counteracts hypoxic ventilatory decline during hemorrhage in order to maintain ventilatory drive, which in turn may contribute to increased sympathetic drive through central coupling mechanisms.

Cardiovascular afferents enter the NTS near the dorsal surface of the brainstem at the floor of the 4th ventricle in the region where the sympathoexcitatory effects of 8-OH-DPAT administration were found to be most robust. The NTS also receives extensive innervation from caudal raphe neurons (Schaffar et al., 1988). Selective lesion of serotonin nerve terminals in the NTS produces similar decreases in sympathetic and ventilatory responses during hemorrhage as seen with the more widespread caudal raphe serotonin cell lesion (Kung and Scrogin, 2011). Rats subjected to lesion of serotonin nerve terminals in the NTS also show a profound reduction in ventilatory and sympathetic responses to chemical hypoxia. In contrast, the same animals show an increase in arterial baroreflex gain. These data suggest that serotonin released in the NTS may contribute to compensation after blood loss by facilitating peripheral chemoreflex responses.

The mechanism by which serotonin is released in the NTS to augment recovery from hemorrhage remains unknown. Studies in our lab and by others suggest that serotonin neurons in the caudal raphe mediate central chemoreception and react to changes in CO₂/H⁺ to promote ventilation (Kung et al., 2010; Teran et al., 2014). Serotonin neurons in the caudal raphe are
activated during hypercapnia, and metabolites of serotonin have been shown to accumulate in the caudal NTS following hypovolemia (Curtis et al., 2013; Haxhiu et al., 2001; Johnson et al., 2005). Immunohistochemical analysis has shown these same serotonin neurons physically wrap around the main blood vessels that perfuse the brainstem and have an ideal location to directly sense changes in CO$_2$/H$^+$ in the arterial blood supply to the brain (Bradley et al., 2002). When isolated and cultured, these neurons show sensitivity to elevated CO$_2$ and H$^+$ (Wang et al., 2001). Moreover, depletion of serotonin in vivo attenuates the ventilatory responses to hypercapnia and peripheral chemoreceptor activation (Hodges et al., 2008; Kung et al., 2010; Kung and Scrogin, 2011). Thus, it is possible that during hemorrhage, as blood flow is reduced, peripheral chemoreceptors become activated to facilitate sympathetic and ventilatory drive, but after prolonged activation these compensatory responses wane (hypoxic ventilatory decline). With sustained blood loss, animals experience metabolic acidosis (hypoventilatory hypercapnia), which may stimulate serotonin neurons in the caudal raphe to release serotonin in the NTS and thereby activate 5-HT$_{1A}$ receptors, which may disrupt the attenuated ventilatory response that develops during sustained peripheral chemoreceptor activation. Studies performed in this dissertation investigated whether endogenous serotonin and exogenous administration of 8-OH-DPAT activate 5-HT$_{1A}$ receptors in the caudal NTS to promote sympathetic compensation from hemorrhage. In addition, studies were performed to determine if this same receptor population is critical for the ventilatory response to hypoxic hypercapnia. A hypoxic hypercapnic stimulus
was used here to mimic the hypoxic acidosis that occurs with hemorrhage. A better understanding of the central mechanisms involved in recovery from blood loss could contribute to the identification of novel therapeutic targets for treating patients with traumatic blood loss.

**Main Hypothesis**

In the current project, studies were performed to examine the OVERALL HYPOTHESIS: *5-HT$_{1A}$ receptor activation in the caudal nucleus tractus solitarius (NTS) augments recovery from hypotensive hemorrhage by facilitating peripheral chemoreceptor activation*. This hypothesis was examined by utilizing the following aims.

**Specific Aim 1:** To determine if endogenous serotonin activates 5-HT$_{1A}$ receptors in the caudal NTS to facilitate sympathetic drive during compensation from hypotensive hemorrhage in unanesthetized rats.

**Rationale**

Current treatments for hemorrhagic shock can cause reperfusion injury and organ damage, making novel treatments critical for improved survival. Lesion of caudal serotonin neurons attenuates sympathetic recovery following hemorrhage and exacerbates metabolic acidosis and tissue hypoxia (Kung et al., 2010). These data suggest that during severe blood loss, serotonin neurons may be critical to the maintenance of peripheral tissue oxygenation during hypovolemia. Selective and discrete lesion of serotonin nerve terminals
within the portion of the NTS that receives afferent input from the cardiovascular system produces virtually the same disruption of ventilatory and sympathetic recovery as the more global caudal raphe lesion (Kung and Scrogin, 2011). However, serotonin neurons release multiple neurotransmitters in addition to serotonin, and multiple serotonin receptor subtypes are expressed in the NTS. It is not known which receptor subtype mediates the ability of endogenous serotonin to facilitate compensatory responses to blood loss. Parallel studies performed in the lab found that the selective 5-HT1A receptor agonist, 8-OH-DPAT, produced the most rapid and robust increase in sympathetic drive and blood pressure when administered into the 4th ventricle near NTS subnuclei that regulate cardiovascular control. Evidence that serotonergic innervation of this region is necessary for normal sympathetic and ventilatory responses to hemorrhage led us to investigate the possibility that endogenous serotonin acts on 5-HT1A receptors in the NTS to modulate sympathetic recovery following hemorrhage. Therefore, we utilized a short hairpin RNA (shRNA) targeting the rat 5-HT1A receptor to enable discrete and selective down regulation of the rat 5-HT1A receptor. This methodology enabled us to determine whether endogenous activation of 5-HT1A receptors in the NTS was critical for normal compensation from blood loss in the unanesthetized rat.

**Experiments**

A) It was determined if decreased 5-HT1A receptor mRNA levels in the caudal NTS attenuated sympathetic drive during recovery from hypotensive hemorrhage.
B) It was determined if decreased 5-HT$_{1A}$ receptor mRNA levels in the caudal NTS augmented metabolic acidosis following hypotensive hemorrhage.

**Specific Aim 2:** To determine if the pressor and sympatho-excitative effects of 8-OH-DPAT observed in unanesthetized, hemorrhaged-rats is dependent upon 5-HT$_{1A}$ receptors in the caudal NTS.

**Rationale**

8-OH-DPAT accelerates sympathetic recovery, increases in venous tone and attenuates metabolic acidosis when given to rats subjected to hypotensive hemorrhage or hemorrhagic shock. The sympathoexcitatory effect of 8-OH-DPAT in hemorrhaged animals is severely attenuated after sinoaortic denervation, suggesting that sensory afferents of the sinoaortic nerve transmit information that attenuates the full extent of reflex responses to hypovolemia, and that 8-OH-DPAT unmask and accelerates the complete reflex response. The effects of 8-OH-DPAT are most robust when drug is administered into the 4th ventricle, near the NTS, where the arterial baroreceptor and chemoreceptor afferents project. However, 8-OH-DPAT has affinity for other receptors expressed in the NTS (e.g. 5-HT$_{7}$ receptors) so the exact location and receptor population which mediates the effects of 8-OH-DPAT are not known. Therefore, we determined if the compensatory responses that are accelerated by 8-OH-DPAT administration were dependent on 5-HT$_{1A}$ receptors located within the
caudal NTS. We used the same AAV vector described in Specific Aim 1 to selectively decrease 5-HT$_{1A}$ receptor expression in the caudal NTS to determine the role of these receptors in the sympathetic or pressor response to 8-OH-DPAT administration.

**Experiments**

A) It was determined if decreased 5-HT$_{1A}$ receptor mRNA in the caudal NTS reduced the sympathoexcitatory and pressor effects of 8-OH-DPAT systemic administration during hypotensive hemorrhage.

**Specific Aim 3:** To determine if acidosis facilitates peripheral chemoreflex responses and whether this effect is dependent on 5-HT$_{1A}$ receptors in the caudal NTS.

**Rationale**

The ability of 8-OH-DPAT to accelerate the compensatory responses to hemorrhage is dependent on the integrity of the sinoaortic nerves that carry baroreceptor and chemoreceptor input to the NTS. Loss of endogenous serotonin input in the NTS increases arterial baroreflex gain during hypotension but impairs peripheral chemoreceptor activation in response to chemical hypoxia. This led us to speculate that the ability of 8-OH-DPAT and endogenous serotonin to improve sympathetic recovery from hemorrhage likely depends on peripheral chemoreceptor activation rather than arterial baroreflex unloading. 5-HT$_{1A}$ receptors typically induce neuronal hyperpolarization. Thus, 8-OH-DPAT (and
endogenous serotonin) may disinhibit full expression of chemoreflex responses during hemorrhage by acting on 5-HT$_{1A}$ receptors in the NTS. We predicted that acidosis would promote serotonin release, which in turn, would facilitate peripheral chemoreflex response to hypoxia. Therefore, we examined the role of NTS 5-HT$_{1A}$ receptors on ventilatory responses to the simultaneous activation of central and peripheral chemoreceptors that is believed to occur during severe hemorrhage. In these studies, chemoreflex activation was induced by altering blood gases to simulate the hypoxia and acidosis that develops during hemorrhage, without altering blood volume. We utilized the same AAV vector as described in Aim 1 to determine if 5-HT$_{1A}$ receptors in the caudal NTS are necessary for augmentation of ventilatory responses to hypoxic hypercapnic gas exposure. In addition, we determined which serotonin cell populations of the caudal raphe were activated by the same hypoxic hypercapnic conditions.

**Experiments**

A) It was determined if decreased 5-HT$_{1A}$ receptor mRNA levels in the caudal NTS inhibits the ability of acidosis to augment the ventilatory response to hypoxia.

B) It was determined if immunoreactive serotonin neurons in the caudal raphe are activated by acidosis.
CHAPTER II

REVIEW OF RELATED LITERATURE

Current treatment for hemorrhagic shock

Severe trauma is the leading cause of death in children and young adults (1-44 years of age) in the United States as well as many other developed countries (Centers for Disease Control and Prevention, 2012). In the United States alone, this accounts for an average of 50,000 deaths per year and costs the government over 400 billion dollars annually (Corso et al., 2006). This monetary figure also accounts for the cost of lost productivity since traumatic injury results in 30% of all life years lost, more than cancer and heart disease combined (Centers for Disease Control and Prevention, 2012). Of those traumatic deaths, 30-56% are due directly to blood loss (Kauvar and Wade, 2005). Hemorrhage also is the most common cause of preventable death during combat and accounts for 50% of all combat casualties (Clifford, 2004). Severely injured trauma patients usually require the resources of a designated trauma center within an hour after injury in order to improve their chance of survival. However, nearly 45 million Americans still do not have immediate access to a trauma center, and morbidity and mortality remain high (Centers for Disease Control and Prevention, 2012).
The current treatment for hemorrhagic shock is primarily directed at resuscitation with fluid volume to maintain perfusion pressure of the vital organs. This consists of an immediate administration of volume expanders that are either crystalloids (saline, Ringer’s lactate, etc) or colloids (blood, blood substitutes, etc.) (Perel et al., 2013). While necessary to avoid hypoperfusion, aggressive fluid resuscitation can also lead to detrimental complications including hemodilution, endothelial damage, and coagulopathy (Cripps et al., 2013).

When volume resuscitation is unable to maintain proper organ perfusion pressure; patients may require the additional administration of vasoconstrictive agents such as catecholamines, including norepinephrine, dopamine, epinephrine, or non-catecholaminergic vasoactive drugs such as phenylephrine, and vasopressin (Hollenberg, 2011). Although these therapeutic interventions can increase vasoconstriction to maintain blood pressure (BP), they can also exacerbate ischemic reperfusion (IR) injury. During hypoperfusion, the peripheral tissues become hypoxic and subsequently undergo anaerobic metabolism. Byproducts of this process can form reactive oxygen species (ROS) upon the reintroduction of blood flow and oxygen. Reactive oxygen species can promote damage of the microvasculature and induce endothelial injury. The endothelium is important for maintaining the integrity of the vasculature, and with disruption of the endothelial layer, introduction of leukocytes and platelets into the vessels can promote inflammation leading to organ failure and death (Verma et al., 2002).
The inflammatory response to hemorrhage and resuscitation also leads to complications during recovery. Within 3 days post-hemorrhage, enhanced gene expression of the proinflammatory cytokines, IL-1β, TGF-β, TNF-α, IL-10, IL-6, and INF-γ develops (Shenkar et al., 1994). The increase of cytokine production correlates directly with acute lung injury following recovery from blood loss. The production of proinflammatory cytokines also promotes disseminated intravascular coagulopathy (DIC), a pathological condition of dysregulated coagulation cascade that can occur during hemorrhage. With administration of resuscitation fluids, coagulation factors become diluted and can lead to hypocoagulation, which is associated with improper clot and fibrin formation that can lead to mortality 24 hours after traumatic injury (Palmer and Martin, 2014). Thus, even with restoration of perfusion pressure, inflammation, endothelial damage, and coagulopathy can eventually lead to death several days after the initial traumatic injury. While advancements in treatment have been made, morbidity and mortality associated with blood loss remain high.

**Autonomic responses to hemorrhage**

Progressive blood loss elicits a complex, multiphasic response from the autonomic nervous system (Figure 1). During the initial stages of blood loss, BP is maintained primarily through the arterial baroreflex (Korner et al., 1990; Schadt and Ludbrook, 1991). Decreased BP reduces activation of stretch-sensitive mechanoreceptors which promotes sympathetically-mediated increases in
Figure 1. Progressive blood loss elicits four distinct phases of autonomic nervous system response. Arterial pressure (AP, top), heart rate, (HR, middle), and renal sympathetic nerve activity (RSNA, bottom) of an unanaesthetized rat subjected to continued blood withdrawal. From left to right, normotensive phase (white box; I), hypotensive phase (light gray-shaded box; II), secondary compensatory phase (medium gray-shaded box; III) and decompensation phase (dark gray-shaded box; IV).
vasoconstriction and reflex tachycardia that help to maintain organ perfusion with oxygenated blood.

After a significant amount of blood is lost (20-30% of total blood volume in humans or 15% total blood volume of a conscious rat), these compensatory responses suddenly abate, resulting in a dramatic decline in BP paralleled by a rapid fall in sympathetic drive (Hasser and Schadt, 1992; Ludbrook and Ventura, 1996; Schadt and Ludbrook, 1991). This drop in BP is reminiscent of a vasovagal syncopal or “fainting” and is thought to be evolutionarily beneficial due to a resulting increase in ventricular filling time and increased cardiac output (CO).

This transient loss of BP is reversed as a secondary sympathetic compensatory phase ensues, characterized by a slow, but even greater increase in sympathetic drive than observed in the initial sympathoexcitatory phase (Haljamae, 1984). During the syncopal-like phase of hemorrhage, when sympathetic drive is at its nadir, blood pools in the highly compliant venous vasculature. This ultimately “traps” the blood within the veins and limits return of venous blood flow back to the heart further decreasing CO. Therefore, when sympathetic drive is re-established, an increase in sympathetically-mediated venoconstriction remobilizes blood back to the heart where it can be redistributed to the arterial vasculature. The second sympathoexcitatory phase is accompanied by the simultaneous release of vasoconstrictive hormones that primarily constrict the arterial side of the vasculature because the majority of their
receptors reside in the arterial vasculature (Liu et al., 2003). Arterial vasoconstriction is less hemodynamically favorable since the majority of the blood is within the venous vasculature and further constriction of the arterial vasculature may actually exacerbate ischemia at the capillary beds (Mellander and Lewis, 1963).

If blood loss progresses, the secondary increase in sympathetic drive eventually wanes, and the subsequent decrease in blood flow to the brain further attenuates sympathetic drive. Also at this time, the vasculature becomes more permeable and less responsive to catecholamines (Liu et al., 2003). Increased permeability of the vasculature disrupts the osmotic gradient and allows fluid and plasma proteins to leak out from the interstitium and augments inflammation by promoting the release of lymphatic molecules like macrophages and leukocytes. If left untreated, this forth stage eventually progresses to end organ failure and death. Patients that arrive to trauma centers during this final stage are very difficult to treat. Therefore, research efforts have focused on prolonging the previous stages of hemorrhage in order to prevent patients from advancing to decompensation.

**Overview of baro-, chemo- and cardiopulmonary reflexes during hypotensive hemorrhage**

The autonomic nervous system is responsible for insuring proper tissue oxygenation and organ perfusion pressure and does so utilizing a myriad of physiological reflexes. One such negative feedback system, termed the arterial
baroreflex, utilizes stretch-sensitive receptors (baroreceptors) to detect changes in pressure to modulate BP. When there is a rise in mean arterial pressure (MAP), detected as increased stretch, baroreceptors are activated and reduce sympathetic outflow to the peripheral vasculature and heart, thereby facilitating a subsequent fall in BP. Conversely, when BP is low, these receptors fire less frequently and subsequent increases in sympathetic drive and reflex tachycardia permit perfusion pressure to rise. The arterial baroreceptors are most sensitive in the physiological range of BP (70 – 150 mmHg) and possess a firing threshold of 60 mmHg. Thus, when BP is less than 60 mmHg, arterial baroreceptors are unable to further modulate perfusion pressure because the arterial vessels are fully dilated (Seagard et al., 1990). In addition to the arterial baroreceptors, low-pressure stretch receptors, termed cardiopulmonary baroreceptors, also respond to changes in pressure. Cardiopulmonary baroreceptors are located in large systemic veins, pulmonary vessels, and the atrium of the heart and are primarily responsible for regulating blood volume through hormone release (Mancia et al., 1986). Because the venous side of the vasculature is highly compliant, increases in stretch of the cardiopulmonary receptors are indicative of changes in blood volume since the majority of the blood is contained within the large veins. Elevations in blood volume are detected as an increase in stretch of the large veins. The subsequent activation of the cardiopulmonary baroreceptors causes cardiopulmonary afferents that project to the brain through the vagus nerve to initiate a reflex reduction in sympathetic nerve activity.
Chemoreceptors include both peripheral chemoreceptors and central chemoreceptors which modulate sympathetic activity and control ventilation. Both receptors groups are sensitive to changes in pCO$_2$ and pH, but only the peripheral chemoreceptors can detect changes in oxygen content in the peripheral circulation. Central chemoreceptors and peripheral chemoreceptors, which alter sympathetic drive and ventilation, are necessary to help maintain MAP and metabolic homeostasis.

**Anatomy of arterial baroreflex**

The primary arterial baroreceptors are specialized stretch receptors located on the aortic arch and the carotid sinuses near the bifurcation of the internal and external carotid arteries. As BP rises, the blood vessels passively expand and activate the arterial baroreceptors to generate an action potential. The action potentials are carried by afferent fibers from the aortic arch and carotid sinus to the caudal NTS by the vagus and glossopharyngeal nerves respectively (Berger, 1979; Ciriello, 1983). The release of excitatory amino acids, presumably glutamate, coincides with the activation of baro-sensitive second order neurons within the NTS to activate excitatory glutamatergic fibers that project to the caudal ventrolateral medulla (CVLM) (Lawrence and Jarrott, 1994; Talman et al., 1984).

The second order neurons in the NTS project to the CVLM and release glutamate to activate inhibitory neurons that release γ-aminobutyric acid (GABA)
to inhibit the rostroventrolateral medulla (RVLM) and suppress tonic sympathetic
drive. It is important to note that these inhibitory neurons within the CVLM are
tonically active and continuously suppress sympathetic drive without
baroreceptor input.

The GABAergic afferents from the CVLM extend short axons bilaterally
just rostral to the RVLM to suppress activity of bulbospinal presympathetic
excitatory neurons that primarily control tonic sympathetic activity. The
bulbospinal neurons have glutamatergic projections to sympathetic preganglionic
neurons located in the intermediolateral nucleus (IML) of the spinal cord
(Pilowsky and Goodchild, 2002). Therefore, when BP increases, the aortic arch
and carotid sinuses distend to increase action potential generation by the arterial
baroreceptors. This in turn, activates second order neurons in the caudal NTS
which activate the CVLM to increase inhibition of the RVLM. Decreased activity
in the RVLM results in decreased sympathetic drive, reduces HR, and ultimately
decreases BP (Figure 2). The NTS also contains excitatory projections to the
nucleus ambiguous (NA) which regulates parasympathetic outflow to the heart.
Activation of the NA promotes vagal stimulation to the heart to reduce HR.
Conversely, when BP decreases, the baroreceptors become less active and the
RVLM becomes disinhibited, which augments sympathetic drive and tachycardia
to increase BP.
Figure 2. Neural pathways of the arterial baroreflex. Sagittal view of a rat brain illustrating projections involved in the arterial baroreflex (Dampney et al., 2002). Arterial baroreceptors generate action potentials in response to increases in BP and activate second order neurons within the NTS. Glutamatergic input to the GABAergic neurons in the CVLM facilitates inhibition of the bulbospinal neurons in the RVLM. This ultimately reduces activation of the sympathetic preganglionic fibers in the IML and suppresses sympathetic outflow. Therefore, activation of the arterial baroreflex attenuates tonic sympathetic activity to reduce vasoconstriction and HR.
Physiological role of the arterial baroreflex and hypotensive hemorrhage

Arterial blood pressure is tightly regulated and maintained (between 85-100 mmHg in humans) to ensure proper oxygenation and nutrient delivery to the vital organs as well as provide waste removal. Arterial baroreceptors are key players in maintaining MAP within this narrow range. The carotid sinus baroreceptors are more sensitive than the baroreceptors located in the aortic arch, due to the larger pressure within the aortic vessel compared to the carotid arteries. Therefore, maximal carotid sinus sensitivity occurs between 70-150 mmHg. Thus, minute to minute variations in pressure effectively alter the firing rate of the baroreceptors which, in turn, maintains BP within a physiological range. In contrast, aortic baroreceptors detect changes in BP at a higher pressure range (100-200 mmHg). It is important to note that the point at which BP is maintained or, “set point,” changes during exercise, chronic hypertension, and heart failure. In addition to changes in pressure, arterial baroreceptors can also detect changes in pulse pressure.

During hemorrhage, the baroreceptors are unloaded as blood is lost and pressure wanes. This results in less activation of the baro-sensitive neurons in the NTS that facilitate inhibition of tonic sympathetic activity. Therefore, excitatory bulbospinal neurons within the RVLM are disinhibited and promote a robust increase in sympathetic drive and tachycardia to maintain BP. Additionally, during blood loss, there is a decrease in pulse pressure. The combination of reduced BP and pulse pressure in hemorrhagic shock results in
an even greater response from the baroreceptors. Vagal activity is also reduced to alleviate parasympathetic activation and increase BP (Wehrwein and Joyner, 2013).

**Anatomy of the peripheral chemoreflex**

Peripheral chemoreceptors are composed of specialized cells strategically located in carotid bodies in the carotid sinus and the aortic bodies within the aortic arch. Carotid bodies are composed of two main cell types, type I or glomus cells are the excitable cells that can directly detect changes in pH, CO₂, and especially O₂ and generate action potentials. Additionally, the carotid bodies contain type II cells (15-20% of the total cell population), which have long processes that physically wrap around the glomus cells and play a more supportive role, similar to glial cells (Lopez-Barneo et al., 2008). Although the exact mechanism by which glomus cells detect changes in oxygen remains to be fully elucidated, the leading hypotheses include the metabolic hypothesis and the membrane hypothesis. First, the metabolic hypothesis suggests that the glomus cells respond to even slight changes in O₂ content because oxygen-sensing proteins within the cytoplasm or mitochondria of the cells become activated during decreases in cellular O₂. During hypoxia, heme-containing proteins, such as cytochrome oxidase, prevent potassium (K⁺) channels from opening. The subsequent reduction of K⁺ influx promotes membrane depolarization, activation of voltage gated calcium (Ca²⁺) channels and ultimately, neurotransmitter
release. Alternatively, the membrane hypothesis proposes that certain K\(^+\) channels, such as high conductance Ca\(^{2+}\) activated channels and low conductance Ca\(^{2+}\) insensitive channels, are able to directly detect changes in O\(_2\) content to activate influx of extracellular Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels (Lopez-Barneo et al., 1988; Peers, 1990). Whether indirectly or directly activated, the influx of Ca\(^{2+}\) promotes membrane depolarization to facilitate the final step in oxygen sensing, and neurotransmitter release.

While numerous neurotransmitters have been associated with peripheral chemoreceptor activation, the most prevalent active neurotransmitters released in the synapse are acetylcholine and adenosine triphosphate (ATP). Upon Ca\(^{2+}\) influx, neurotransmitters are released from the glomus cell into the synapse and activate the afferent nerve fibers that project to the medulla. Additionally, the carotid body is a highly dopaminergic structure, and it is thought that dopamine (DA) is released in an autocrine fashion to reduce activation of the carotid body in a negative feedback loop (Lopez-Barneo et al., 2008).

Chemoreceptor afferent fibers then relay this information through the carotid sinus and glossopharyngeal nerves that synapse in a more caudal portion of the NTS, separate from where baroreceptor afferents project in the commissural NTS (Figure 3). These sensory afferents release excitatory amino acid neurotransmitters, such as glutamate, to facilitate direct activation of the RVLM to increase sympathetic drive. Additionally, the NTS promotes increases
Figure 3. Neural pathways of the peripheral chemoreceptors. Sagittal image of a rat brain illustrating projections involved in the peripheral chemoreceptor activation (Dampney et al., 2002). Peripheral chemoreceptors in the carotid and aortic bodies are activated by pH, CO₂ and decreased O₂ (hypoxia). Activation of the peripheral chemoreceptors stimulate second order neurons in the caudal NTS. Excitatory chemo-sensitive neurons in the NTS directly activate excitatory input into the RVLM to stimulate sympathetic drive. Additionally, activation of the peripheral chemoreceptors increases activity in the rVRG to enhance ventilation by downstream stimulation of the phrenic motor nucleus (PMN). Therefore, activation of the peripheral chemoreflex augments tonic sympathetic activity and facilitates ventilation.
Physiological role of the peripheral chemoreflex and hypotensive hemorrhage

Although peripheral chemoreceptors become activated by increases in CO₂ and decreases in pH; they are primarily activated by hypoxia. Glomus cells within the carotid body can be activated either indirectly by O₂-sensitive proteins or directly by K⁺ channels to promote membrane depolarization. Depolarization causes Ca²⁺ influx through voltage dependent Ca²⁺ channels, which facilitate the release of excitatory amino acid neurotransmitters that excite the sensory afferents that project to second order neurons in the NTS. Upon activation of the NTS, direct projections from the NTS to the RVLM augment sympathetically mediated increases in vasoconstriction. Additionally, ventilation is increased by activation of neurons of the phrenic motor nucleus in the cervical spinal cord. Furthermore, peripheral chemoreceptor activation facilitates coronary vasodilation and bradycardia through vagal activation of the parasympathetic system. Thus, the traditional peripheral chemoreflex response is associated with increased sympathetically mediated vasoconstriction, increased ventilation, and
bradycardia. The carotid bodies are a highly vascularized structure that receive an enormous amount of blood flow (10 times the flow per unit mass compared to the rest of the cerebral circulation), but have a relatively low metabolic demand (1-2 ml/100g/min O₂ consumption) (Barnett et al., 1988; De Burgh Daly et al., 1954; Obeso et al., 1989). Therefore, during hemorrhage, the reduced blood flow past the carotid bodies is sensed as reduced blood oxygen content and promotes the peripheral chemoreflex to boost sympathetic drive and increase ventilation to ameliorate reductions in BP. During hemorrhage, bradycardia is masked by input from the arterial baroreceptors that suppress vagal input to the heart.

**Peripheral chemoreceptor and baroreceptor interactions**

Peripheral chemoreceptor activation results in increased ventilation, exaggerated vasoconstriction, and varying effects on HR. In contrast, activation of the arterial baroreflex elicits vasodilation, bradycardia, and reduced BP. Since baroreceptors and chemoreceptors have opposing effects on ventilation and sympathetic nerve discharge, it is plausible that these reflexes antagonize each other. Specifically, there is evidence that elevated BP associated with arterial baroreceptor activation attenuates the sympathetic and ventilatory responses that result during peripheral chemoreceptor activation. Conversely, peripheral chemoreceptor activation is potentiated by hypotension (Heistad et al., 1974; Mifflin, 1993).
Central chemoreceptors

In contrast to peripheral chemoreceptors, central chemoreceptors are cells located in the brainstem that are exclusively sensitive to changes in CO$_2$ and H$^+$ ion content. Activation of central chemoreceptors facilitates sympathoexcitation and augments ventilation during respiratory or metabolic acidosis via projections to the neural circuitry in the brainstem and spinal cord involved in sympathetic and ventilatory drive.

The partial pressure of arterial blood CO$_2$ content (PaCO$_2$) is tightly controlled and maintained near 40 mmHg in humans. The pH of the arterial blood is closely regulated and typically ranges between 7.35-7.45. Even slight disturbances in the cellular environment produce dramatic alterations in ventilation in order to maintain homeostasis. In humans, ventilation can increase by as much as 50% in response to slight increases in PaCO$_2$ (1.5 mmHg) or decreases in pH (0.01) (Smith et al., 1988). Increases in CO$_2$ and H$^+$ ions promote a robust increase in ventilation, which buffers the blood by causing the release of CO$_2$ in the lungs. This occurs because bicarbonate (HCO$_3^-$) and H$^+$ ions are quickly converted by carbonic anhydrase (CA) into water and CO$_2$. Moreover, CO$_2$ is easily able to permeate lipid membranes, unlike H$^+$ ions, which are unable to diffuse across the blood-brain barrier (BBB). Thus, H$^+$ ions require a transport mechanism, such as the Na$^+$/H$^+$ exchanger to relay plasma pH information to the brain, but this process takes significantly longer than CO$_2$ diffusion (Adler et al., 1990). Alternatively, CO$_2$ can readily cross the BBB and be
converted back into H+ ions to acidify brain tissues and activate the central chemoreceptors (Figure 4).

Besides possessing the intrinsic ability to detect changes in CO₂/H⁺ ions, the central chemoreceptors also must possess a direct connection to the respiratory central pattern generator (CPG) to induce increased ventilation in response to increased demand for buffer. The central pattern generator refers to the semi-autonomous neural network within the pons and medulla that drives ventilatory function. The CPG contains the ventral respiratory group (VRG) composed of a bilateral group of inspiratory and expiratory neurons that have been divided into the rostral ventral respiratory group (rVRG), caudal ventral respiratory group (cVRG), Pre- Bötzinger complex (PreBötC) and Bötzinger complex (BötC). Activity of the VRG is regulated by afferent input from the NTS and pontine nuclei (Kölliker-Fuse nucleus and parabrachial nucleus) that regulate phase transitions between inspiration and expiration (Abdala et al., 2009; Alheid and McCrimmon, 2008; Smith et al., 2009; Smith et al., 2013). Behavioral responses also influence the CPG through input from the cortex, cerebellum, basal ganglia, and hypothalamus (Pattinson et al., 2009a; Pattinson et al., 2009b). The VRG contains projections to motor neurons that control the upper airway muscle and innervate the diaphragm and thoracic respiratory muscles.
Figure 4. Schematic representation of how CO₂ and H⁺ ions in plasma activate central chemoreceptors. CO₂ is able to readily cross the blood brain barrier (BBB). Conversely, H⁺ ions require the aid of a transporter, such as the Na+/H⁺ exchanger to cross the BBB more slowly. During hemorrhage, when peripheral tissue becomes hypoxic and undergoes anaerobic metabolism, H⁺ ions can be converted in the blood by carbonic anhydrase to CO₂, which can quickly cross the BBB and be converted back into H⁺ ions to acidify brain tissue and activate the central chemoreceptors. Simultaneously, ventilatory drive increases such that most of the H⁺ is blown off in the lungs as CO₂. The remaining H⁺ accumulates in the blood which eventually perfuses the brain. Thus, during blood loss, central chemoreceptors would presumably be activated more so by the local metabolic production of H⁺ and H⁺ transport, rather than PaCO₂ which remains low during the hyperventilatory response to hemorrhage.
During homeostasis, inspiratory drive is initiated in the PreBötc and facilitated by the rVRG. Inhibitory GABAergic and glycinergic interneurons from PreBötc suppress expiratory drive from the Bötc and cVRG. The PreBötc also provides excitatory glutamatergic input to the rVRG. The rVRG contains the main cluster of bulbospinal premotor neurons that relay inspiratory drive to the phrenic motor neurons that innervate the diaphragm to control and initiate inspiration (Smith et al., 1991). Bulbospinal neurons in the rVRG are also inhibited by interneurons in the cVRG and Bötc regions. Conversely, expiration is driven by Bötc through the inhibition of PreBötc and rVRG. The Bötc activates cVRG, which contains bulbospinal expiratory neurons that excite thoracic and lumbar motor neurons to facilitate expiration by engaging the abdominal muscles. Although the rhythmic drive to motor neurons can persist without sensory feedback; these respiratory networks are heavily influenced by central chemoreceptors.

The exact location of the central chemoreceptors is not entirely clear and remains a topic of debate. Central chemoreceptors were thought to be confined to the ventrolateral surface of the medulla because direct application of acidic solutions to the CVLM and RVLM augmented ventilation while selective blockade of neurotransmission in these regions via lesion, cooling, or chemicals reduced the ventilatory and sympathetic responses to hypercapnia (Hanna et al., 1979). More recently, additional sites of central chemosensation within the brainstem have been identified. These include the NTS (Dean et al., 1990), locus coeruleus (Pineda and Aghajanian, 1997), serotonin neurons within the
medullary raphe (Wang et al., 1998), glutamatergic neurons in the retrotrapezoid nucleus (RTN) (Mulkey et al., 2004), and orexinergic neurons in the hypothalamus (Williams et al., 2007). Although several potential central chemoreceptor sites have been investigated, attention has been primarily focused on the serotonergic neurons in the medullary raphe and glutamatergic neurons of the RTN. The evidence supporting the involvement of serotoninergic neurons of the medullary raphe in the central chemoreflex will be explored further in the section entitled “serotonin and central chemoreception,” located on page 64.

The RTN is a nucleus located near the ventral surface of the rostral medulla and is known to mediate tonic ventilatory drive, but is also thought to modulate ventilation in response to changes in CO$_2$ (Mulkey et al., 2004). Within the RTN exists a bilateral cluster of glutamatergic, non-purinergic neurons (~1000 neurons total) that can be identified by their unique expression of the transcription factor, paired-like homobox2b (Phox2b). This transcription factor is known to regulate neuron formation and differentiation in brain regions important to cardiorespiratory function (Dauger et al., 2003; Tiveron et al., 2003). Subsequently, mutations within the Phox2b transcription factor gene result in autonomic dysfunction and are linked to congenital central hypoventilation syndrome. Moreover, mice that lack Phox2b neurons within the RTN experience pronounced hypoventilation and reduced chemoreflex responses (Dubreuil et al., 2008).
The small subset of neurons proposed to be central chemoreceptors in the RTN have been extensively studied \textit{in vitro} and \textit{in vivo} over the last decade. Brainstem slice recordings have shown that these particular neurons are stimulated by acidification and inactivate when pH is increased (Lazarenko et al., 2009; Mulkey et al., 2004; Stornetta et al., 2006). Additionally, focal acidification of the RTN via microdialysis produced a 25\% increase in ventilation in awake rats (Li et al., 1999). Moreover, rats exposed to increasing concentrations of CO$_2$ gas were found to have increased c-Fos expression in the RTN indicating that these neurons are activated during hypercapnia (Sato et al., 1992). Recently, a more selective activation of these neurons was found to promote increases in ventilation \textit{in vivo}. Studies by Abbott et al. utilized a lentiviral delivery system to express light-activated cation channel, Channel Rhodopsin 2 (ChR2) in RTN neurons in rats. Channel expression was driven by a Phox2-responsive promoter enabling the use of photostimulation to selectively activate only the neurons within the RTN that expressed Phox2b. Results of these experiments concluded that direct excitation of these neurons augments breathing frequency and amplitude \textit{in vivo} (Abbott et al., 2009).

The RTN also projects to the VRG to facilitate ventilation directly. Tract tracing methods have elucidated connections of the RTN to the ventrolateral medulla, caudal and lateral portions of the NTS, and the dorsolateral pons. Specifically, the RTN has direct projections to regions associated with the CPG including the PreBötC (Rosin et al., 2006). If glutamatergic neurons within the RTN are intrinsically sensitive to changes in CO$_2$/H$^+$, then they are also able to
initiate alterations in ventilation directly through these connections to maintain PaCO$_2$ within a normal physiological range. Interestingly, the RTN receives synaptic input from other putative chemoreceptors located in the medullary raphe and NTS (Takakura et al., 2006). Therefore, it is possible that additional/alternative central chemoreceptors, such as the serotonergic neurons in the medullary raphe, and/or input from cells in the NTS that are responsive to peripheral chemoreceptor activation facilitate RTN central chemosensitivity in vivo.

**Nucleus tractus solitarius**

The nucleus tractus solitarius (NTS) is an important sensory integration center located in the dorsal medulla within the brainstem. The NTS is the initial site to which a multitude of sensory afferents, including the arterial baroreceptors, project. The NTS is involved in a vast range of autonomic functions from sensations of tastes and intestinal mobility to cardiovascular and ventilatory function. In general, the NTS contains important second order neurons that receive sensory inputs critical in initiating the autonomic responses that maintain cardiorespiratory homeostasis in the face of pathological or environmental changes.
Anatomical features of the NTS

The NTS is composed of multiple subnuclei that are orientated rostro-caudally along the solitary tract. The solitary tract is the bundle of cranial nerves (facial, glossopharyngeal, and vagus) that carry visceral sensory information to the CNS (Berger, 1979; Ciriello, 1983; Mifflin, 1992). Therefore, sensory information is organized in this rostro-caudal orientation such that the more rostral regions convey information from gustatory and somatic processes, while the more caudal region receives input from cardiovascular, renal, digestive and respiratory organs. The more caudal portion of the NTS is known to be involved in compensation from hypotensive hemorrhage and will be the primary focus of this section.

As previously mentioned, the NTS receives afferent projections from the arterial baroreceptors and peripheral chemoreceptors. The arterial baroreceptors project to the more medial portion of the NTS to modulate sympathoexcitation via projections to the CVLM. Additionally, the peripheral chemoreceptors project to the more caudal regions of the NTS to modulate cardiorespiratory parameters in response to changes in O₂ through direct projections to the RVLM and VRG (see section, overview of baro-, chemo- and cardiopulmonary reflexes during hypotensive hemorrhage on page 20).

Additionally, the NTS can modulate cardiac function through glutamatergic projections to cardiac vagal pre-ganglionic neurons (CVPN) located in the dorsal motor nucleus of the vagus (DMN) and NA. Activation of
CVPNs initiate negative chronotropic, ionotropic, and dromotropic effects depending on their origin. Specifically, CVPNs located in the DMN have negative dromotropic, ionotropic and chronotropic effects on the heart (Nosaka et al., 1982; Stuesse, 1982; Takanaga et al., 2003). Interestingly, DMN CVPNs do not appear to be modulated by arterial baroreceptor input, unlike CVPNs located in the NA. The CVPNs of the NA facilitate bradycardia in response to arterial pulse pressure changes and are reduced during the inspiratory phase of ventilation.

The NTS also projects to the paraventricular nucleus of the hypothalamus (PVN). The PVN is an integration center for autonomic and endocrine function that is particularly important in maintaining cardiovascular homeostasis. The PVN is known to regulate BP in response to stress, primarily through activation of the hypothalamic-pituitary-adrenal (HPA) axis (Badoer and Merolli, 1998; Herman et al., 1996). The PVN also has direct projections to regions important for cardiorespiratory responses to hemorrhage, including the RVLM, spinal cord, phrenic motor nucleus, PreBötC, and the dorsomedial, medial, and commissural portions of the NTS (Affleck et al., 2012; Kc et al., 2002a; Saper et al., 1976).

Interestingly, exposure to hypoxia enhances c-Fos expression in NTS neurons that project to the PVN, and these projections were found to be mostly catecholaminergic (King et al., 2012). Hypotensive hemorrhage and hypercapnia have also been shown to increase activation of the PVN (Badoer and Merolli, 1998; Kc et al., 2002b). Moreover, lesion of the PVN has been shown to block the pressor response to chemical hypoxia in the unanesthetized rat (Olivan et al., 2001). Based on these findings, it appears that the PVN plays a pivotal role in
peripheral and/or central chemoreceptor activation, possibly through the input received from the NTS.

Additionally, NTS provides direct catecholaminergic and peptidergic projections to the central nucleus of the amygdala (CeA), a subnucleus of the amygdala found to be important in the modulation of BP during exposure to fearful stimuli (Zardetto-Smith and Gray, 1990). In turn, the CeA provides inhibitory GABAergic projections that synapse onto second order baroreceptor neurons within the NTS. Thus, CeA activation in response to fear/anxiety can attenuate arterial baroreceptor activation to promote increases in BP (Saha, 2005). The CeA also modulates BP during stress via direct projections to C1 adrenergic regions in the RVLM that promote sympathoexcitation (Saha et al., 2005).

As mentioned previously, the NTS can augment ventilation through direct projections to the VRG. Additionally, the NTS projects to pontine nuclei to modulate ventilation. The pontine ventilator nuclei are located in the parabrachial area at the junction between the midbrain and the pons and are composed of three regions: the Kölliker-Fuse nucleus (KFN), lateral parabrachial nucleus (LPBN) and the medial parabrachial nucleus (MPBN). Both the KFN and the LPBN receive projections from the caudal NTS and are involved in cardiorespiratory functions (Herbert et al., 1990). Lesion of either the KFN or LPBN has no effect on normal breathing in room air, but elicits an impaired ventilatory response to hypoxia and hypercapnia in the unanesthetized rat. Interestingly, KFN and LPBN lesions also produce altered behavior of the rats
during hypercapnic gas exposure. Specifically, control animals were observed to spend more time sniffing, moving, gasping, and generally were more active than animals subjected to KFN and LPBN lesion (Mizusawa et al., 1995). Since the ventilatory response in either region has been shown to be heavily impaired by anesthetics, it appears as though consciousness and higher brain function is important in the ventilatory response to changes in CO$_2$. Moreover, consciousness may be necessary for behavioral responses to sensory information generated by changes in blood gases that are integrated within the NTS.

Neurotransmission in the NTS

As mentioned previously, the more caudal portion of the NTS receives input from the arterial baroreceptor and the peripheral chemoreceptor afferents carried by the solitary tract. Neurotransmitter release from these afferent projections modulate sympathetic drive and ventilation. Although it is known that an excitatory amino acid neurotransmitter is released from these afferents, numerous additional neurotransmitters, gases, and peptides are also thought to be involved in modulating the arterial baroreflex and peripheral chemoreflex responses.

Many investigators have found evidence that glutamate acts as the primary excitatory amino acid neurotransmitter released from the baroreceptor afferents to activate second order neurons within the NTS. Microinjection of the
NMDA glutamate receptor antagonist, (2R)-amino-5-phosphonovaleric acid (AP-5) into the NTS of conscious rats did not change baseline BP but did reduce baroreflex gain in response to phenylephrine-induced hypertension (Machado, 2001; Machado and Bonagamba, 1992). The utilization of alternative glutamate receptor antagonists that were specific for metabotropic glutamate receptors or non-NMDA glutamate receptors (AMPA or Kainate receptors) ultimately revealed that glutamate had varying effects on BP depending on the specific glutamate receptor population that was manipulated and whether the animal was anesthetized. In general, glutamate neurotransmission modulates beat-to-beat BP control through a non-NMDA glutamate receptor population, whereas activation of the arterial baroreceptors by larger changes in BP facilitate glutamate binding of the NMDA glutamate receptors.

Similarly, Substance P (SP) has been identified as one of the main neurotransmitters released in the NTS in response to baroreceptor activation (Helke et al., 1980). Aortic nerve afferent fibers release SP in the NTS in response to increased BP (Potts and Fuchs, 2001). Moreover, NTS microinjections of exogenous SP or SP antagonist were found to enhance and blunt the arterial baroreflex respectively, but only in response to loading (activation) of the baroreceptors (Seagard et al., 2000). Selective destruction of cells in the NTS that express the primary SP receptor, neurokinin-1 (NK-1), using a SP-conjugated saponin toxin, impaired baroreflex sensitivity (Riley et al., 2002). Thus, SP also appears to contribute to arterial baroreceptor activation through NK-1 receptors in the NTS.
Retrograde tracing studies have also identified a population of inhibitory interneurons in the NTS that are activated by the arterial baroreflex and project to the CVLM to modulate RVLM activity. These neurons are located more rostrally and are distinct from the interneurons in the NTS that are tonically active. At least a portion of these baro-sensitive interneurons are GABAergic (Izzo et al., 1992). Several investigators have studied the role of these GABAergic neurons in the NTS and found that their selective activation facilitates the pressor effect associated with inhibition of the arterial baroreflex (Sved and Sved, 1990). Similarly, this response was reversed with direct injection of an antagonist to the GABA\(_A\) receptors, gabazine (Zubcevic and Potts, 2010). Therefore, GABA appears to be the primary neurotransmitter involved in baroreflex inhibition.

Glutamate is also thought to be the primary excitatory amino acid released in the NTS to facilitate ventilatory and BP effects during peripheral chemoreceptor activation. Peripheral chemoreceptors are also activated by systemic KCN injection that elicits a transient hypoxic state in the carotid body cells. Consequently, increased ventilation and BP, as well as bradycardia, result in response to simultaneous activation of sympathetic and parasympathetic systems. Similar responses in conscious rats have been observed following microinjections of L-Glutamate directly into the NTS (Colombari et al., 1998). It appears that glutamate modulation of HR develops through NMDA glutamate receptor activation since direct microinjection the NMDA receptor glutamate antagonist, AP-5, eliminates the bradycardia associated with peripheral chemoreceptor activation (Accorsi-Mendonca et al., 2009).
The pressor effect of peripheral chemoreceptor activation is also dependent on glutamate neurotransmission, but this process is thought to be mediated through a non-NMDA ionotropic glutamate receptor population in the NTS. When Machado et al. microinjected the non-selective AMPA and Kainate antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX), in the NTS and administered KCN to activate the peripheral chemoreceptors, there was only a partial attenuation of the pressor effect associated with peripheral chemoreceptor activation. This suggests that an alternative glutamate receptor subtype or another excitatory neurotransmitter besides glutamate is also involved in the pressor response to peripheral chemoreceptor activation (Machado and Bonagamba, 1992).

Interestingly, separate studies found that NTS microinjections of ATP mimicked peripheral chemoreceptor activation, similar to results seen with direct injection of L-Glutamate (de Paula et al., 2004). Moreover, when the non-selective purinergic receptor antagonist, suramin, was administered, a significant attenuation of the bradycardiac response to chemical hypoxia was observed (Paton et al., 2002). More recent studies microinjected both a non-selective ionotropic glutamate receptor antagonist, kynurenic acid, and purinergic receptor P2X2 antagonist, pyridoxal phosphate-6-azophrenyl-2',4'-disulfonic acid (PPADS), during chemical hypoxia. The result was significantly impaired sympathoxcitatory and pressor responses to peripheral chemoreceptor activation. In summary, NTS microinjection studies suggest that ATP and glutamate may be co-transmitters involved in peripheral chemoreceptor activation in the NTS. It
appears that glutamate, and possibly ATP, facilitate the pressor effect of peripheral chemoreflex activation through increases in sympathetic drive via non-NMDA and purinergic receptors. Additionally, it is likely that glutamate binds NMDA receptors in the NTS to mediate the bradycardic component of peripheral chemoreceptor activation through subsequent cardiac vagal activation.

Increased sympathetic and ventilatory drive during peripheral chemoreceptor activation can be modulated by a myriad of neurotransmitters. Noradrenergic structures in the brain, including the A1 and A2 regions of the medulla, show increased c-Fos expression in response to chemical hypoxia and activation of the carotid sinus nerve (Buller et al., 1999; Erickson and Millhorn, 1994; Smith et al., 1995; Teppema et al., 1997). The role of norepinephrine (NE) in peripheral chemoreceptor activation was further supported by in vivo studies that found excitation or inhibition of these same regions could modulate cardiorespiratory responses to peripheral chemoreceptor activation (Hayward, 2001; Perez et al., 1998). Anatomically, the caudal NTS projects to other central noradrenergic structures, and noradrenergic cells identified in the NTS make up the A2 nucleus (Dahlstrom and Fuxe, 1964; Jacobowitz and Palkovits, 1974). Microinjection of NE into the caudal NTS produces a dose-dependent decrease in the pressor response to KCN injection in awake rats, suggesting that NE exerts an inhibitory effect on the pressor/sympathoexcitatory response to peripheral chemoreceptor activation (Silva de Oliveira et al., 2007). Both $\alpha_1$ and $\alpha_2$ adrenergic receptors are expressed in the NTS, but the expression of $\alpha_1$ adrenergic receptors is more prevalent in the medial NTS, while $\alpha_2$ adrenergic
receptors are expressed primarily in the more caudal portions of the caudal NTS (Young 1985). Microinjection of the selective $\alpha_1$AR agonists, phenylephrine or methoxamine, near the medial NTS elicits a dose-dependent increase in BP and HR. In contrast, dose-dependent decreases in BP and HR are produced by microinjection of $\alpha_2$AR agonist, clonidine, in the same region (Kubo et al., 1987; Tsukamoto et al., 2002; Zandberg et al., 1979). Similar results are seen with microinjection of clonidine in the commissural NTS of rats. Moreover, the effects of clonidine can be reversed by microinjection of $\alpha_2$AR antagonist yohimbine (Bhuiyan et al., 2009). In general, $\alpha_1$- and $\alpha_2$-adrenergic receptors are thought to have an inhibitory/modulatory role on both the arterial baroreflex and peripheral chemoreflex.

It is also important to note that the physiological response to hypoxia-induced peripheral chemoreceptor activation is biphasic. The initial augmentation of ventilation is followed by a decline or “roll off” in ventilation termed hypoxic ventilatory decline (HVD). It is likely that glutamate contributes to the immediate increase in ventilation during hypoxia exposure, but the mechanism by which HVD occurs remains to be fully elucidated. One neurotransmitter thought to be involved in HVD is GABA. The NTS receives GABAergic innervation from other brain regions, such as the PVN; but the major source of GABA in the NTS is from interneurons. Most GABAergic interneurons are excited by second order neurons that become activated by afferent fibers from the solitary tract. Consequently, GABA’s role in the inhibition of the NTS is, to some degree, regulated by excitatory drive. It has been proposed that with
prolonged peripheral chemoreceptor activation, and thus increased glutamate accumulation, GABA synthesis increases to facilitate HVD. Data from microdialysis studies of Tabata et al. showed that rats exposed to sustained hypoxia had significantly elevated GABA concentrations in the NTS 15 minutes after the start of gas exposure. Animals in which the carotid bodies have been denervated do not accumulate GABA upon exposure to hypoxia. Therefore, it is plausible that with prolonged peripheral chemoreceptor activation GABA levels increase, and, as a consequence, ventilatory drive becomes suppressed.

Separate studies have found that increased GABA concentrations in the commissural NTS correlates with impaired peripheral chemoreceptor activation. Specifically, microinjection of the GABA reuptake inhibitor, nipecotic acid, reduces the ventilatory, BP, and sympathetic responses to peripheral chemoreceptor activation. Furthermore, this effect is reversed by GABA receptors antagonists, bicuculline. Similar to the results seen with blockade of GABA reuptake, microinjection of GABA receptor agonists, muscimol, attenuates the autonomic responses to peripheral chemoreceptor activation. Together, these studies provide significant evidence that GABA neurotransmission is facilitated by excitatory second order neurons in the NTS to reduce the peripheral chemoreflex response during sustained hypoxia.

Another neurotransmitter that may contribute to HVD, is DA. It has been well established that glomus cells of the carotid bodies are highly dopaminergic structures that utilize DA release to inhibit peripheral chemoreceptor activation in an autocrine fashion (Gonzalez et al., 1994; Prabhakar, 1994). Additionally, DA
is found in peripheral chemoreceptor afferent fibers that terminate in the NTS. Like GABA, DA has also been shown to accumulate in the NTS during sustained hypoxia, and its accumulation is dependent on intact carotid sinus nerves (Goiny 1991). Dopamine is thought to activate D₂-like receptors in the caudal NTS (Qian et al., 1997; Yokoyama et al., 1994). The D₂ receptors are inhibitory and have been co-localized within the NTS with synaptophysin, a presynaptic vesicle protein. Thus, DA release in the NTS can attenuate excitatory glutamatergic synaptic transmission via the activation of presynaptic D₂ receptors located in the caudal NTS. Furthermore, DA release during sustained hypoxia may enhance or coincide with HVD.

Previous studies from our laboratory and others have found that serotonin also plays a critical role in the physiological responses to peripheral chemoreceptor activation. The NTS receives extensive serotonin innervation from the nodose ganglion as well as the caudal raphe nuclei (Nosjean et al., 1990; Thor and Helke, 1987a). Earlier studies found that serotonin turnover was increased in the NTS of rats that received systemic injection of phenylephrine, and that this effect was abolished by sinoaortic denervation (Bhaskaran and Freed, 1988). It was also previously shown that low dose microinjection of serotonin in mediocaudal and commissural NTS produced cardiovascular responses typically associated with baroreceptor activation, specifically decreased HR and BP. In a study from Orer et al., the selective serotonin neurotoxin, 5,7-DHT, was utilized to lesion serotonin nerve terminals in the NTS. They showed a moderate, but significant increase in BP in unanesthetized, freely
moving rats (Orer et al., 1991). Previous studies from our laboratory utilized the same neurotoxin to selectively destroy the serotonergic nerve terminals in the dorsomedial medulla (including the NTS) in rats. Loss of serotonin innervation in the NTS enhanced arterial baroreceptor reflex gain but reduced the sympathetic and ventilatory response to peripheral chemoreceptor activation by systemic KCN injection (Kung and Scrogin, 2011). Although several serotonin receptor subtypes have been identified in the NTS, 5-HT$_{1A}$, 5-HT$_{2}$, and 5-HT$_{3}$ receptors have been the most extensively studied to date. In general, that 5-HT$_{2}$ and 5-HT$_{1A}$ receptors in the NTS are thought to mediate the depressor effect of arterial baroreflex activation, while 5-HT$_{3}$ receptors located on vagal afferent fibers facilitate hypertensive responses during baroreflex activation induced by systemic phenylephrine (Itoh et al., 1992; Itoh and Bunag, 1991; Merahi et al., 1992). Interestingly, partial and full 5-HT$_{1A}$ receptor agonists have been shown to increase ventilation in rats following spinal cord injury and in intact cats and rats, although the location of these effects remains obscure (Garner et al., 1989; Mendelson et al., 1990; Teng et al., 2003).

**Overview of central serotonergic system**

Serotonin, or 5-Hydroxytryptamine, is a monoamine neurotransmitter biochemically synthesized from tryptophan via tryptophan hydroxylase (TPH). The majority of serotonin (approximately 90%) is produced and stored in enterochromaffin cells located in the lining of the gut, while the remaining
serotonin is synthesized in the CNS. Serotonin modulates numerous physiological and behavioral functions, such as mood, sleep, appetite, vasoconstriction, and pain processing. Serotonin has the capacity to regulate so many biological functions owing to the widespread distribution of its molecularly diverse receptor subtypes throughout the CNS.

Anatomy of serotonergic system

The majority of serotonin innervation in the CNS originates from a group of neurons termed the raphe nuclei. This cluster of nuclei is located medially throughout the rostrocaudal extent of the brainstem. Although the raphe nuclei are made up of a heterogeneous population of neurons, the main constituents of the region are serotonergic.

There are two major divisions of raphe nuclei (Figure 5). The more rostral group of raphe nuclei (B5-B8) are located in the midbrain and rostral pons and project almost exclusively to the forebrain to modulate pathways involved in mood, appetite, and sleep. The caudal raphe nuclei (B1-3) are located within the caudal pons and medulla oblongata and project within the brainstem as well as to regions within the spinal cord to regulate cardiorespiratory function and pain processing. In 1964, Dalhstrom and Fuxe identified and divided serotonin nuclei within the raphe into 9 groups (B1-9) (Dahlstrom and Fuxe, 1964).
Figure 5. Sagittal view of a rat brain depicting serotonin innervation throughout the CNS. The more rostral serotonin nuclei, B5 (pontine median raphe), B6 (pontine dorsal raphe), B7 (midbrain dorsal raphe), and B8 (midbrain median raphe) project rostrally towards the forebrain to mediate functions related to mood, sleep, and appetite. The more caudal raphe, B1 (raphe pallidus), B2 (raphe obscurus), and B3 (raphe magnus have projections within the medulla and spinal cord which modulate cardiorespiratory function and pain processing (Cooper JR, 2003).
The rostral group is composed of three nuclei and is responsible for 85% of all serotonin innervation within the brain. The caudal linear nucleus (CLi) is the most rostral of the raphe nuclei, but contains relatively few serotonin neurons compared to the other raphe nuclei. The dorsal raphe (DR-B7) is the next most rostral of the nuclei. The DR contains the largest number of serotonin neurons, the majority of which are located in the rostral portion of the DR and also utilize SP as a co-transmitter with serotonin (Hornung, 2003). The most caudal nucleus of the rostral group is the median raphe (MR-B8). The majority of serotonin synthesizing neurons are located in the midline (>80%), while the lateral extensions of the MR rarely express serotonin. Caudal extensions of the DR and MR located in the pons are termed B6 and B5 respectively. Both the DR and MR receive glutamatergic afferent projections from the limbic system in the forebrain including the cingulate cortex, ventral tegmental area, and several nuclei of the hypothalamus (Behzadi et al., 1990; Peyron et al., 1998). While the rostral raphe nuclei project to a myriad of regions in the forebrain, there is very little overlap of projections from the DR and MR. The DR projections provide afferent input to the amygdala, hypothalamus, olfactory bulb, cortex, and basal ganglia (Van de Kar 1979). In contrast, the MR project to more midline structures which include the hippocampus, caudal raphe nuclei, laterodorsal tegmental nucleus, dorsal raphe nucleus, interpeduncular nucleus, medial mammillary body, and other medial sites (Vertes 1999). In general, the rostral raphe project throughout the forebrain to mediate many behavioral functions, such as arousal, aggression,
and anxiety (Monti, 2011; Pucilowski and Kostowski, 1983; Zangrossi and Graeff, 2014).

The caudal raphe are composed of the raphe magnus (RM-B3), which is the largest serotonergic group of the caudal raphe, the raphe obscurus (RO-B2), and the raphe pallidus (RP-B1, smallest group of serotonergic neurons). The caudal raphe project to other regions within the brainstem and to the spinal cord to regulate a variety of autonomic functions. The RM is mainly involved in pain processing as it has projections (most of which are serotonergic) to the dorsal horn of the spinal cord. Studies have identified an excitatory projection from the periaqueductal gray (PAG) region to the RM which promotes the release of opioid neurotransmitters in the spinal cord to cause analgesia (Mason, 2001). The RO has been shown to augment ventilation and has reciprocal projections with the PreBötC and hypoglossal motoneurons. Glutamate injections into the RO or direct activation of the RO by optogenetic means both result in a dramatic increase in ventilation (Depuy et al., 2011; Ptak et al., 2009). The RP has been shown to be involved in regulating tachycardia and increased BP in response to emotional stressors and provides excitatory input to the sympathetic neurons that innervate brown adipose tissue to stimulate thermogenesis (Morrison et al., 2012; Pham-Le et al., 2011).

The NTS receives extensive serotonin innervation from all three of the caudal raphe, the DR, and peripherally from the nodose ganglia, in which the cell bodies of the vagal sensory afferent nerves are located (Gaudin-Chazal et al., 1982; Nosjean et al., 1990; Schaffar et al., 1988; Thor and Helke, 1987b). The
highest density of serotonin innervation is located in the commissural and
dorsomedial band of the parvocellular subdivision of the NTS and to a lesser
degree in the remaining portion of commissural NTS and lateral and medial
subdivisions of the NTS. Only sporadic serotonin immunoreactive fibers are
observed in the ventrolateral subnucleus and remainder of the parvocellular
portions of the NTS (Maley and Elde, 1982). It is also important to note that
projections from the caudal raphe also release SP as a co-transmitter with
serotonin in the NTS, dorsal motor nucleus of the vagus (DMX), area postrema,
hypoglossal nucleus and the ventral horn of the cervical spinal cord (Thor and
Helke, 1987a; Thor et al., 1988). There are considerably fewer serotonin and SP
double-labeled varicosities from the nodes and petrosal ganglia in the NTS
compared to the spinal cord or hypoglossal nucleus. Instead, serotonin and SP
are more likely to be co-localized in varicosities arising from the RM, RO, and RP
(Thor and Helke, 1989).

**Serotonin receptors**

To date, 7 classes of serotonin receptors have been identified, each with
multiple receptor subtypes (14 total). The majority of serotonin receptors are G-
protein coupled receptors (GPCRs), with the exception of the ionotropic 5-HT₃
receptor. The first group of serotonin receptors are the 5-HT₁ family, comprised
of 5 receptor subtypes (5-HT₁A, 5-HT₁B, 5-HT₁D, 5-HT₁E, and 5-HT₁F), which are
typically inhibitory in nature. Specifically, these receptors preferentially couple to
5-HT\textsubscript{1A} to inhibit adenylyl cyclase (AC) as well as to activate K\textsuperscript{+} channels that promote hyperpolarization. The 5-HT\textsubscript{1A} receptor is the most extensively studied receptor of this family due to its wide CNS distribution and prominent role in various mood disorders. Both, the 5-HT\textsubscript{1A} and 5-HT\textsubscript{1B} receptors have been localized to the NTS, but the exact role of these receptors within the NTS remains unknown (Manaker and Verderame, 1990).

The 5-HT\textsubscript{2} receptor family is composed of 3 receptor subtypes (5-HT\textsubscript{2A}, 5-HT\textsubscript{2B}, and 5-HT\textsubscript{2C}) and promotes depolarization by preferentially coupling to G\textsubscript{aq/11} to activate phospholipase C (PLC) and increase inositol phosphatase 3 (IP\textsubscript{3}) levels. This leads to increased Ca\textsuperscript{2+} to promote vascular constriction and neurotransmission. Cells of the NTS express both 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors. The 5-HT\textsubscript{2A} receptors have been shown to be involved in the depressor and bradycardic responses of arterial baroreflex function (Comet et al., 2007; Pompeiano et al., 1994).

The ionotropic 5-HT\textsubscript{3} receptor is composed of 5 subunits (5-HT\textsubscript{3A/B/C/D/E}), but must contain at least one 5-HT\textsubscript{3A} subunit to be functional. Upon ligand binding, the channels are permeable to cations such as, Na\textsuperscript{+}, Ca\textsuperscript{2+}, and K\textsuperscript{+} and typically promote depolarization and facilitate neurotransmission. The 5-HT\textsubscript{3} receptor is highly expressed within the NTS on vagal afferent terminals (Leslie et al., 1990; Pratt and Bowery, 1989). Activation of these receptors promotes release of glutamate within the NTS to facilitate vagal excitatory input to the NTS (Jeggo et al., 2005).
The remaining receptors, 5-HT$_4$, 5-ht$_5$, 5-ht$_6$, and 5-HT$_7$ are less well characterized, but it is recognized that 5-ht$_5$ receptors couple to G$_{ai}$, while the rest couple to G$_{as}$ and activate AC, and thus protein kinase A (PKA) to facilitate depolarization. The NTS also expresses 5-HT$_4$ and 5-HT$_7$ receptors. Although these receptors have not been as extensively studied, some laboratories have found that activation of the 5-HT$_4$ receptor in the NTS results in an attenuated bradycardic response to cardiopulmonary reflex activation in anesthetized rats (Edwards and Paton, 1999). Additionally, 5-HT$_7$ receptors located in the NTS have been shown to increase cardiac vagal drive in anesthetized rats (Kellett et al., 2005).

**Major function of serotonin**

Serotonin has been investigated over many decades and has been identified as a key neurotransmitter in a variety of physiological functions including appetite, mood, arousal, thermoregulation, and autonomic functions. Although the role of serotonin in cognitive and emotional functions has been well documented, its role in cardiovascular function has not been as well characterized. Injection of serotonin directly into the NTS results in a dose-dependent increase in BP that can be blocked by local and systemic injection of serotonin antagonists. Additionally, administration of a selective serotonin reuptake inhibitor (SSRI) augments the serotonin-dependent increase in BP (Wolf et al., 1981). Similar results were reproduced in unanaesthetized rats.
(Callera et al., 1997). More recently, a study by Curtis et al., found increased serotonin metabolite accumulation in the caudal NTS of rats exposed to polyethylene glycol (PEG) induced hypovolemia (Curtis et al., 2013).

Serotonin and 5-HT$_{1A}$ receptors in hemorrhage

General functions of serotonin in hemorrhage

Several laboratories have provided evidence that central release of serotonin is involved in the sympatholytic phase of hemorrhage resulting in a dramatic fall in BP. Studies performed in anesthetized cats and rats found that depletion of serotonin resulted in a delayed hypotensive response to hypovolemic shock. Furthermore, central administration of a non–selective serotonin receptor antagonist, methysergide, produced similar effects (Blum and Spath, 1986; Elam et al., 1985; Morgan et al., 1988). Direct injections of the 5-HT$_{1A}$ receptor antagonist, WAY-100635, in the RVLM resulted in a delayed hypotensive and sympathoinhibitory responses to blood loss in anaesthetized rats. Thus, central serotonin release was thought to contribute to syncopal-like responses to blood loss through activation of 5-HT$_{1A}$ receptors located in the RVLM (Dean and Bago, 2002). However, additional studies have provided conflicting evidence for the role of central serotonin to sympathoinhibitory responses that follow blood loss. Unanesthetized animals subjected to prior depletion of serotonin demonstrated normal phasic responses to blood loss,
suggesting that anesthesia likely contributed to previous observations that implicated serotonin release in sympathoinhibition (Evans et al., 1992).

More recent studies from our laboratory have investigated the role of the serotonin neurons of the caudal raphe in hypotensive hemorrhage. Utilizing the immediate early gene, c-Fos, as a marker for neuronal activation, serotonin neuronal activation was assessed during hemorrhage in unanesthetized, freely moving rats. Serotonin neurons within the caudal raphe, including the RO and RM, were activated during hypotensive hemorrhage but not during hydralazine-induced hypotension (Kung et al., 2010). Studies from our laboratory also found that selective destruction of the caudal hindbrain serotonin neurons resulted in an attenuation of sympathetic and ventilatory recovery from hypotensive hemorrhage rather than attenuated sympathoinhibition to blood loss as had been observed in previous studies. In addition, rats subjected to serotonin depletion showed greater metabolic acidosis as a result of hypotensive hemorrhage despite normal BP and HR responses. Metabolic acidosis is indicative of peripheral tissue hypoxia. Thus, oxygen delivery to peripheral tissue was reduced by serotonin depletion. Oxygen delivery is dependent upon CO and arterial blood oxygenation. Interestingly, the partial pressure of O₂ in arterial blood (PaO₂) was significantly higher in serotonin depleted animals. These data suggest that the rats depleted of serotonin had impaired oxygen delivery to the peripheral tissues, possibly due to reduced CO. Since BP is determined by CO and TPR, the data further suggest that after serotonin lesion normal BP responses to blood loss are maintained by detrimental increases in peripheral
resistance rather than more hemodynamically favorable increases in CO. Together, these studies suggested that endogenous serotonin release from the caudal raphe contributes to sympathoexcitation and increased ventilation during compensation from hypotensive hemorrhage, and that in the absence of serotonin BP may be maintained by a potentially more dangerous increase in TPR that further reduces tissue perfusion.

The mechanism by which serotonin neurons in the caudal raphe become activated during blood loss remains unknown. The recovery of sympathetic and ventilation following blood withdrawal was found to rise in parallel with the PaCO$_2$, suggesting that central chemoreceptor activation may contribute to compensation from blood loss. Moreover, rats subjected to serotonin depletion were found to have reduced sympathetic and ventilatory responses to hyperoxic-hypercapnic gas exposure. Hyperoxia prevents activation of peripheral chemoreceptors. Therefore, these data suggest an important role for serotonin neurons in central chemoreceptor responses to acidosis in unanesthetized animals.

Following the initial study by Kung et al., evidence supported the view that serotonin neurons in the caudal hindbrain facilitated chemosensory input in the brainstem to modulate sympathetic and ventilatory drive. But it was unclear where serotonin was acting to mediate these effects. The NTS is an important sensory integration center in the brainstem and receives extensive serotonin innervation from the caudal raphe neurons. Therefore, additional studies were conducted to selectively destroy serotonin nerve terminals in the dorsomedial
medulla, including regions of the NTS. Serotonin nerve terminal lesion in the
dorsal midline brainstem also reduced sympathetic and ventilatory responses to
compensation from hypotensive hemorrhage in unanesthetized, freely moving
rats (Kung and Scrogin, 2011). Additionally, the lesion reduced the sympathetic
and ventilatory responses to peripheral chemoreceptor activation by systemic
KCN injections but enhanced arterial baroreflex gain during pharmacologically
induced hypotension. Results from the serotonin nerve terminal lesion study
were consistent with the previous studies, and further supported the view that
serotonin facilitates sympathetic and ventilatory recovery during blood loss by
facilitating chemoreflexes within the NTS.

5-HT\textsubscript{1A} receptor activation in hemorrhage

Although previous studies have identified a sympathoexcitatory role for
central serotonin release in the NTS during hypotensive hemorrhage, the
receptor population mediating these effects remains unknown. The previously
mentioned studies found that administration of the non-selective serotonin
receptor antagonist, methysergide, delayed the hypotensive phase of
hemorrhage (Elam et al., 1985; Morgan et al., 1988). However, methysergide
also acts as an agonist at 5-HT\textsubscript{1A/B/D} receptors (Hoyer et al., 1994). Studies from
our laboratory found that injection of methysergide into the lateral
cerebroventricle delayed the hypotension, bradycardia, and sympathoinhibition
associated with progressive blood loss in unanesthetized rats (Scrogin et al.,
It was also found that the partial 5-HT$_{1A}$ receptor agonist, buspirone, and the more selective 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT mimicked the effects of methysergide in a dose-dependent manner (Osei-Owusu and Scrogin, 2004). Systemic injection of the potent 5-HT$_{1A}$ receptor antagonist, WAY-100635, dose-dependently blocked the ability of methysergide to delay the sympathoinhibitory effect of hemorrhage, whereas a selective 5-HT$_{2}$ receptor antagonist had virtually no effect.

To better localize the 5-HT$_{1A}$ receptor population that mediated these effects, 8-OH-DPAT was injected in various rostro-caudal locations along the ventricular system of rats subjected to hypotensive hemorrhage. Injections into the 4$^{th}$ ventricle produced the most robust responses with the shortest latency. The results suggested that 5-HT$_{1A}$ receptors located in the hindbrain contributed to compensation from blood loss (Scrogin, 2003). The portion of the NTS that receives cardiovascular and chemoreceptor afferents forms the ventral floor of the 4$^{th}$ ventricle. This area also expresses 5-HT$_{1A}$ receptors. Denervation of the baroreceptor and chemoreceptor afferents that innervate the NTS was found to attenuate the sympathoexcitatory and pressor effects of 8-OH-DPAT compared to intact controls (Osei-Owusu and Scrogin, 2006). This implies that baroreceptor, and/or peripheral chemoreceptor input into the NTS is necessary for sympathoexcitatory and pressor responses elicited by 5-HT$_{1A}$ receptor activation in a hypovolemic rat. This is intriguing because the sympathoexcitatory and pressor effects of 8-OH-DPAT are only observed in a hypovolemic rat. Activation of 5-HT$_{1A}$ receptors actually induces
sympathoinhibition in euvolemic rats. Therefore, it is plausible that 5-HT$^{1A}$ receptors modulate a pathway that is quiescent in the euvolemic rats but is activated during severe blood loss.

Subsequent studies utilizing a more severe shock model (~50% estimated blood volume withdrawn) showed that 8-OH-DPAT administration increased mean circulatory filling pressure (MCFP) in hemorrhaged rats (Tiniakov and Scrogin, 2006). Mean circulatory filling pressure is the mean pressure that exists when CO stops and the blood redistributes throughout the vasculature. Thus, MCFP is a measure of how full the circulatory system is and is determined by blood volume and venous tone, since the veins are more compliant than the arterial vasculature. Given that the amount of blood withdrawn during hemorrhage was not different between 8-OH-DPAT and saline-control treated rats, it was assumed that 8-OH-DPAT increased MCFP through increased venous tone. An increase in venous tone should cause an increase in venous return and BP if right arterial pressure is maintained. In fact, systemic administration of 8-OH-DPAT to hypovolemic animals was also found to improve metabolic acidosis following severe blood, indicating that 5-HT$^{1A}$ receptor activation improves oxygen delivery to peripheral tissue in the hemorrhaged animal. Indeed, it was determined that 8-OH-DPAT administration in hemorrhaged rats increased BP exclusively by increasing CO. Neither HR nor TPR were affected by 8-OH-DPAT administration. Thus, 8-OH-DPAT may selectively promote vеноconstriction to ultimately increase venous return and CO
and ameliorate the progression of metabolic acidosis in hypovolemic rats (Tiniakov et al., 2007).

It is also plausible that 8-OH-DPAT may mobilize blood stores within the venous vasculature to increase MCFP. The splanchnic vasculature holds 33% of blood volume in euvoletic animals (Greenway and Lister, 1974). Due to the high compliance of the splanchnic venous vasculature, a relatively large portion of this blood remains unstressed and contributes very little to central venous pressure. Sympathetic-mediated constriction of the splanchnic veins can result in up to 60% of blood re-mobilization back into the circulation to increase perfusion pressure (Reilly et al., 2001). Our laboratory also showed that bilateral denervation of the splanchnic sympathetic nerves in the rat did not alter the initial pressor effect of 8-OH-DPAT but did abolish the ability of 8-OH-DPAT to increase MCFP and improve acid-base balance. Interestingly, denervation greatly shortened the pressor effect of 8-OH-DPAT, as did ganglionic blockade. Together, the data indicate that 8-OH-DPAT, when given following hemorrhagic shock, promotes a sympathetic-dependent increase in the splanchnic venous tone that increases CO and thereby reduces metabolic acidosis (Tiniakov et al., 2012). Endogenous serotonin released from the caudal hindbrain raphe produces very similar effects to administration of the 5-HT\textsubscript{1A} receptor agonist, 8-OH-DPAT in hypovolemic animals. These findings suggest that 8-OH-DPAT and endogenous serotonin release may both facilitate sympathetic and ventilatory compensation through activation of 5-HT\textsubscript{1A} receptors located within the NTS.
Serotonin and central chemoreceptors

As discussed previously, the serotonergic neuronal population within the mid-line medullary raphe are considered putative central chemoreceptors. Central chemoreceptors are defined by their intrinsic sensitivity to PaCO$_2$/H$^+$ and direct connection to a respiratory network that mediates ventilation. Over the years, there have been various (often conflicting) results from studies investigating whether serotonin neurons of the caudal raphe are central chemoreceptors or modulate chemosensitivity.

In general, serotonin has not been shown to be necessary for homeostatic ventilatory rhythmogenesis in adults, but it does increase the frequency and amplitude of phrenic nerve bursts during hypercapnia (Corcoran et al., 2013). Hypercapnia has also been shown to induce c-Fos expression in raphe serotonin neurons (Haxhiu et al., 2001; Johnson et al., 2005), and to augment serotonin release within the dorsomedial medulla (Kanamaru et al., 2013). Hypercapnia was shown to increase the firing rate of serotonin neurons in the raphe of freely moving cats (Veasey et al., 1995). Moreover, numerous laboratories have shown that rodents display reduced chemosensitivity when serotonin neurons have been genetically altered (Barrett et al., 2012; Buchanan and Richerson, 2010; Hodges et al., 2011; Hodges et al., 2008; Penatti et al., 2011a), pharmacologically lesioned (Nattie et al., 2004), or selectively silenced (Ray et al., 2011). Dietary restriction of tryptophan also reduces central chemosensitivity (Penatti et al., 2011b). Together, these studies indicate that serotonin modulates or facilitates central chemoreflex activation.
One compelling piece of evidence that serotonin neurons are central chemoreceptors is their ideal location for the direct assessment of how efficiently the lungs oxygenate the blood supply. The PaCO$_2$ is an excellent reflection of the aveolar ventilation, and immunohistochemical analysis has shown that serotonin immunoreactive caudal raphe neuronal dendrites physically wrap around the major arterial blood vessels in the brain (Bradley et al., 2002). Therefore, it is plausible that these neurons are able to penetrate the blood vessels to directly and accurately assess PaCO$_2$ and H$^+$ in the arterial blood supply to the brain. This idyllic location of serotonin neurons is comparable to the peripheral chemoreceptors located in carotid and aortic bodies, which enable them to directly measure the oxygen content of the blood immediately after departing the heart.

Furthermore, when the same serotonin neurons are removed and cultured, they are found to be directly sensitive to changes in CO$_2$ and H$^+$ (Wang et al., 1998; Wang et al., 2001). Specifically, they are more active when CO$_2$ is elevated and pH is decreased. Conversely, when pH increases or CO$_2$ decreases, these neurons become quiet. This intrinsic ability to be activated by changes in CO$_2$ and pH strongly suggest that these neurons are chemosensitive. Caudal raphe serotonin neurons not only provide afferent projections to numerous components of the respiratory network including, PreBötC and hypoglossal motorneurons, but they have also been shown to directly stimulate respiratory motor output. Patch clamp recording studies by Ptak et al., found that focal administration of a glutamate receptor agonist in the RO caused an
increase in firing rate of the serotonin neurons that was proportionate to an
increase in firing of the hypoglossal nerve (Ptak et al., 2009). And for the first
time, a study has recently shown that specific activation of serotonin neurons via
optogenetic-mediated activation in vivo caused an increase in phrenic nerve
bursting frequency and amplitude (Depuy et al., 2011).

Serotonin receptor subtypes involved in this central chemoreceptor
response have been linked to 5-HT$_{2A/C}$, 5-HT$_{4A}$, and 5-HT$_{1A}$ receptor activation.
When 5-HT$_2$ receptor agonists are administered via the cerebroventricles, a
subsequent increase in ventilation occurs which can be reversed by 5-HT$_2$
receptor antagonist administration (Ray et al., 2011). Similarly, a 5-HT$_{4A}$ receptor
agonist has been shown to increase respiratory motor output. Systemic
administration of 8-OH-DPAT also restores ventilation after fentanyl-induced
apnea (Manzke et al., 2003; Zhuang et al., 2012). As mentioned, 5-HT$_{1A}$
receptors are inhibitory, and their ability to promote ventilation suggests a
potential role for 5-HT$_{1A}$ receptor-mediated disinhibition of respiratory centers
through suppression of GABAergic or glycinergic interneurons (Corcoran et al.,
2014).

Despite evidence from the aforementioned studies, some investigators do
not believe that serotonin neurons play a role in central chemoreception in vivo
because serotonin neurons in the RO were unresponsive to hyperoxic
hypercapnia in anaesthetized rats (Depuy et al., 2011). Similarly, other studies
have found that intact medullary serotonin neurons are not chemosensitive by in
vivo recordings in anaesthetized rats (Mulkey et al., 2004; Takakura and Moreira,
2013). However, it has been well documented that the hypercapnic ventilatory response (HCVR) is significantly impaired by isoflurane and other anesthetics. In fact, studies from Depuy et al., found that 8% CO\textsubscript{2} only increased ventilation by 35% in anesthetized rats. In contrast, studies performed in unanaesthetized rats found 7% CO\textsubscript{2} produce a dramatic 250% increase in ventilation (Davis et al., 2006; Hodges et al., 2008).

Similarly, arguments have been made against serotonin neurons as central chemoreceptors because they are less sensitive to CO\textsubscript{2} during sleep. Therefore, some have suggested that the responses of serotonin activation are due to arousal rather than central chemosensitivity. However, it has long been known that HCVR is reduced during slow wave sleep and further reduced during REM, but the mechanism is unknown (Bulow, 1963). Additionally, the firing of serotonin neurons is highest during wakefulness, and virtually absent during REM sleep. Combined with the fact that serotonin neurons are important for arousal from sleep during elevated CO\textsubscript{2}, it plausible that consciousness is necessary for serotonin neurons to be fully active and augment the HCVR.

Another argument against the medullary raphe as central chemoreceptors includes a study by Mulkey et al., that utilized TASK knockout mice. It had been previously determined that TASK1/3 channels are the acid sensitive ion channels directly linked to chemosensitivity of the medullary raphe in vitro. Therefore, when TASK channel knockout mice were found to have a normal HCVR, it was presumed that serotonin neurons in the medullary raphe were not central chemoreceptors. However, there are several plausible explanations for why
these results do not exclude serotonin neurons as central chemoreceptors. Specifically, brain slice recordings were performed in P7-P12 mice despite the fact serotonin neurons are not fully developed until P21 (Wang and Richerson, 1999). It is well known that age affects chemosensitivity and that in earlier stages of development chemosensation is mediated more by central chemoreceptors in the LC and NTS (Conrad et al., 2009; Stunden et al., 2001). More importantly, TASK channels become inactive with most anesthetics, and thus serotonin neurons may not be chemosensitive when studied in an anesthetized animal model (Conway and Cotten, 2012; Patel et al., 1999; Talley and Bayliss, 2002).

In light of the most recent studies, there is a compelling amount of evidence suggesting that medullary serotonin neurons may act as central chemoreceptors or at least contribute to part of the ventilatory response to hypercapnia. Additionally, serotonin neurons are most active during wakefulness, and silenced during sleep. Therefore, studies examining the contribution of serotonin neurons to the central chemoreflex in awake animals are necessary for a better understanding of the ventilatory response to hypercapnia.
CHAPTER III

5-HT<sub>1A</sub> RECEPTORS IN THE NUCLEUS TRACTUS SOLITARIUS FACILITATE SYMPATHETIC RECOVERY FOLLOWING HYPOTENSIVE HEMORRHAGE

Abstract

Serotonin neurons in the caudal raphe are activated in response to hypotensive hemorrhage. Moreover, selective destruction of the same neuronal population results in attenuated sympathetic and ventilatory responses to blood loss. The caudal raphe provide extensive serotonin innervation of the nucleus tractus solitarius (NTS), the lesion of which also attenuates ventilatory and sympathetic responses to hemorrhage and peripheral chemoreceptor stimulation. Multiple serotonin receptor subtypes are expressed in the NTS, but it is not known which receptor subtype mediates compensatory responses during blood loss. However, our prior studies found that systemic administration of the 5-HT<sub>1A</sub> receptor agonist, 8-OH-DPAT, improves recovery from hypotensive hemorrhage by stimulating sympathetically-mediated venoconstriction and CO. Thus, we hypothesize that serotonin released in the NTS during hemorrhage activates 5-HT<sub>1A</sub> receptors that facilitate sympathetic recovery. We utilized viral mediated knockdown of the rat 5-HT<sub>1A</sub> receptor in the caudal NTS to examine the role of 5-HT<sub>1A</sub> receptors on compensation following hemorrhage. Genes encoding a
5-HT$_{1A}$ shRNA or scrambled version of the same sequence were incorporated into adeno-associated viral (AAV) vectors and injected into the caudal NTS of rats. Five weeks after viral injection, rats treated with the vector encoding the 5-HT$_{1A}$ shRNA showed decreased recovery of renal sympathetic nerve activity following hemorrhage compared to controls (-6.4 ± 12.9 vs. +42.6 ± 15.6% of baseline, 30 min after start of hemorrhage, P<0.05). They also showed greater lactate levels 60 min after the start of hemorrhage (2.77 ± 0.5 vs 1.34 ± 0.2 mmol/L, P<0.05). The 5-HT$_{1A}$ mRNA expression in the commissural NTS was directly correlated with renal sympathetic nerve activity and inversely correlated with lactate 50 minutes after hemorrhage termination (P< 0.01, P< 0.05 respectively). These data indicate that serotonin released in the caudal NTS during hemorrhage activates 5-HT$_{1A}$ receptors to facilitate sympathetic-mediated compensation and tissue perfusion following severe blood loss.

Introduction

Progressive blood loss elicits a complex, multiphasic response of the autonomic nervous system, but the mechanisms that regulate these responses remain ambiguous. Initially, blood pressure is maintained primarily through sympathetically-mediated increases in vasoconstriction and tachycardia (Korner et al., 1990; Schadt and Ludbrook, 1991). After significant blood loss, these compensatory responses suddenly abate, resulting in a rapid and dramatic decline in BP (Hasser and Schadt, 1992; Ludbrook and Ventura, 1996). As blood loss continues, a secondary, sympathoexcitatory phase ensues, in which
venoconstriction mobilizes blood pooled in the highly compliant venous vasculature. The resulting increase in venous return increases CO and maintains BP (Haljamae, 1984). This phase can only be temporarily sustained in the absence of volume resuscitation and with continued blood loss, eventually gives way to decompensation, characterized by a decline of sympathetic drive and a more permeable and less responsive vasculature (Liu et al., 2003). Elucidating the pathways that contribute to the secondary sympathoexcitatory response to blood loss could lead to novel methods of therapy to manipulate and prolong the sympathetically-mediated venoconstriction, and thereby slow the progression to decompensation.

Serotonergic neurons in the caudal raphe of rats become activated during blood loss (Kung et al., 2010). Selective lesion of those same serotonin neurons results in attenuated sympathetic and ventilatory recovery following hypotensive hemorrhage. Rats subjected to lesion of caudal raphe serotonin neurons also develop greater metabolic acidosis with hemorrhage, despite having elevated arterial blood oxygen and normal BP recovery (Kung et al., 2010). Together, these data suggest that serotonin neurons in the caudal raphe are necessary for normal compensation from blood loss, quite possibly by orchestrating a beneficial hemodynamic pattern in which perfusion pressure recovers more by venoconstriction and increased venous return rather than by arterial constriction which may further exacerbate tissue hypoxia.

Caudal medullary serotonin neurons have numerous projections within the medulla and brainstem, including the NTS (Schaffar et al., 1988). These
projections densely innervate the medial and commissural subnuclei of the NTS, where baroreceptor and peripheral chemoreceptor afferents terminate. We found that lesion of the serotonin nerve terminals in the dorsomedial brainstem (including the medial and commissural NTS) significantly impaired sympathetic and ventilatory recovery from blood loss in conscious rats. The lesion also attenuated ventilatory and sympathetic responses to peripheral chemoreflex activation (Kung and Scrogin, 2011). Given the similar effects of caudal serotonin cell body lesion and discrete lesion of serotonin nerve terminal lesion in the dorsomedial brainstem in hemorrhaged animals, it is plausible that endogenous serotonin acts in the NTS during hemorrhage to augment sympathetic drive and ventilation, possibly by enhancing peripheral chemoreceptor responsiveness.

It is not known which serotonergic receptor population mediates these compensatory effects. A myriad of serotonin receptor subtypes are expressed in the NTS including the 5-HT\textsubscript{1A} receptor (Edwards and Paton, 1999; Gustafson et al., 1996; Leslie et al., 1990; Manaker and Verderame, 1990; Pompeiano et al., 1994). Previous experiments have shown that the selective 5-HT\textsubscript{1A} receptor agonist, 8-OH-DPAT, elicits a sympathetically mediated increase in BP when administered centrally or systemically to conscious, hypovolemic rats (Scrogin, 2003). This pressor effect is due almost exclusively to an increase in venous tone with virtually no effect on peripheral resistance (Tiniakov et al., 2007). Furthermore, the sympathoexcitatory effect of 8-OH-DPAT is dependent on intact sinoaortic nerve afferents which terminate in the medial and commissural
subnuclei of the NTS (Osei-Owusu and Scrogin, 2006). Since, 8-OH-DPAT appears to mimic the hemodynamically beneficial effects of endogenous serotonin release during blood loss, we predicted that 5-HT$_{1A}$ receptor activation in the NTS would be necessary for normal compensation from hypotensive hemorrhage.

Therefore, in the current study we tested the hypothesis that 5-HT$_{1A}$ receptors in the caudal NTS are necessary for normal recovery of sympathetic activity following severe hemorrhage. Moreover, we determined whether 5-HT$_{1A}$ receptor activation in the NTS contributes to peripheral tissue oxygenation. Here we used an AAV vector to selectively reduce gene expression of the 5-HT$_{1A}$ receptors within the NTS to test these hypotheses.

**Methods**

*Short hairpin RNA sequence screening*

Five short hairpin RNA (shRNA) sequences targeting various regions of rat 5-HT$_{1A}$ receptor mRNA were cloned into a humanized Renilla reniformis green fluorescent protein (hrGFP) expression vector (courtesy of Dr. Nichole Dudek). The plasmid contained a mouse RNA polymerase III U6 promoter upstream of the shRNA sequence, and a hrGFP marker gene driven by a separate Cytomegalovirus (CMV) promoter. The 5 shRNA sequences and the control shRNA sequence targeting the rat tryptophan hydroxylase 2 (TPH2) gene are provided in Table 1.
Table 1. shRNA sequences screened in HEK 293 cells.

<table>
<thead>
<tr>
<th>shRNA</th>
<th>Sequence 5’ → 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT\textsubscript{1A} shRNA #1</td>
<td>GTGAAAGGAAGACGGGTGAA</td>
</tr>
<tr>
<td>5-HT\textsubscript{1A} shRNA #2</td>
<td>CCGAGTGGGCAACTCCAAA</td>
</tr>
<tr>
<td>5-HT\textsubscript{1A} shRNA #3</td>
<td>GCTCAATCCGGTTATTTAT</td>
</tr>
<tr>
<td>5-HT\textsubscript{1A} shRNA #4</td>
<td>GAGGTGCACCGAGTGGGCAACTCCAAAGA</td>
</tr>
<tr>
<td>5-HT\textsubscript{1A} shRNA #5</td>
<td>GGCTATCACCGACCCCTAT</td>
</tr>
<tr>
<td>TPH2 shRNA</td>
<td>CCACCATTGTGACGCTGAA</td>
</tr>
</tbody>
</table>

The sequences for the 5 shRNA sequences targeting various portions of the rat 5-HT\textsubscript{1A} receptor (5-HT\textsubscript{1A} shRNA #1-5) and the control shRNA targeting the rat TPH2 gene (TPH2shRNA).
The full length rat 5-HT$_{1A}$ receptor gene (gift from Dr. Paul Albert) was cloned into the mammalian expression vector mCherry-C1-I74T3 (donated by Dr. Roger Y. Tsien) by Dr. Dudek to produce a rat 5-HT$_{1A}$ receptor mCherry fusion protein (mCherry-5-HT$_{1A}$R) that was used to examine efficiency of shRNA-mediated knockdown.

Human embryonic kidney (HEK) 293 cells were grown at 37°C in Dulbecco’s Modified Eagle Medium (DMEM; Hyclone™, ThermoScientific) with high glucose (4.5g/L), L-Glutamine, and Sodium Pyruvate. Medium was supplemented with 1.2g Sodium Bicarbonate, 1% nonessential amino acids, 10% fetal bovine serum (FBS; Cellgro), 100 units/mL penicillin (Cellgro) and 100µg/ML streptomycin (Cellgro) in the presence of 5% CO$_2$. HEK 293 cells were grown in six-well plates (9.6 cm$^2$) to 80% confluency, then were co-transfected with 0.4µg mCherry-5-HT$_{1A}$R and 1.2 µg of the shRNA-hrGFP encoding plasmid using the transfection reagent, TurboFect (ThermoScientific) according to manufacturer’s guidelines. Three days post transfection, the efficacy of each shRNA sequence was determined via fluorescent microscopy by comparing the intensity of red fluorescence of cells treated with each shRNA sequence to fluorescence of cells treated with a control shRNA vector. Fluorescence intensity was determined using Image J software (NIH, USA). Afterwards, cells were lysed and harvested in Trizol™ (Invitrogen).

5-HT$_{1A}$ receptor protein and mRNA expression were determined by Western blot and semi-quantitative PCR respectively (methods described below). The most efficient shRNA sequence and a scrambled version of the same
sequence without homology to any known sequence in the rat genome were incorporated into serotype 2/8 adeno-associated viral (AAV) vectors through an outside vendor (Gene Transfer Vector Core, University of Iowa, USA).

*Semi Quantitative Reverse Transcriptase-Polymerase Chain Reaction*

Total RNA was isolated from transfected cell lysates (Trizol, Invitrogen) and reverse transcribed into cDNA (First Strand cDNA Synthesis, Fermentas) according to the manufacturer’s guidelines. Specific primers for GAPDH and 5-HT$_{1A}$ receptor were used to amplify cDNA from extracted RNA (Table 2; Integrated DNA Technologies). PCR reactions were prepared with PCR buffer, 200µM dNTPS, 1mM MgSO$_4$, 1-4µL cDNA, 20µM forward primer, 20µM reverse primers, 1U KOD Hot Start Polymerase then up to 50µL with PCR water (Novagen). PCR cycling conditions for 5-HT$_{1A}$ receptor were as follows: 94°C for 1 min, followed by 30 cycles of 94°C for 15s/45° for 30 s/ 72°C for 45 s and a final extension period of 10 min at 72°C. Similar PCR cycling conditions were used for GAPDH: 94°C for 1 min, followed by 32 cycles of 94°C for 15s/58° for 30 s/ 72°C and a final extension at 72°C for 10 min. PCR products were run on a 1% agarose gel at 100 volts along with a 1Kbp DNA molecular weight marker (GeneScript USA Inc). Images were acquired with a Kodak Image Station 4000mm Pro. Band density was calculated using Image J (NIH, USA) and individual 5-HT$_{1A}$R bands were normalized to the GAPDH band.
### Table 2. Reverse transcriptase PCR primers.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>T (°C)</th>
<th>Cycle</th>
<th>Forward Primer (5’→3’)</th>
<th>Reverse Primer (5’→3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>58</td>
<td>32</td>
<td>CAA GGT CAT CCA TGA CAA CTT TG</td>
<td>GTC CAC CAC CCT GTT GCT GTA G</td>
<td>500</td>
</tr>
<tr>
<td>5-HT1A</td>
<td>45</td>
<td>30</td>
<td>TCC AGA ATG TGG CCA ACT AT</td>
<td>CCT TCT TTT CCA CCT TCC TG</td>
<td>510</td>
</tr>
</tbody>
</table>

Forward and reverse primers specific for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 5-HT1A receptor. Primer specific temperature used for annealing phase of RT-PCR (T), number of cycles of PCR performed, and product length are also shown.
Immunoblotting

Total protein was extracted from transfected HEK 293 cell lysates according to manufacturer’s guidelines (Trizol, Invitrogen). Protein concentration was determined by BCA assay (Pierce) using bovine serum albumin (BSA) as standard. Protein (10µg) was then separated by electrophoresis on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel for 1.5 hours at 150 volts, then transferred to a nitrocellulose membrane (BioRad) for 2 hours at 100 volts. The membrane was blocked in 5% nonfat dry milk and 0.1% Tween-20 in Tris-buffered saline (TBST) for 1 hour, prior to overnight incubation with a custom rabbit anti-5-HT$_{1A}$ receptor antibody (1:10,000, Pacific Immunological), or mouse anti-actin (1:40,000, MP Biomedicals Inc.) in 1% nonfat dry milk at 4˚C. Membranes were washed with TBST (3 times for 10 minutes) and incubated with HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (1:2000 and 1:5000 respectively, Pierce) for 1 hour at room temperature. The membrane was washed a final time with TBST (3 times for 10 minutes) then protein bands were developed with Super-Signal West Pico substrate (Themo) and exposed on autoradiographic film. The band density was quantified with Image J (NIH, USA), and normalized to actin.

Animals

Experiments were performed on 44 Male Sprague-Dawley rats (275 to 300 g; Harlan, Indianapolis, IN), which were acclimated to the institutional animal facility under standard conditions (22°C ambient temperature, 12:12 hour light cycle) with food and water available ad libitum for at least 1 week prior to
surgery. All experimental protocols were approved by the Institutional Animal Care and Use Committee and conducted in compliance with the Principles of Laboratory Animal Care, as adopted and promulgated by the United States National Institutes of Health.

*Surgical procedures*

**AAV Vector injections**

Prior to surgery, AAV vectors were diluted in viral storage buffer (400nM NaCl, 20 mM Tris base) to $1 \times 10^{12}$ vg/ml and dialyzed in 0.9% saline (Slide-a-Lyzer, ThermoScientific) according in manufacturer’s guidelines. Briefly, 10-25 µL of virus was dialyzed for 10-25 min in 500 mL 0.9% saline on ice. After dialysis, heparin (1000 units/ml) was added in a 1:7 ratio (heparin: virus v/v) to facilitate the initial internalization step of viral infection (Smith et al., 2004). Rats were anesthetized with ketamine/xylazine (85/13mg/kg, im) and placed in a stereotaxic apparatus with the nose bar positioned 11 mm below a flat skull plane. Throughout the surgery, animals were monitored and supplemental doses of ketamine (10 mg/supplemental injection, im) were administered as necessary to maintain a surgical plane of anesthesia. The back of the skull was exposed and a small portion of the occipital bone was removed in order to visualize the dorsal surface of the brainstem at the level of calamus scriptorius. A glass micropipette pulled to an outer diameter of 20-40 µm, was filled with virus encoding the 5-HT$_{1A}$R shRNA or scrambled version of the same sequence. Using an oil pressure injection system, AAV vectors were infused bilaterally (750 nL/site) at a rate of 200 nL/min, 0.5 mm ventral to calamus scriptorius at two
rostrocaudal planes along the NTS: +0.5 and 0 mm relative to calamus scriptorus. Injections were made 0.7 mm lateral from the midline for the rostral set of injections, and 0.25 mm lateral from the midline for the caudal set of injections for a total of 4 injections targeting the commissural and medial NTS. Prior to the injection, the glass pipette remained in the tissue for 5 minutes after insertion to allow the tissue to equilibrate. Similarly, following each injection, the injector remained in tissue for 5 minutes after infusion to ensure that the virus had completely exited the injector tip before removal so as to avoid viral spread to alternative brain regions dorsal to the injection site. Following injection, the neck muscle and skin were closed in separate layers and rats were given buprenorphine (50µg/kg, sc) every 8 hours, for 2 days post-surgery along with ampicillin (150 mg/kg sc) twice per day for 3 days. Animals were then allowed to recover for four weeks prior to hemorrhage experiments.

Vascular catheter and renal sympathetic recording electrode implantation

The day before hemorrhage experiments, rats were anesthetized with isoflurane (5% for induction and 1.5-2.5% for maintenance) in 100% O₂ (1L/min) and instrumented with bilateral femoral arterial catheters (PE-50 fused with PE-10 tubing) to enable simultaneous blood withdrawal and direct measurement of arterial pressure. A third catheter was inserted in the femoral vein to enable blood sampling for venous blood gas determinations and for systemic injections. All catheters were externalized at the nape of the neck and secured with 3-0 silk suture. After catheter implantation, a segment of the left renal sympathetic nerve was isolated through a left flank incision. A fiber bundle was placed on a bipolar
electrode made from Teflon-coated stainless steel (bare diameter = 0.005 in; A-M Systems, Everett, WA) and isolated in Kwiksil silicon (World Precision Instruments). The electrode lead wires were tunneled under the skin to exit at the nape of the neck. Rats were given ampicillin (150 mg/kg sc) antibiotic and ketoprofen (5mg/kg sc) for analgesia and allowed to recover overnight. Experiments were conducted the day after surgery.

Experimental Protocols

Hypotensive hemorrhage

All rats were subjected to hypotensive hemorrhage conducted as described previously (Kung and Scrogin, 2011). Briefly, arterial lines were connected to a withdrawal pump and an arterial pressure transducer through an overhead swivel system while the rats rested, unrestrained in their home cage. The recording electrode and venous line were connected to appropriate sampling lines through the same overhead swivel system. Rats were habituated for at least one hour, after which blood withdrawal commenced at a rate of 3.2 mL/min/kg for 6 minutes, and then 0.52 mL/min/kg for an additional 4 minutes, to provide a total withdrawal of 21% of estimated blood volume over 10 minutes. Data acquisition began 20 minutes prior to the start of blood withdrawal and continued throughout the course of hemorrhage and for an additional 50 minute recovery period following termination of blood withdrawal. Femoral venous blood samples (150 µL) were taken 20 minutes prior to hemorrhage, and 10 and 60 minutes after the start of blood withdrawal to measure hematocrit, venous blood gases and acid-base status with an i-STAT 1 analyzer (i-STAT, East Windsor,
Plasma protein concentrations were determined by a handheld clinical refractometer (ATAGO U.S.A. Inc.). At the end of the experiment, rats were given hexamethonium chloride (30 mg/kg iv) to block post-ganglionic sympathetic activity and allow determination of background noise in the renal sympathetic recording electrode. A subset of the rats were then quickly anesthetized with sodium pentobarbital (100mg/kg, iv), and decapitated. Brains were quickly extracted and flash frozen in cold isopentane and stored at -80°C for later qPCR assessment of receptor gene expression. The remaining rats were anesthetized with sodium pentobarbital (100mg/kg iv) then quickly perfused transcardially with 90 ml of sodium nitrate (0.1M) in 6.7 mM phosphate buffer followed by 90 ml 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS). Brains were then removed and post-fixed for 1 hour. The brainstem was blocked and post-fixed overnight. The following day, brains were transferred to 30% sucrose solution and stored at 4°C until sectioning. Brains were sectioned on a freezing microtome in 40 µm sections and collected serially into 6 wells filled with cryoprotectant (30% sucrose and 30% ethylene glycol in 0.1 M PB) and stored at -20° until immunohistochemical analysis was performed.

**Spontaneous baroreflex sensitivity**

Five minute segments of baseline BP and HR data were recorded prior to hemorrhage and analyzed with Nevrokarid SA-BRS software version 3.2.4 to determine spontaneous baroreflex gain using the sequence method (Padley et al., 2005). Spontaneous baroreflex gain was determined as the average slope of linear regressions obtained from at least 3 sequences. Sequences included in
the analysis had to satisfy the following constraints: three or more consecutive interbeat intervals (IBI) with variation in the same direction, >0.5 ms that correlated \((r^2 > 0.85)\) with systolic, diastolic, or mean arterial BP variations of >0.5 mmHg, with a three-beat delay. Parameters used to select sequences were set based on evidence obtained from our prior work that they retrieved the most sequences (Henze et al., 2008). A 128-point fast-Fourier transformation with a smoothed Hamming window was used to conduct cross-spectral analysis on IBI and BP. The relationship between IBI and BP variability was determined as the square root of the ratio of IBI and BP power spectra, with 50% overlap and zero padding of eight. Values were reported as the \(\alpha\)-index in the low-frequency (LF, 0.06 – 0.6 Hz) and high-frequency (HF, 0.6- 3.0 Hz) domains (Pinna et al., 2002). BP variability was determined in the time domain as the standard deviation of systolic, mean, or diastolic BP. Blood pressure variability was also determined in the LF domain. HR variability in the LF and HF domains was analyzed using the IBI power spectra criteria described above (Kuwahara et al., 1994). Group means for each variable were compared by Student’s t-test.

**Data acquisition**

Arterial pressure, HR, and renal sympathetic nerve activity (RSNA) were recorded on a Macintosh G4 Powerbook with PowerLab data acquisition software (Chart version 5.2.1; ADinstruments, Colorado Springs, CO). Arterial pressure was measured with a disposable pressure transducer (Transpac IV; Abbott Labs, North Chicago, IL) and a PowerLab bridge Amplifier (ADinstruments). Heart rate was calculated using peak-to-peak detection of the
pulse pressure wave. Sympathetic activity was sampled (4,000 Hz) and amplified (10,000x) with a PowerLab Bioamplifier (ADinstruments). The raw signal was filtered (1-1,000 Hz), rectified, and integrated over a 20-ms time constant. Background noise, determined as the signal that remained after hexmethonium chloride injection, was subtracted from all values. Only data from recordings with a signal to noise ratio greater than 2:1 were included in the data analysis.

_Tissue Microdissection_

Brains were transferred to a Leica CM 3050 S Cryostat (-15 to -17°C). Five hundred µm thick sections were cut and 0.75 and 1 mm diameter micropunches were taken from areas of interest determined using the stereotaxic rat brain atlas of Paxinos and Watson (Watson, 1982). Bilateral punches were taken from the commissural- and medial NTS (mNTS) as well as the hypoglossal nucleus -14.3 to -13.8, -13.3 to -12.3, and -14.8 to -13.3 mm relative to Bregma respectively. Micropunches from RO were obtained by two midline punches from -13.3 to -11.3 mm from Bregma. Dorsal raphe samples were obtained from a single punch -9.3mm to -6.8mm from Bregma. The tissue was homogenized immediately in 1.0 mL TRIzol® Reagent (Invitrogen) using a glass mortar and pestle on ice. Samples were transferred to microcentrifuge tubes for RNA extraction.
**Tissue RNA Isolation and cDNA synthesis**

Total RNA was extracted according to manufacturers’ protocols (Invitrogen) with the addition of GlycoBlue™ Coprecipitant (15mg/mL) to facilitate nucleic acid precipitation (Invitrogen). RNA purity and concentration were assessed using a NanoDrop 8000 Spectrophotometer (Thermo Scientific). Samples were treated with DNase I enzyme (1U/1µg RNA) (Fermentas) to remove remaining genomic DNA and cDNA was synthesized (First Strand cDNA Synthesis Kit, Fermentas) according to the manufacturers’ instructions using 200 ng of total RNA from each sample. Each sample was processed in duplicate except that the reverse transcription enzyme was omitted in one of the duplicates as a negative control.

**Quantitative Real-Time PCR**

Messenger RNA levels of the target 5-HT_{1A} receptor were determined from tissue obtained from the virus injection site, i.e., mNTS and commissural NTS, as well as the RO, which is the caudal raphe nucleus with the greatest density of serotonergic projections to the NTS. This latter analysis was performed in order to rule out the possibility that any observed physiological effect of treatment was due to retrograde transport of virus and subsequent knocked down of 5-HT_{1A} autoreceptors. Samples of DR were also assessed as a positive control for 5-HT_{1A} gene expression. The α-adrenergic receptor (αAR_{2A}) were also assessed in mNTS and commissural NTS to assess potential off target or compensatory effects of 1ARshRNA expression. Real-time
quantitative PCR (qPCR) was performed on amplified cDNA using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Template cDNA (2ng) corresponding to the transcripts of interest were amplified using primers (500nM) for both the sense and antisense strands. SsoAdvanced™ SYBR® Green Supermix (2X) diluted to 1X with DEPC-treated water was added to all samples. Samples were run in triplicate and in parallel with a negative control in which water was substituted for template. The following general PCR protocol was used with various annealing/extension temperatures: 95°C for 30 sec, 40 cycles of 95°C for 5 sec and 56-63°C for 30 sec. Melt curves from 65°C to 95°C were included to confirm that only a single product was formed for each primer pair. CFX Manager Software was used to determine relative mRNA expression levels using the 2-ΔΔCT method (Livak and Schmittgen, 2001). Results are presented as relative mRNA expression normalized to relative GAPDH mRNA and compared with average group values obtained from rats injected with the scrambled shRNA that were run in the same cohort (defined as 1.0-fold). Forward and reverse primers were screened and designed using http://bioinfo.ut.ee/primer3-0.4.0/ and are shown in Table 3. The Basic Local Alignment Search Tool (BLAST) from the NCBI website: (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch) was applied to ensure that all amplified sequences shared no sequence homology with any other cDNA in the database. Efficiency of each primer pair was determined by the slope of the standard curve and kept between 90 and 110%.
Table 3. Primers for qPCR analysis.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>T (°C)</th>
<th>Primer Efficiency (%)</th>
<th>Forward Primer (5’ → 3’)</th>
<th>Reverse Primer (5’ → 3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>56/61</td>
<td>99/96</td>
<td>GAC ATG CCG CCT GGA GAA AC AGC CCA GGA TGC CCT TTA GT</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>5-HT1A</td>
<td>56</td>
<td>89</td>
<td>AAG AAG AGC CTG AAC GGA CAG AGG AAG GTG CTC TTT GG</td>
<td>171</td>
<td></td>
</tr>
<tr>
<td>ADR2A</td>
<td>63</td>
<td>98</td>
<td>CGG CAA TAG CAG CTG GAA CG TTG CCA AAC ACG GTG AAC AG</td>
<td>129</td>
<td></td>
</tr>
</tbody>
</table>

Forward and reverse primers are shown for GAPDH, 5-HT1A (5-HT1A), and α-adrenergic2A receptor (ARD2A). Annealing temperature (T), primer efficiency, and product length are also shown.
**Immunohistochemistry**

Serotonin immunoreactivity in caudal raphe nuclei was determined in a subset of rats injected with AAV vectors to rule out an effect of infection of caudal raphe serotonin cells with virus as a cause of observed physiological effects. One out of every six 40 µm sections were washed with 0.05 M potassium phosphate buffered saline (KBPS, 6 times for 10 minutes) then incubated in rabbit anti-serotonin primary antibody (1:80,000 in 0.4% Triton X-100 in KBPS; Immunostar) for one hour at room temperature followed by a 48 hour incubation at 4°C. Sections were then washed with 0.05 M KPBS (10 times for 6 minutes) then incubated in goat anti-rabbit Dylight-649 (1:500, Jackson ImmunoResearch) for 3 hours at 37°C. Following secondary antibody incubation, sections were washed a final time (10 times for 6 minutes), mounted on gelatin-coated slides, and coverslipped using Fluromount mounting media (Sigma). Once coverslipped, sections were immediately stored at 4° for 14 days prior to imaging.

**Statistical Analysis**

Two-way ANOVA with repeated measures were used to determine the effects of viral injection on cardiovascular and sympathetic measures as well as blood gas variables over time. Holm-Šidák post-hoc tests were used to determine group differences at specific time points (i.e. every minute for the first 10 minutes, then every 5 minutes until the end of the recovery period). P values of <0.05 were considered significant. Effect of viral injections on gene expression was determined by unpaired Student’s t-tests. When group data
failed the Shipiro-Wilk normality test, the Mann-Whitney U Rank Sum Test was used instead. All qPCR results are expressed as the group mean ± SEM.

**Results**

**shRNA screening**

Images of mCherry and hrGFP positive HEK293 cells are shown after transfection with 5 unique sequences targeting various portions of the rat 5-HT\textsubscript{1A} receptor gene (1ARshRNA #1-5) or an shRNA sequence targeting the rat tryptophan hydroxylase 2 gene (TPHshRNA) as a control (Figure 6). Of the 5 sequences screened, only 1ARshRNA #2 significantly decreased expression of the 5-HT\textsubscript{1A} mCherry fusion protein as shown by reduced fluorescence intensity compared to cells transfected with TPHshRNA control (P < 0.01).

Semi-quantitative PCR also showed a decrease in 5-HT\textsubscript{1A} receptor message in cells transfected with 1AshRNA #2 (Figure 7A; P < 0.05). Our custom designed polyclonal rabbit 5-HT\textsubscript{1A} rabbit antibody identified a ~46 Kd protein that was present in HEK293 cells transfected with the full length rat 5-HT\textsubscript{1A} receptor, but not in untransfected cells (Figure 7B). This size is consistent with the expected size of the unmodified rat 5-HT\textsubscript{1A} receptor. Whole cell lysates from cells transfected with the full length 5-HT\textsubscript{1A} receptor and 1AshRNA#2 showed almost no labeled protein of this size, while cells transfected with the receptor and the shRNA sequence targeting TPH continued to express the identified protein. 1AshRNA#2 produced a 97% knockdown of 5-HT\textsubscript{1A} receptor protein compared to the TPHshRNA (P <0.01). Together, these experiments
Figure 6. *In vitro* screening of shRNA sequences targeting the rat 5-HT$_{1A}$ receptor. HEK-293 cells co-transfected with Cherry-5-HT$_{1A}$R and one of 5 different 1ARshRNA sequences or a control sequence targeting the tryptophan hydroxylase gene, TPHshRNA (left). Each sequence was compared to its own separate control. Average fluorescent intensity (FI) of mCherry determined from each plate are also shown (right). Independent experiments were repeated 3 times on separate occasions. Values are group means ± SEM. Student’s t-test was used to analyze FI of ScramshRNA vs 1ARshRNA#1-5. Only 1ARshRNA #2 had a significant reduction in FI compared to TPHshRNA [df = 4, t = 7.60, **P <0.01]
Figure 7. The 5-HT\textsubscript{1A} receptor shRNA #2 reduces 5-HT\textsubscript{1A} receptor mRNA and protein expression \textit{in vitro}. A) The cell lysates from HEK 293 cells transfected with 1ARshRNA #2 and TPHshRNA were used to determine relative 5-HT\textsubscript{1A}R mRNA levels via semi-quantitative RT-PCR (normalized to GAPDH). Representative blot of protein obtained from cells transfected with TPHshRNA and 1ARshRNA #2 (left) and mean densitometry data of 5-HT\textsubscript{1A}R mRNA from 6 independent experiments (right). Student’s t-test was used to compare TPHshRNA vs 1ARshRNA, *P<0.05. B) Immunoblot illustrating custom 5-HT\textsubscript{1A}R antibody specificity in untransfected (UT) and transfected HEK-293 cells. C) Representative immunoblot of 5-HT\textsubscript{1A}R protein levels in cells transfected with 1ARshRNA #2 or TPHshRNA transfected cells (top) and mean densitometry data (bottom) of 5-HT\textsubscript{1A}R protein from 3 independent experiments which show reduced 5-HT\textsubscript{1A}R protein expression in cells treated with 1ARshRNA #2 vs TPHshRNA. Student’s t-test [df = 4, t = 4.71, **P <0.01].
identified one shRNA vector (1ARshRNA #2) that was found to efficiently knock down 5-HT$_{1A}$ receptor expression in cultured cells.

Four weeks after *in vivo* injection of viruses encoding hrGFP and the efficient 1ARshRNA sequence or a scrambled version of the sequence, the hrGFP transgene was highly expressed in the NTS of injected rats (Figures 8 and 9). Evidence of hrGFP expression in the caudal raphe regions was identified, but was not extensive (Figure 9). However, there was no apparent hrGFP expression in serotonin-positive cells of the caudal raphe region. Nor was there hrGFP expression in or around the DR, an area enriched with 5-HT$_{1A}$ receptor (data not shown). Approximately 50% and 30% reductions in 5HT$_{1A}$ receptor mRNA levels were found in the commissural NTS and mNTS respectively in rats injected with 1ARshRNA compared to ScramshRNA (Figure 10A-C, P < 0.01). The $\alpha$AR$_{2A}$ receptor levels did not differ between groups in either the commissural NTS or mNTS injection sites (Figure 10D).

**Effects of 5-HT$_{1A}$ receptor knockdown on cardiovascular variables during hypotensive hemorrhage**

Treatment had no effect on body weight (358 ± 5 vs. 368 ± 5 g for ScramshRNA and 1ARshRNA respectively) prior to the start of hemorrhage. All rats subjected to acute hypovolemic hemorrhage showed a characteristic multiphasic response to blood withdrawal (Figures 11 and 12). Specifically, both groups displayed a short normotensive phase characterized by augmented
**Figure 8. AAV injection sites.** A) Schematic, modified from Mitchell et al. 1975, shows dorsal view of the rat brain stem modified with numbers to indicate location of injection sites of AAV.  
B) Location of injection sites relative to calamus scriptorius (top), and schematic, modified from Scott et al, 2000, showing coronal brainstem sections and location of injection in medial NTS (middle panel) and commissural NTS (lower panel).  
C) Fluorescent micrographs demonstrating neuronal infection (hrGFP expression) in rats injected with AAVs encoding either the scrambled shRNA (left) or an shRNA targeting the rat 5-HT$_{1A}$ receptor (right).  
mNTS, medial NTS; cNTS, commissural NTS; AP, area postrema; DMVX, dorsal motor nucleus of the vagus; XII, hypoglossal nucleus.
Figure 9. Representative coronal rat brain sections after immunohistochemical label of serotonin. Green fluorescence indicates hrGFP expression or AAV infection and red fluorescence represents serotonin (5-HT) expression. Cells infected with virus encoding the ScramshRNA or the 1ARshRNA were surrounded by 5-HT-positive nerve terminals in the commissural NTS (left panels). 5-HT immunoreactive cells of the RO (center panels) and those of the RM (right panels) were not infected with virus.
Figure 10. 5-HT<sub>1A</sub> receptor shRNA selectively and discretely reduces relative 5-HT<sub>1A</sub> receptor gene expression at the target site in vivo.
Representative qPCR amplification curves for ScramshRNA (blue) and 1ARshRNA (red) samples from RO (A) and commissural NTS (cNTS, B) show knockdown in cNTS but not RO. C) Relative 5-HT<sub>1A</sub> mRNA levels were normalized to GAPDH and calculated as fold change from the average 5-HT<sub>1A</sub> expression of the corresponding brain regions in the rats injected with ScramshRNA. Values are group means ± SEM. Student’s t-test was used to assess differences in 5-HT<sub>1A</sub> receptor mRNA levels between ScramshRNA vs 1ARshRNA in different brain regions. 5-HT<sub>1A</sub> receptor expression was significantly decreased in the commissural NTS [df = 17, t = 6.66, P < 0.01] and mNTS [df = 17, t = 4.71, P < 0.01], but not in the hypoglossal nucleus [df = 16, t = 0.60, P = 0.56], RO [df = 15, t = 1.23, P = 0.24] or DR [df = 13, t = 0.29, P = 0.78]. D) Relative mRNA expression of αAR<sub>2A</sub> receptor in cNTS and mNTS. Values are group means ± SEM. Mann-Whitney U and Student’s t-test revealed no significant differences on the relative αAR<sub>2A</sub> receptor population in the cNTS [U = 22, t = 62, p = 0.54] and mNTS [df = 13, t = -1.04, p = 0.32] respectively in 1ARshRNA vs ScramshRNA rats.
Figure 11. Representative sample recordings during hypotensive hemorrhage in ScramshRNA- and 1ARshRNA-injected rats. Representative sample recordings (2.5 seconds) of arterial pressure (AP), raw renal sympathetic nerve activity (RSNA) and integrated activity (int. RSNA) in individual ScramshRNA (A) and 1ARshRNA injected rats (B). Data are shown prior to hemorrhage (baseline), 3 minutes after start of hemorrhage (peak compensation), and 8, 20 and 60 minutes after start of hemorrhage, and after injection with the ganglionic blocker hexamethonium chloride (Hex).
Figure 12. Mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (RSNA) during hemorrhage and subsequent recovery in ScramshRNA- and 1ARshRNA-injected rats. The MA, HR and RSNA during hemorrhage (gray shaded area) and subsequent recovery in rats injected with AAV encoding ScramshRNA or 1ARshRNA. Values are group means ± SEM. Number of subjects for ScramshRNA were 8 for RSNA and 16 for MAP and HR measurements respectively. Group number for rats treated with 1ARshRNA were 6 for RSNA and 15 for MAP and HR respectively.

Two-way ANOVA with repeated measures that examined the effect of ShRNA treatment and Time on cardiovascular variables revealed an interaction between ShRNA treatment and Time [F (1,144) = 3.06, P < 0.001] on RSNA responses. Post-hoc Holm-Sidak analyses performed at each time point revealed a significant reduction of RSNA among 1AshRNA-treated rats compared to ScramshRNA-treated controls throughout recovery, but not during hemorrhage itself. *P <0.05 and ** P< 0.01 vs. ScramshRNA treated rats.
sympathetic activity and reflex tachycardia. After withdrawal of approximately 9-12 ml/kg of estimated blood volume, a rapid decline in BP, HR, and RSNA developed in both groups. After termination of blood withdrawal, BP and HR recovered over the next 10-15 minutes before reaching a plateau. Treatment with the 1ARshRNA did not affect either baseline BP or HR, or the response to, and subsequent recovery from blood withdrawal (Figure 11). Among animals treated with the ScramshRNA, RSNA recovered following termination of blood withdrawal in parallel with BP and HR. The RSNA of rats treated with ScramshRNA exceeded baseline levels and stabilized at a plateau approximately 40-50% above baseline. In contrast, RSNA of rats treated with 1ARshRNA did not fully return to baseline levels and stabilized at levels approximately 20% below baseline for the remainder of the experiment. Rats treated with the 1ARshRNA had reduced RSNA (relative to their own baseline) compared to rats treated with the ScramshRNA during recovery (P<0.01).

Spontaneous baroreflex activity did not differ between the two treatment groups (Table 4). Similarly, hematocrit and protein plasma levels were comparable between groups throughout the experiment (Table 5). Lactate levels were significantly elevated in the 1ARshRNA group compared to the ScramshRNA group 60 minutes after the start of hemorrhage (Figure 13, P <0.01), but there were no further differences in the acid-base balance or blood gas variables between groups (Table 6).
Table 4. Spontaneous baroreflex sensitivity data from baseline recordings in 1ARshRNA and ScramshRNA injected rats

<table>
<thead>
<tr>
<th>shRNA treatment</th>
<th>Up Sequences (ms/mmHg)</th>
<th>Down Sequences (ms/mmHg)</th>
<th>All Sequences (ms/mmHg)</th>
<th>Alpha (LF)</th>
<th>Alpha (HF)</th>
<th>LF Power (mmHg²)</th>
<th>BP SDNN</th>
<th>LF RRI (nu)</th>
<th>HF RRI (nu)</th>
<th>LF - to - HF ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scram (10)</td>
<td>1.89 ± 0.32</td>
<td>1.49 ± 0.21</td>
<td>1.76 ± 0.26</td>
<td>0.83 ± 0.10</td>
<td>1.98 ± 0.37</td>
<td>13.95 ± 1.54</td>
<td>4.54 ± 0.47</td>
<td>43.22 ± 5.42</td>
<td>41.76 ± 4.82</td>
<td>1.35 ± 0.32</td>
</tr>
<tr>
<td>SBP</td>
<td>2.18 ± 0.32</td>
<td>2.06 ± 0.26</td>
<td>2.13 ± 0.27</td>
<td>0.90 ± 0.11</td>
<td>2.36 ± 0.38</td>
<td>11.88 ± 1.07</td>
<td>4.21 ± 0.43</td>
<td>43.22 ± 5.42</td>
<td>41.76 ± 4.82</td>
<td>1.35 ± 0.32</td>
</tr>
<tr>
<td>MBP</td>
<td>2.30 ± 0.28</td>
<td>1.93 ± 0.25</td>
<td>2.13 ± 0.25</td>
<td>0.91 ± 0.12</td>
<td>2.20 ± 0.30</td>
<td>11.29 ± 0.68</td>
<td>4.21 ± 0.40</td>
<td>43.22 ± 5.42</td>
<td>41.76 ± 4.82</td>
<td>1.35 ± 0.32</td>
</tr>
<tr>
<td>DBP</td>
<td>2.56 ± 0.15</td>
<td>2.03 ± 0.11</td>
<td>2.25 ± 0.10</td>
<td>0.67 ± 0.06</td>
<td>2.67 ± 0.29</td>
<td>11.29 ± 1.88</td>
<td>4.09 ± 0.40</td>
<td>27.71 ± 6.50</td>
<td>57.76 ± 7.50</td>
<td>0.65 ± 0.28</td>
</tr>
<tr>
<td>1AR (6)</td>
<td>2.28 ± 0.18</td>
<td>1.88 ± 0.62</td>
<td>2.55 ± 0.14</td>
<td>0.61 ± 0.04</td>
<td>2.87 ± 0.51</td>
<td>13.55 ± 2.52</td>
<td>4.33 ± 0.48</td>
<td>27.71 ± 6.50</td>
<td>57.76 ± 7.50</td>
<td>0.65 ± 0.28</td>
</tr>
<tr>
<td>SBP</td>
<td>2.54 ± 0.13</td>
<td>2.46 ± 0.22</td>
<td>2.20 ± 0.32</td>
<td>0.65 ± 0.06</td>
<td>3.18 ± 0.39</td>
<td>11.84 ± 2.02</td>
<td>4.09 ± 0.42</td>
<td>27.71 ± 6.50</td>
<td>57.76 ± 7.50</td>
<td>0.65 ± 0.28</td>
</tr>
<tr>
<td>MBP</td>
<td>2.56 ± 0.15</td>
<td>2.03 ± 0.11</td>
<td>2.25 ± 0.10</td>
<td>0.67 ± 0.06</td>
<td>2.67 ± 0.29</td>
<td>11.29 ± 1.88</td>
<td>4.09 ± 0.40</td>
<td>27.71 ± 6.50</td>
<td>57.76 ± 7.50</td>
<td>0.65 ± 0.28</td>
</tr>
</tbody>
</table>

Spectral analyses were performed in the low frequency (LF) and high frequency (HF) domains. Number of animals is shown in parentheses. Systolic (SBP), mean (MBP), and diastolic (DBP) blood pressure. Data are group means ± SEM. Student’s t-test were used to compare group means for ScramshRNA and 1ARshRNA treated groups. No significant differences were identified.
Table 5. Hematocrit and plasma protein concentrations before and after hypotensive hemorrhage in ScramshRNA and 1ARshRNA rats

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Hematocrit (%)</th>
<th>Plasma Protein (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SCRshRNA</strong></td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td></td>
<td>43 ± 1</td>
<td>6.3 ± 0.15</td>
</tr>
<tr>
<td>10 min</td>
<td></td>
<td>33 ± 1”</td>
<td>4.7 ± 0.1”</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>33 ± 1”</td>
<td>4.9 ± 0.1”</td>
</tr>
<tr>
<td><strong>1ARshRNA</strong></td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td></td>
<td>41 ± 2</td>
<td>6.2 ± 0.1</td>
</tr>
<tr>
<td>10 min</td>
<td></td>
<td>31 ± 2”</td>
<td>4.6 ± 0.1”</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>30 ± 2”</td>
<td>4.9 ± 0.1”</td>
</tr>
</tbody>
</table>

Values are group means ± SEM. Two-way ANOVA with repeated measures were used to examine the effect of ShRNA treatment and Time on hematocrit and protein plasma. No interactions were found. The main effect of Time were followed up by Holm-Šidák post-hoc analysis. Both plasma protein concentrations [F (2,21) = 246, P<0.001] and hematocrit levels [F (2, 21) = 531, P < 0.01] fell by 10 minutes after start of hemorrhage and both remained below baseline by 60 minutes after start of hemorrhage **P < 0.01 vs 0 minutes within group.
Table 6. Effects of hemorrhage on venous blood gas variables and acid-base status in ScramshRNA- and 1ArshRNA-injected rats

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>pH</th>
<th>pCO₂ (mmHg)</th>
<th>pO₂ (mmHg)</th>
<th>BE (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCRshRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>13</td>
<td>7.49 ± 0.01</td>
<td>43.31 ± 0.44</td>
<td>34.85 ± 1.58</td>
<td>10.00 ± 0.66</td>
</tr>
<tr>
<td>10 min</td>
<td>13</td>
<td>7.44 ± 0.01*</td>
<td>42.35 ± 0.82</td>
<td>26.62 ± 1.38*</td>
<td>4.46 ± 0.55*</td>
</tr>
<tr>
<td>60 min</td>
<td>13</td>
<td>7.46 ± 0.01**ː†</td>
<td>46.19 ± 0.54**ː‡</td>
<td>31.69 ± 1.14**ː‡</td>
<td>9.00 ± 0.78**ː‡</td>
</tr>
<tr>
<td>1ARshRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>13</td>
<td>7.50 ± 0.01</td>
<td>42.52 ± 0.66</td>
<td>34.85 ± 1.49</td>
<td>10.08 ± 0.70</td>
</tr>
<tr>
<td>10 min</td>
<td>13</td>
<td>7.44 ± 0.01*</td>
<td>41.89 ± 0.96</td>
<td>27.92 ± 1.14*</td>
<td>4.08 ± 1.06*</td>
</tr>
<tr>
<td>60 min</td>
<td>13</td>
<td>7.45 ± 0.01**ː†</td>
<td>46.40 ± 0.76**ː‡</td>
<td>31.85 ± 1.64**ː‡</td>
<td>8.00 ± 1.12**ː‡</td>
</tr>
</tbody>
</table>

Values are group means ± SEM. Two-way ANOVA with repeated measures were used to compare ShRNA Treatment and Time on blood gas variables and acid-base status. There were no group differences observed at any time point. Main effects of Time only on pH [F (2, 25) = 57.91, P < 0.001], pCO₂ [F (2, 25) = 36.33, P < 0.001], pO₂ [F (2, 25) = 19.88, P < 0.001] and BE [F (2, 25) = 121.82, P < 0.001] were all due to reductions in all variables with hemorrhage and/or subsequent recovery by 60 min. The main effects of Time were followed up by post-hoc analysis using Holm-Šidák, * P < 0.05 vs 0 min within group, ** P < 0.01 vs 0 min within group, † P < 0.05 vs 10 min, and ‡ P < 0.01 vs 10 min within group.
Figure 13. Lactate levels at baseline, after hemorrhage termination and recovery. Data were analyzed by 2-way ANOVA with ShRNA treatment and repeated measures of Time as factors. There were significant effects of shRNA treatment \(F (1,25) = 5.16, P < 0.05\) due to higher lactate levels overall in 1ARshRNA treated rats. A main effect of Time \(F (2,25) = 62.1, P < 0.01\) was due to the rise in lactate with hemorrhage, and the subsequent fall with recovery.
5-HT₁A receptor knockdown correlates with sympathetic recovery and acid-base status following hypotensive hemorrhage

5-HT₁A mRNA expression in 1ARshRNA-treated rats ranged from 20-80% of levels found in rats treated with ScramshRNA. To determine if the degree of 5-HT₁A receptor knockdown was related to sympathetic compensation from hemorrhage, RSNA and lactate levels determined 60 min after the start of hemorrhage were correlated with 5-HT₁A mRNA gene expression. Significant positive correlations between 5-HT₁A receptor mRNA and RSNA in both commissural NTS and mNTS injection sites were observed (Figure 14A; Spearman coefficient = 0.90 and 0.79, r² = 0.82 and 0.63, P < 0.01). Lactate was negatively correlated with 5-HT₁A receptor mRNA in the commissural NTS, but not in the mNTS (Figure 14B; Spearman coefficient -0.82 vs -0.23, r² = 0.67 vs 0.05, P= 0.01 vs P= 0.42).

Discussion

In the present study, we investigated the role of 5-HT₁A receptor expression in the commissural NTS and mNTS on compensation from hypotensive hemorrhage in unanesthetized rats. We found that rats with 30-50% knockdown of 5-HT₁A receptors in the commissural NTS and mNTS showed an attenuated recovery of sympathetic activity following a moderate level and rate of blood loss (~21% of estimated blood volume over 10 minutes), but showed no differences in BP or HR responses to hemorrhage. The extent of 5-HT₁A
Figure 14. Relationship of 5-HT_{1A} receptor mRNA levels in the commissural NTS to sympathetic recovery and lactate levels. 5-HT_{1A} receptor gene expression in the commissural NTS is directly related to sympathetic recovery and inversely related to lactate levels. A) A Spearman correlation between relative 5-HT_{1A} receptor mRNA expression and RSNA 60 min after the start of hemorrhage in commissural NTS (cNTS; left) and mNTS (right). B) Spearman correlation data between the relative mRNA levels of 5-HT_{1A}R and lactate production 60 minutes after the start of hemorrhage in the cNTS (left) and mNTS (right).
receptor knockdown was also related to the level of lactic acidosis and RSNA activity 50 minutes after termination of hemorrhage. This relationship was most robust when receptor expression in the more caudal commissural subnucleus of the NTS was examined. Together, the data indicate that sufficient expression of 5-HT<sub>1A</sub> receptors in the commissural NTS is necessary for normal sympathetic compensation following moderate blood loss, and that this receptor population contributes to oxygen delivery to peripheral tissue during compensation from hypotensive hemorrhage.

These results extend our previous findings that selective destruction of the serotonin neurons in the caudal raphe attenuates sympathetic recovery following hypotensive hemorrhage and exaggerates metabolic acidosis. Such lesions reduced serotonin innervation of the caudal NTS by 50% and the RVLM by almost 90%. Despite the use of the same protocol for hemorrhage, the caudal raphe lesioned animals from the prior study showed more severe lactic acidosis as well as reduced arterial blood pH. In contrast, animals subjected to 5-HT<sub>1A</sub> receptor knockdown in the current study did not show significant deficits in pH buffering. Clinical evidence indicates that pH does not correlate with lactate until lactate values exceed 5.0 mM, as was seen in the caudal raphe lesion study (Mizock and Falk, 1992). It is not known why the animals in this experiment experienced less severe tissue hypoxia than in our prior study given that the blood withdrawal protocols were the same. However, in the prior study, animals were also implanted with diaphragmatic EMG electrodes, which required a more invasive surgical procedure that could have potentially affected ventilation,
possibly resulting in an impaired buffering through expiration of CO₂.
Nevertheless, lactate is the first and best indication of deficits in tissue perfusion, and the present data indicate that 5-HT₁A receptors in the NTS contribute to neural responses that facilitate tissue perfusion following hemorrhage.

Alternatively, the greater metabolic acidosis seen with caudal raphe serotonin cell lesion may indicate that serotonin acting on additional receptor populations beyond the 5-HT₁A receptors of the NTS may also contribute to sympathetic-dependent changes in tissue perfusion. A more recent study from our laboratory would suggest that this is not likely the case since discrete lesion of serotonin nerve terminals in the caudal NTS also attenuates sympathetic recovery to the same extent found in the present study. However, blood gases and acid base balance were not examined in the NTS lesion study.

Rats injected with 1ARshRNA displayed the normal, multi-phasic response to blood loss, suggesting that 5-HT₁A receptors in the NTS are probably not involved in the initial sympathoexcitatory response or in the sympatholytic phase of hypotensive hemorrhage. Rather, 5-HT₁A receptor activation in the NTS appears to be necessary for normal secondary sympathoexcitatory compensation from blood loss. The presence of exaggerated lactic acidosis during this secondary sympathoexcitatory phase in 1ARshRNA-treated rats indicates that a deficit in oxygen delivery to peripheral tissue developed following termination of blood withdrawal in these animals. Furthermore, the correlation of lactic acid as well as sympathetic recovery with the extent of 5-HT₁A receptor knockdown suggests that sympathetic drive aids in peripheral tissue
oxygenation. Reduced oxygen delivery results from reduced oxygen in arterial blood and/or deficits in perfusion of peripheral tissue. In this study, we did not measure arterial blood gases. However, in the caudal serotonin cell lesion study PaO₂ was actually increased in lesioned animals, indicating that peripheral tissue hypoxia observed in this prior study was due to reduced perfusion rather than to a deficit in blood oxygenation. Here, we focused on blood gases measured in venous blood since they better reflect peripheral metabolic acidosis. No group differences were found in either PvO₂ or PvCO₂ at any point, suggesting that arterial blood oxygen may have been similar between groups during and after hemorrhage. While we cannot rule out the possibility that tissue O₂ extraction differed between groups and thus that PaO₂ differed between groups, given our prior results that extensive caudal serotonin cell lesion did not impair blood oxygenation, it seems more likely that the greater tissue hypoxia observed in 1ARshRNA-treated rats was due to reduced perfusion of peripheral tissue.

Our findings that BP responses were not different between groups despite evidence of perfusion deficits suggests that serotonin acts on 5-HT₁A receptors in the NTS to maintain CO. In the absence of these mechanisms, other potentially more damaging mechanisms of vasoconstriction may contribute to the maintenance of the BP. Toung et al, performed elegant studies that examined the degree to which circulating vasoconstrictor hormones and sympathetic activity contribute to arterial and venous constriction during blood loss. They noted that sympathetic activity contributes substantially more to venous constriction, while increases in angiotensin II and vasopressin contribute to BP
maintenance primarily by increasing arterial vasoconstriction (Toung et al., 2000). Together, these data suggest that endogenous serotonin released during hemorrhage acts on 5-HT$_{1A}$ receptors in the NTS to maintain CO through increased venoconstriction; this hypothesis remains to be tested. Nevertheless, this hypothesis is further supported by our prior studies which showed that administration of the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, into the 4$^{th}$ cerebroventricle of unanesthetized rats increased venous tone, CO and indices of tissue oxygenation. Moreover, these effects were found to be dependent upon sympathetic activity.

It is unclear how 5-HT$_{1A}$ receptors in the caudal NTS contribute to sympathoexcitation following hemorrhage. The decrease in 5-HT$_{1A}$ receptor mRNA levels had no effect on any index of spontaneous arterial baroreflex sensitivity. Therefore, it is unlikely that deficits in baroreflex sensitivity contributed to the attenuated sympathetic response in 1ARshRNA-treated rats during hemorrhage. Indeed, our prior study showed that serotonin nerve terminal lesion actually improved baroreflex sensitivity. The current study further extends these earlier findings by demonstrating that the enhanced baroreflex sensitivity that accompanies deficits in serotonin innervation of the dorsomedial portion of the caudal brainstem are likely not due to deficits in 5-HT$_{1A}$ receptor activation.

Of interest were findings that the 5-HT$_{1A}$ receptor gene expression in the commissural NTS correlated better with lactate levels or sympathetic recovery 50 minutes after termination of hemorrhage than did receptor expression in the mNTS. The commissural NTS receives a far greater density of nerve terminals
from peripheral chemoreceptors than the medial NTS, which itself receives more arterial baroreceptor terminals than the commissural NTS (Finley and Katz, 1992; Housley et al., 1987; Mifflin, 1992). Our prior work showed that the sympathoexcitatory effect of 5-HT$_{1A}$ receptor agonist administration in hemorrhaged animals was severely attenuated following sinoaortic denervation, indicating that 5-HT$_{1A}$ receptor-mediated increases in sympathetic activity after hemorrhage are dependent on arterial baroreceptors and/or peripheral chemoreceptor input (Osei-Owusu and Scrogin, 2006). Together, these findings are highly suggestive of the possibility that 5-HT$_{1A}$ receptors facilitate peripheral chemoreceptor afferent activation of sympathetic drive during hemorrhage.

A limitation of this study is the possibility that our results are due to unintentional effects of viral injection due either to down regulation of 5-HT$_{1A}$ receptor populations remote from the site of injection or by altering gene expression of entirely different receptors that could influence sympathetic drive during hemorrhage. Post-mortem assessments of 5-HT$_{1A}$ receptor gene expression confirmed that knockdown of the receptor was localized to the injection sites. We did not observe significant reductions in gene expression ventral to the injection site in the hypoglossal nucleus. Nor did we observe decreases in 5-HT$_{1A}$ receptor gene expression in sites remote from the injection that are enriched in 5-HT$_{1A}$ receptor such as the DR. We also assessed the possibility that retrograde transport of the virus and subsequent expression of the shRNA in serotonin cells that project to the NTS from the RO and RM might have contributed to deficits in compensation during hemorrhage by reducing
expression of 5-HT$_{1A}$ autoreceptors. While we noted some hrGFP expression around the serotoninergic cells of the RO and RM, we did not see co-localization of hrGFP and serotonin label in either of these regions. Furthermore, 5-HT$_{1A}$ receptor gene expression in the RO, which provides the densest serotoninergic projections to the caudal NTS, was not affected by 1ARshRNA treatment.

We also explored the possibility that the attenuated sympathetic recovery exhibited by rats injected with virus encoding the 1ARshRNA was due to compensatory gene expression of other receptors within the NTS. Previous investigations have found that αAR$_{2A}$ receptors in the caudal NTS facilitate central integration of the peripheral chemoreflex (Hayward, 2001). Therefore, we examined gene expression of the rat αAR$_{2A}$ in the NTS and found no effect of treatment.

Results from this first study were incorporated into a working model that illustrates the major findings of Aim 1 (Figure 15). In summary, results from the current study identified a role for 5-HT$_{1A}$ receptor activation in the sympathetic recovery that follows hypotensive hemorrhage. Additionally, we found that 5-HT$_{1A}$ receptor activation in the caudal NTS facilitates peripheral tissue perfusion. Although the mechanism by which 5-HT$_{1A}$ receptor activation promotes recovery from blood loss remains unknown, it is recognized that activation of 5-HT$_{1A}$ receptors hyperpolarizes neurons. Thus 5-HT$_{1A}$ receptor activation may disinhibit neurons that normally suppress recovery of sympathetic drive during compensation from hypotensive hemorrhage. We speculate that as blood loss progresses, serotonin is released in the NTS where it activates 5-HT$_{1A}$ receptors,
which in turn, disinhibit inhibitory interneurons that suppress sympathetic drive to the venous vasculature that mobilizes blood stores. The precise mechanism(s) that stimulate serotonin release in the NTS remain unknown.
Results from Aim 1 identified a role for 5-HT$_{1A}$ receptor activation in the sympathetic recovery from hemorrhage and facilitates peripheral tissue oxygenation. Since activation of 5-HT$_{1A}$ receptors produces hyperpolarization of neurons, it is plausible that 5-HT$_{1A}$ receptor activation may disinhibit GABA neurons that normally suppress recovery of sympathetic drive during compensation from hypotensive hemorrhage. Therefore, during blood loss serotonin is released in the NTS where it activates 5-HT$_{1A}$ receptors, which in turn, disinhibit GABA interneurons that suppress sympathetic. The precise mechanism(s) that stimulate serotonin release in the NTS remain unknown.
CHAPTER IV

5-HT$_1$A RECEPTORS IN THE NUCLEUS TRACTUS SOLITARIUS ARE RESPONSIBLE, IN PART, FOR THE SYMPATHOEXCITATORY AND PRESSOR EFFECTS OF 8-OH-DPAT ADMINISTRATION DURING HYPOTENSIVE HEMORRHAGE

Abstract

In prior work outside of this dissertation, we demonstrated that administration of the full 5-HT$_1$A receptor agonist, 8-OH-DPAT, promotes a sympathetically-mediated increase in BP in rats subjected to hypotensive hemorrhage or more severe hemorrhagic shock. The sympathoexcitatory effect of 8-OH-DPAT was found to be more pronounced when the drug was administered centrally into the 4$^{th}$ cerebroventricle and was dependent upon intact sinoaortic nerve innervation. Arterial baroreceptors and peripheral chemoreceptor afferents of the sinoaortic nerves innervate the dorsomedial brainstem in the caudal NTS near the site where 8-OH-DPAT was found to have the most pronounced hemodynamic effects. These findings led us to hypothesize that 8-OH-DPAT activates 5-HT$_1$A receptors located in the caudal NTS to mediate its BP and sympathoexcitatory effects in hypovolemic rats. In this study, we injected the 5-HT$_1$A shRNA AAV or the scrambled AAV control
virus into the caudal NTS of male Sprague Dawley rats to test this hypothesis. After 4 weeks, rats treated with the 1ARshRNA-encoding AAV showed a slowed pressor response to 8-OH-DPAT compared to rats injected with virus encoding the scrambled shRNA (68 ± 4 vs 87 ± 3 mmHg, 10 min after start of hemorrhage, P<0.01). 5-HT\textsubscript{1A} receptor gene expression in the commissural NTS, but not the medial NTS or underlying hypoglossal nucleus, was inversely correlated with the latency of BP to recover to 75% of baseline after 8-OH-DPAT injection. Successful knockdown of the 5HT\textsubscript{1A} receptor also attenuated the sympathoexcitatory effect of 8-OH-DPAT (-41.6 ± 13.6 vs 77.1 ± 22.0 % of baseline, 1 minute after 8-OH-DPAT administration, P < 0.05). The sympathoexcitatory response to 8-OH-DPAT was directly correlated with 5-HT\textsubscript{1A} receptor expression in the NTS (P < 0.05). Together, these data indicate that the pressor and sympathoexcitatory effects of 8-OH-DPAT administration observed in hypovolemic rats are due, at least in part, to activation of 5-HT\textsubscript{1A} receptors in the caudal NTS.

**Introduction**

Previous studies from our laboratory have identified a role for 5-HT\textsubscript{1A} receptor agonists to facilitate compensation from blood loss. As detailed in Chapter 2, the administration of 5-HT\textsubscript{1A} receptor agonists, including the full agonist, 8-OH-DPAT, produces a hemodynamically favorable outcome when administered to rats during hypotensive hemorrhage. Moreover, the sympathoexcitatory and pressor effects of 8-OH-DPAT are reversed by
Subsequent studies utilizing a more severe hemorrhagic shock model (≈50% estimated blood volume) showed that 8-OH-DPAT increased venous tone (as measured by mean circulatory filling pressure [MCFP]) and venous return, and reversed lactic acidosis in unanesthetized rats (Tiniakov et al., 2007; Tiniakov and Scrogin, 2006). Increases in BP following 8-OH-DPAT administration were due almost exclusively to an increase in CO, with virtually no effect on TPR. From these studies we concluded that 8-OH-DPAT promotes sympathetic drive to preferentially augment venoconstriction and mobilize venous blood back to the heart, and thereby improve tissue perfusion. Mobilization of venous blood stores during hemorrhage is primarily mediated by the vascular beds of the splanchnic region (Greenway and Lister, 1974; Reilly et al., 2001). Accordingly, the rise in MCFP mediated by 8-OH-DPAT is abolished by sympathetic denervation of the splanchnic vasculature, as is the protective effect of 8-OH-DPAT against lactic acidosis (Tiniakov et al., 2012).

Prior work also suggests that 8-OH-DPAT accelerates recovery from hemorrhage by an agonist action on 5-HT₁A receptors. In these studies, the selective 5-HT₁A receptor antagonist, WAY-100635, given prior to hemorrhage, dose-dependently inhibited the ability of 8-OH-DPAT to slow the hypotensive and sympatholytic response to blood loss. However, WAY-100635 and 8-OH-DPAT have both been shown to possess affinity for other receptor types including αAR₂A receptors (Bonaventure et al., 2004; Sprouse et al., 2004). The αAR₂A
receptors located in the caudal NTS have been implicated in sympathetic and ventilatory responses to chemoreflex activation by chemical hypoxia (Hayward, 2001). A more rostral $\alpha\text{AR}_{2A}$ receptor population located in the RVLM is also known to inhibit sympathetic activity by suppression of bulbospinal barosensitive neurons (Allen and Guyenet, 1993; Hayar and Guyenet, 1999). 8-OH-DPAT also has significant agonist activity on 5-HT$_7$ receptors which are also expressed in the NTS (Gustafson et al., 1996; Sprouse et al., 2004; To et al., 1995). Therefore, it is conceivable that 8-OH-DPAT mediates its actions during hemorrhage through either an agonist or antagonist effect on 5-HT$_7$ or $\alpha\text{AR}_{2A}$ receptors.

It is not clear where in the brainstem 8-OH-DPAT mediates its sympathoexcitatory effects in hemorrhaged animals. Our prior work showed that 8-OH-DPAT produced the most robust compensatory effects when injected into the cisterna magna, just above the floor of the 4$^{th}$ ventricle where the dorsal surface of the caudal NTS is located (Scrogin, 2003). Evidence that the caudal NTS receives sinoaortic afferent input and that severing these same afferents attenuates the sympathetic and BP effects of 8-OH-DPAT led us to speculate that 8-OH-DPAT might act within the NTS to mediate its beneficial effects in hypovolemic animals. Moreover, our prior work suggests that endogenous serotonin, released during hemorrhage, also contributes to sympathetic compensation following hemorrhage in a manner parallel to that of 8-OH-DPAT (Kung et al., 2010). Our evidence that serotonin nerve terminal lesion in the NTS had the opposite effect of 8-OH-DPAT (i.e., delayed sympathetic recovery) led us
to further speculate that both endogenous serotonin and 8-OH-DPAT act on the same receptor population to mediate their effects (Kung and Scrogin, 2011). Therefore, we proposed that 8-OH-DPAT acts on 5-HT$_{1A}$ receptors in the caudal NTS to mediate its pressor and sympathoexcitatory effects during hypotensive hemorrhage.

In unpublished studies, we noted that 8-OH-DPAT did not affect BP in hemorrhaged rats under any anesthesia so far tested including: urethane, $\alpha$-chloralose, isoflurane, sodium pentobarbital, ketamine, chloral hydrate or thiobarbital. The mechanisms behind the insensitivity to 8-OH-DPAT in anesthetized rats is unclear. Nevertheless this anesthetic effect has complicated efforts to identify the specific brain region where 8-OH-DPAT mediates its action, particularly since the caudal hindbrain is difficult to access by chronic cannulation. This complication, together with the evidence that 8-OH-DPAT has affinity for several different receptor subtypes led us to utilize highly specific shRNA knockdown technology as an approach to determine the location and receptor population on which 8-OH-DPAT acts to mediate its effects. Here, we utilized AAV delivery of the 5-HT$_{1A}$ shRNA to selectively and discretely reduce 5-HT$_{1A}$ receptor expression in the caudal NTS in order to determine whether 8-OH-DPAT acts on the 5-HT$_{1A}$ receptors to mediate its pressor and sympathetic effects in unanesthetized hemorrhaged rats.
Methods

Animals

Experiments were performed on 27 Male Sprague-Dawley rats (275 to 300 g; Harlan, Indianapolis, IN) which were acclimated to the institutional animal facility under standard conditions (22°C ambient temperature, 12:12 hour light cycle) with food and water available ad libitum for at least 1 week prior to surgery. All experimental protocols were approved by the Institutional Animal Care and Use Committee and conducted in compliance with the Principles of Laboratory Animal Care, as adopted and promulgated by the United States National Institutes of Health.

Surgical procedures

AAV injections

Prior to surgery, AAV vectors were diluted in viral storage buffer [400nM NaCl, 20 mM Tris base, (1x10^{12} vg/ml)] and dialyzed in 0.9% saline (Slide-a-Lyzer, ThermoScientific) as described in Chapter 3. Rats were anesthetized with ketamine/xylazine (85/13mg/kg, im) and placed in a stereotaxic apparatus with the nose bar positioned 11 mm below a flat skull plane then AAV vectors were injected as described previously in Chapter 3. Animals were then allowed to recover for four weeks prior to hemorrhage experiments.
Vascular catheter and renal sympathetic recording electrode implantation

The day before hemorrhage experiments, rats were anesthetized with isoflurane (5% for induction and 1.5-2.5% for maintenance) in 100% O₂ (1L min⁻¹) and instrumented with catheters and a renal sympathetic recording electrode as described in Chapter 3. The electrode lead wires were tunneled under the skin to exit at the nape of the neck. Rats were given ampicillin (150 mg/kg sc) antibiotic and ketoprofen (5 mg/kg sc) for analgesia and allowed to recover overnight. Experiments were conducted the day after surgery.

Experimental Protocols

Hypotensive hemorrhage

All rats were subjected to hypotensive hemorrhage conducted as described previously (Kung and Scrogin, 2011) and in detail in Chapter 3 with modifications described below. Briefly, arterial lines (PE-50 tubing fused with PE-10) were connected to a withdrawal pump and an arterial pressure transducer through an overhead swivel system while the unrestrained rats rested in their home cage. The recording electrode and venous line were connected to appropriate lead wires and tubing through the same overhead swivel system. Rats were habituated for at least one hour, after which blood withdrawal commenced at a rate of 3.2 mL/min/kg for 6 minutes, and then 0.52 mL/min/kg for an additional 4 minutes, to provide a total withdrawal of 21% of estimated blood volume over 10 minutes. Precisely 7 minutes after the start of hemorrhage, 8-OH-DPAT was administered (30 nMoles/kg, iv) followed by a 150
µL bolus of isotonic saline. Data acquisition began 20 minutes prior to the start of blood withdrawal and continued throughout the course of hemorrhage and for an additional 50 minute recovery period following termination of blood withdrawal. In a subset of rats, femoral venous blood samples (150 µL) were taken 20 minutes prior to hemorrhage, as well as 10 and 60 minutes after the start of blood withdrawal to measure hematocrit, venous blood gases and acid-base status with an i-STAT 1 Analyzer (i-STAT, East Windsor, NJ). Plasma protein concentrations were determined by a handheld clinical refractometer (ATAGO U.S.A. Inc.). At the end of the experiment, rats were given hexamethonium chloride (30 mg/kg iv) to enable determination of background noise in the renal sympathetic recording electrode. The majority of animals were quickly anesthetized with sodium pentobarbital (100 mg/kg, iv), and decapitated. Their brains were quickly extracted and flash frozen in cold isopentane and stored at -80°C for qPCR assessment of receptor gene expression. A small subset of animals were anesthetized with sodium pentobarbital (100 mg/kg iv) then quickly perfused transcardially with 90 ml of sodium nitrate (0.1M) in 6.7 mM phosphate buffer followed by 90 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were then removed and post-fixed for 1 hour. The brainstem was blocked and post-fixed overnight. The following day, brains were transferred to 30% sucrose solution and stored at 4°C until sectioning. Brains were sectioned on a freezing microtome in 40 µm sections and collected serially into 6 wells filled with cryoprotectant (30% sucrose and 30% ethylene glycol in 0.1 M PB) and stored at -20°C prior to histological assessment of the injection sites.
Spontaneous baroreflex sensitivity

Five minute segments of baseline BP and HR data were recorded prior to hemorrhage and analyzed with Nevrokard SA-BRS software version 3.2.4 to determine spontaneous baroreflex gain using the sequence method as described in Chapter 3.

Data acquisition

Arterial pressure, HR, and renal sympathetic nerve activity (RSNA) were recorded on a Macintosh G4 Powerbook with PowerLab data acquisition software (Chart version 5.2.1; ADinstruments, Colorado Springs, CO) and analyzed as described in Chapter 3.

Tissue Microdissection

Brains were transferred to a Leica CM 3050 S Cryostat (-15 to -17°C). Five hundred µm thick sections were cut and 0.75 and 1 mm diameter micropunches were taken from areas of interest (listed below) localized according to the stereotaxic rat brain atlas of Paxinos and Watson as described in Chapter 3 (Watson, 1982). The tissue was homogenized immediately in 1.0 mL TRIzol® Reagent (Invitrogen) using a glass mortar and pestle on ice. Samples were transferred to microcentrifuge tubes for RNA extraction.

Tissue homogenization and immunoblotting

Total protein was extracted from a subset of micropunch samples using a homogenizing buffer that consisted of 50 mM Tris buffer (pH 7.6) with sodium
chloride (0.01 M) and the addition of a proteinase inhibitor cocktail (Roche). Protein concentration from the micropunch samples was determined by BCA assay (Pierce) as described in Chapter 3. Immunoblotting protocols were also performed in the same manner as described in Chapter 3. In addition, hrGFP protein expression was also determined in micropunch samples by incubating the membranes overnight with a mouse anti-hrGFP antibody (1:500, Assay Designs) in 1% nonfat dry milk at 4°C. Membranes were then incubated with HRP-conjugated anti-mouse secondary antibody (1:2000, Pierce) for 1 hour at room temperature. The membrane was washed a final time with TBST (3 times for 10 minutes) then protein bands were developed with Super-Signal West Pico substrate (Themo) and exposed on autoradiographic film. The band densities of 5-HT$_{1A}$ receptor and hrGFP were quantified with Image J (NIH, USA), and normalized to actin.

*Tissue RNA Isolation and cDNA synthesis*

Total RNA was extracted, genomic DNA removed, and then synthesized into cDNA according to protocols described in Chapter 3.

*Quantitative Real-Time PCR*

Messenger RNA levels of the 5-HT$_{1A}$ receptor were determined from tissue obtained from the commissural NTS, mNTS, RO, hypoglossal nucleus, and DR as described in Chapter 3. The 5-HT$_7$ receptor and αAR$_{2A}$ receptors were also assessed in mNTS and commissural NTS to rule out off target, or compensatory effects of shRNA treatment. Real-time qPCR was performed on
amplified cDNA using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The following general PCR protocol was used with various annealing/extension temperatures: 95°C for 30 sec, 40 cycles of 95°C for 5 sec and 56-63°C for 30 sec. Melt curves from 65°C to 95°C were included to confirm that only a single product was formed for each primer pair. Samples were prepared and analyzed as described in Chapter 3. Primer sequences used for amplification and annealing temperatures for each gene are listed in Table 7. Results are presented as relative mRNA expression normalized to relative GAPDH mRNA.

Statistical Analysis

Two-way ANOVA with repeated measures were used to determine the effects of viral injection on cardiovascular and sympathetic measures as well as and on blood gas parameters over time. Holm-Šidák post-hoc tests were used to determine group differences every minute for the first 10 minutes, then every 5 minutes until the end of the recovery period. P values of < 0.05 were considered significant. PCR data from rats treated with the 1ARshRNA-encoding virus were normalized to the group average of rats injected with the ScramshRNA that were in the same qPCR cohort. Typically, two animals of each shRNA group were run in the same cohort. Significance was determined by unpaired Student’s t-tests. When group data failed the Shipiro-Wilk normality test, the Mann-Whitney Rank Sum Test was used instead. All qPCR results are expressed as the group mean ± SEM.
Table 7. PCR conditions for 8-OH-DPAT-treated rats

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>T (°C)</th>
<th>Primer Efficiency (%)</th>
<th>Forward Primer (5’ → 3’)</th>
<th>Reverse Primer (5’ → 3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>56/61</td>
<td>99/96</td>
<td>GAC ATG CCG CCT GGA GAA AC</td>
<td>AGC CCA GGA TGC CCT TTA GT</td>
<td>94</td>
</tr>
<tr>
<td>5-HT1A</td>
<td>56</td>
<td>89</td>
<td>AAG AAG AGC CTG AAC GGA</td>
<td>CAG AGG AAG GTG CTC TTT GG</td>
<td>171</td>
</tr>
<tr>
<td>5-HT7</td>
<td>61</td>
<td>100</td>
<td>GTG GAT CTT CGG CCA CTT CT</td>
<td>CCA AGG TAC CTG TCG ATG CTG</td>
<td>108</td>
</tr>
<tr>
<td>ADR2A</td>
<td>63</td>
<td>98</td>
<td>CGG CAA TAG CAG CTG GAA CG</td>
<td>TTG CCA AAC ACG GTG AAC AG</td>
<td>129</td>
</tr>
</tbody>
</table>

Primers for qPCR analysis. Forward and reverse primers are shown for GAPDH, 5-HT1A, 5-HT2A receptor (5-HT2A), 5-HT7 receptor (5-HT7) and α-adrenergic2A receptor (ARD2A). Annealing temperature (T), primer efficiency, and product length are also shown.
Results

Prior to the start of hemorrhage experiments, there was no significant difference in body weights between ScramshRNA and 1ARshRNA injected rats (Table 8). Similarly, hematocrit and plasma protein values were not significantly different between groups. Nor did indices of spontaneous baroreflex sensitivity differ between groups (Table 9). Both groups displayed the typical multiphasic response to blood loss (Figure 16). Specifically, BP was maintained with the onset of blood withdrawal while sympathetic drive and HR increased. After 9-12 ml/kg of estimated blood volume had been withdrawn, a syncopal-like phase ensued, characterized by a dramatic decline in BP, HR, and RSNA. Seven minutes after the initiation of blood withdrawal, systemic administration of 8-OH-DPAT (30 nM/kg, iv) produced a pressor effect in both treatment groups, but the effect was more pronounced in the ScramshRNA-treated rats compared to rats treated with the 1ARshRNA-encoding virus (P <0.01). Specifically, the BP response to 8-OH-DPAT was slower the first 3 minutes after injection in 1ARshRNA-treated rats compared to ScramshRNA animals. However, BP in both groups eventually plateaued to a level that was no longer different. 8-OH-DPAT raised HR in both groups as well. While, the response was attenuated in rats treated with 1ARshRNA, the difference was not significant (P = 0.09). Renal sympathetic nerve activity rose dramatically after 8-OH-DPAT in rats treated with the ScramshRNA. Though the response tended to be less robust in rats treated
Table 8. Body weight and blood volume indices for 8-OH-DPAT-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Hemorrhage BW (g)</th>
<th>Hematocrit (%)</th>
<th>Plasma Protein (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCRshRNA</td>
<td>N (3-12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>383 ± 9</td>
<td>45 ± 1</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>10 min</td>
<td>35 ± 2**</td>
<td>35 ± 2**</td>
<td>4.8 ± 0.1**</td>
</tr>
<tr>
<td>60 min</td>
<td>34 ± 1**</td>
<td>34 ± 1**</td>
<td>4.8 ± 0.1**</td>
</tr>
<tr>
<td>1ARshRNA</td>
<td>N (4-13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>387 ± 7</td>
<td>43 ± 1</td>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td>10 min</td>
<td>34 ± 1**</td>
<td>34 ± 1**</td>
<td>4.7 ± 0.1**</td>
</tr>
<tr>
<td>60 min</td>
<td>33± 1**</td>
<td>33± 1**</td>
<td>4.9 ± 0.1**</td>
</tr>
</tbody>
</table>

Body weight (BW), hematocrit, and plasma protein levels before (0 min) after hemorrhage termination and 8-OH-DPAT injection (10 min) and 50 min after termination of hemorrhage (60 min) in rats treated with the scrambled shRNA encoding virus (ScramshRNA) or the 1ARshRNA-encoding virus. The number of ScramshRNA and 1ARshRNA per group are 12 and 13 respectively for BW measurements and 3 and 4 respectively for hematocrit and protein samples. Values are group means ± SEM. A 2-way ANOVA with repeated measures was used to examine the factors of Treatment and Time over the course of hemorrhage on hematocrit and protein plasma concentrations. No interaction was found, but a main effect of Time in both groups was followed up by Holm-Sidak post-hoc analysis. Both plasma protein concentrations [F (1,6) = 108, P < 0.001] and hematocrit levels [F (1,6) = 156, P <0.001] fell by 10 minutes after the start of hemorrhage and both remained below baseline at the end of the recovery period, ** P < 0.01 vs 0 minutes within group.
Table 9. Spontaneous baroreflex indices in 8-OH-DPAT-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Up Sequences (ms/mmHg)</th>
<th>Down Sequences (ms/mmHg)</th>
<th>All Sequences (ms/mmHg)</th>
<th>Alpha (LF)</th>
<th>Alpha (HF)</th>
<th>LF Power (mmHg²)</th>
<th>BP SDNN</th>
<th>LF RRI (nu)</th>
<th>HF RRI (nu)</th>
<th>LF/HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScramshRNA (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP</td>
<td>1.56 ± 0.18</td>
<td>1.44 ± 0.18</td>
<td>1.52 ± 0.17</td>
<td>0.48 ± 0.07</td>
<td>1.83 ± 0.36</td>
<td>21.08 ± 5.36</td>
<td>4.28 ± 0.43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP</td>
<td>1.74 ± 0.17</td>
<td>1.69 ± 0.18</td>
<td>1.72 ± 0.17</td>
<td>0.50 ± 0.07</td>
<td>2.31 ± 0.40</td>
<td>22.16 ± 5.84</td>
<td>3.63 ± 0.18</td>
<td>17.89 ± 2.95</td>
<td>46.61 ± 7.31</td>
<td>0.47 ± 0.11</td>
</tr>
<tr>
<td>DBP</td>
<td>1.81 ± 0.22</td>
<td>1.70 ± 0.19</td>
<td>1.75 ± 0.21</td>
<td>0.51 ± 0.07</td>
<td>2.08 ± 0.31</td>
<td>22.53 ± 5.90</td>
<td>3.43 ± 0.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1ARshRNA (9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP</td>
<td>1.89 ± 0.19</td>
<td>1.73 ± 0.27</td>
<td>1.87 ± 0.23</td>
<td>0.54 ± 0.09</td>
<td>2.21 ± 0.37</td>
<td>22.04 ± 6.90</td>
<td>3.76 ± 0.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP</td>
<td>2.23 ± 0.19</td>
<td>2.31 ± 0.31</td>
<td>2.28 ± 0.26</td>
<td>0.56 ± 0.09</td>
<td>3.12 ± 0.30</td>
<td>22.56 ± 7.43</td>
<td>3.49 ± 0.28</td>
<td>16.65 ± 3.45</td>
<td>46.82 ± 7.60</td>
<td>0.44 ± 0.10</td>
</tr>
<tr>
<td>DBP</td>
<td>2.02 ± 0.14</td>
<td>1.88 ± 0.13</td>
<td>1.99 ± 0.11</td>
<td>0.56 ± 0.09</td>
<td>2.73 ± 0.21</td>
<td>22.54 ± 7.46</td>
<td>3.46 ± 0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Spontaneous baroreflex sensitivity (SBS) determined from baseline BP and HR recordings in rats treated with virus encoding the 1ARshRNA or ScramshRNAs. Spectral analyses were performed in the low frequency (LF) and high frequency (HF) domains. SDNN, standard deviation of normal beats; RRI, R-R interval (detected as peak to peak BP); nu, normalized units. Number of animals is shown in parentheses. Systolic (SBP), mean (MBP), and diastolic (DBP) blood pressure. Data are group means ± SEM. Student’s t-test were used to compare group means for ScramshRNA and 1ARshRNA treated groups. No significant differences were identified.
Figure 16. Effect of ScramshRNA and 1ARshRNA expression in the NTS on cardiovascular responses to 8-OH-DPAT in hemorrhaged rats. Mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA) during hemorrhage (shaded in gray), and subsequent recovery following 8-OH-DPAT administration (indicated by arrow) of ScramshRNA (black circles) and 1ARshRNA (open circles) injected rats. Values are group means ± SEM. Number of subjects for ScramshRNA were 5 for RSNA and 11 for BP and HR recordings. The 1ARshRNA subject number was 6 for RNSA and 13 for BP and HR measurements. Data were analyzed by 2-way ANOVA with repeated measures using ShRNA treatment and Time as factors. A significant interaction between ShRNA treatment and Time on BP during the first 10 minutes of hemorrhage was revealed [F (1, 207), = 2.44, P < 0.01]. Post-hoc analysis with Holm-Šidák performed at each time point showed a lower BP in 1ARshRNA treated rats immediately following 8-OH-DPAT administration.
with the 1ARshRNA-encoding virus, the group difference was not significant at any time point when all animals were included in the analysis (P = 0.27).

In the small subset of animals tested (n = 6), there was no effect of shRNA treatment on acid-base balance or lactic acidosis with hemorrhage (Table 10). However, a 2-way ANOVA with repeated measures using Treatment and Time as factors revealed a significant interaction on P\textsubscript{vO2}. Post-hoc analysis revealed that 1ARshRNA-treated rats had significantly decreased P\textsubscript{vO2} immediately after 8-OH-DPAT administration (10 min time point).

In most subjects, viral transduction was considered successful as determined by the presence of hrGFP fluorescence in neurons of the caudal NTS when tissue was saved for histological analysis, or by evidence of hrGFP mRNA expression in micropunch samples. Since, hrGFP expression was driven by a different promoter than the shRNA expression, hrGFP expression was not used for normalization of shRNA expression. Instead, it was used as a qualitative measure to determine success or failure of viral transduction. In a subset of animals in which we collected brains for histological examination, we observed rare occasions of off target hrGFP expression in which the center of the injection site was not located within the NTS, but instead, was identified more ventrally within the hypoglossal nucleus (Figure 17A vs. B). On rare occasions we noted a complete lack of hrGFP expression in neurons (Figure 17C). Instead, significant gliosis with some hrGFP expression limited almost exclusively to glia was observed. Also, in one 1ARshRNA-treated rat, we failed to observe any hrGFP expression in any tissue samples. In some animals, brain tissue was used for
Table 10. Venous blood gas and acid-base status for 8-OH-DPAT-treated rats

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>pH</th>
<th>p$_v$CO$_2$ (mmHg)</th>
<th>p$_v$O$_2$ (mmHg)</th>
<th>BE (mmol/L)</th>
<th>Lactate (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ScramshRNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td></td>
<td>7.50</td>
<td>43.47 ± 0.99</td>
<td>36.67 ± 2.19</td>
<td>11.33 ± 1.86</td>
<td>0.71 ± 0.14</td>
</tr>
<tr>
<td>10 min</td>
<td></td>
<td>7.48</td>
<td>41.17 ± 1.23</td>
<td>35.33 ± 0.88</td>
<td>8.67 ± 2.19 ‡</td>
<td>1.86 ± 0.41 ‡</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>7.47</td>
<td>46.87 ± 1.24 †,#</td>
<td>32.33 ± 1.67</td>
<td>9.00 ± 0.58 †</td>
<td>1.05 ± 0.18 †,§</td>
</tr>
<tr>
<td><strong>1ARshRNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td></td>
<td>7.52</td>
<td>43.16 ± 0.46</td>
<td>32.60 ± 1.83</td>
<td>11.8 ± 0.74</td>
<td>0.56 ± 0.05</td>
</tr>
<tr>
<td>10 min</td>
<td></td>
<td>7.48</td>
<td>40.88 ± 1.95</td>
<td>30.20 ± 1.63 †</td>
<td>7.00 ± 0.89 †</td>
<td>2.63 ± 0.41 ‡</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>7.47</td>
<td>48.6 ± 1.53 †,#</td>
<td>33.8 ± 0.74</td>
<td>10.00 ± 1.30 †</td>
<td>1.62 ± 0.12 †,§</td>
</tr>
</tbody>
</table>

Effects of hemorrhage and 8-OH-DPAT administration on venous blood gas variables and acid-base status for rats treated with virus encoding the scrambled shRNA (ScramshRNA) or the 1ARshRNA. Values are group means ± SEM at baseline (0 minutes), 10 and 60 minutes after the start of hemorrhage. A 2-way ANOVA with repeated measures was utilized to examine the factors of Treatment and Time on blood gas variables and acid-base status. A significant interaction was found between Treatment and Time on p$_v$O$_2$ [F (1,2) = 4.03, P < 0.05]. Post-hoc analysis by Holm-Šidák revealed a significantly reduction in p$_v$O$_2$ among 1ARshRNA injected rats compared to rats treated with the ScramshRNA 10 minutes after the start of hemorrhage, but not at baseline or the end of the recovery period (60 minutes). * P < 0.05 vs ScramshRNA. There were no other significant interactions, but a main effect of Time was found for all parameters due to reductions in all variables with hemorrhage and/or subsequent recovery by 60 minutes. †P < 0.05 vs 0 minutes within group, ‡P < 0.01 vs 0 minutes within group, §P < 0.05 vs 10 minutes within group, and #P < 0.01 vs 10 minutes within group.
Figure 17. Coronal rat brain sections demonstrating variations in hrGFP expression in AAV-treated rats. A) Successful injection centered in the NTS, B) off target injection centered in the hypoglossal nucleus. C) Absence of neuronal expression of hrGFP expression with significant gliosis and glial hrGFP expression (magnified inset).
Western blot analysis of 5-HT$_{1A}$ receptor expression. However, the lack of specificity of the 5-HT$_{1A}$ antibody in tissue prevented us from making determinations of 5-HT$_{1A}$ receptor protein levels. However, hrGFP expression could be confirmed in these samples by Western blot. In some cases there was insufficient tissue for gene expression studies after failed attempts at Western blot determination of 5-HT$_{1A}$ receptor protein. Therefore, confirmation of the degree of knockdown was not possible in some regions of a subset of animals.

Among those animals for which gene expression data were obtained, 5-HT$_{1A}$ receptor mRNA levels were decreased in 1ARshRNA-treated rats by ~50% in the commissural NTS (Figure 18). Transduction in the mNTS was more variable and achieved only a modest (~20%) reduction in 5-HT$_{1A}$ receptor gene expression. Average receptor expression in the hypoglossal nucleus was also reduced by approximately 20% in 1ARshRNA-treated rats, but overall the expression was not different from ScramshRNA-treated rats (Figure 18C). There was no effect of treatment with the 1ARshRNA-encoding virus on 5-HT$_{1A}$ receptor mRNA levels in the RO or DR.

Latency to recover 75% of baseline BP was inversely correlated with 5-HT$_{1A}$ receptor expression in both the commissural NTS and mNTS injection sites (Figure 18A and B; Spearman coefficient = -0.81 and -0.56, $r^2$ = -0.66 and -0.31, $P < 0.01$ and $P < 0.05$ respectively). Despite the tendency for reduced 5-HT$_{1A}$ receptor expression in the hypoglossal nucleus of 1ARshRNA-injected rats, relative 5-HT$_{1A}$ receptor expression in the hypoglossal nucleus did not correlate with the time to recover 75% of baseline BP (Figure 18D; Spearman coefficient
Figure 18. Relationship of 5-HT\textsubscript{1A} receptor mRNA levels and latency to recover 75\% of baseline blood pressure after hypotensive hemorrhage. Relative 5-HT\textsubscript{1A} receptor mRNA levels correlations with time to recover 75\% of baseline blood pressure following 8-OH-DPAT injection. A) Spearman correlation of 5-HT\textsubscript{1A} receptor mRNA levels in commissural (A) and mNTS (B) and time to recover 75\% of baseline blood pressure following 8-OH-DPAT injection. C) Relative 5-HT\textsubscript{1A} receptor expression in the cNTS, mNTS, hypoglossal nucleus, RO, and DR of ScramshRNA and 1ARshRNA- injected rats. D) Correlation between relative 5-HT\textsubscript{1A} receptor expression in hypoglossal nucleus and time to recover 75\% of baseline BP. Values are group means ± SEM. An unpaired Student’s t-test revealed a significant decrease in 5-HT\textsubscript{1A} receptor expression in the cNTS of 1ARshRNA injected rats [df = 12, t = 2.52, P < 0.05] but not in the mNTS injection sites (df = 15, t = 1.50, P = 0.15), hypoglossal nucleus (df = 13, t = 1.94, P = 0.07), RO (df = 11, t = - 0.23, P = 0.82) or DR (df = 12, t = - 0.41, P = 0.69).
-0.31, r² = -0.10, P = 0.317). Though fewer samples were available for rats that had adequate renal sympathetic recordings, there was a clear direct correlation between the 8-OH-DPAT-induced rise in RSNA (one minute after injection) and 5-HT₁A receptor expression in the mNTS. Likewise, there was a tendency for the sympathoexcitatory effect of 8-OH-DPAT administration to correlate with 5-HT₁A receptor expression in the commissural NTS (Figure 19; Pearson correlation coefficient = 0.728, r² = 0.57, P < 0.05 and 0.74, r² = 0.55, P = 0.057). Neither expression of 5-HT₇ receptors nor αAR₂A was affected by 1ARshRNA treatment (Figure 20).

Given the apparent lack of viral transduction of the NTS in a subset of animals, cardiovascular parameters were reanalyzed after exclusion of two 1ARshRNA-treated animals that showed no evidence of hrGFP within the NTS. Among animals that showed significant infection within either the commissural or mNTS, there still was a clear deficit in 8-OH-DPAT-induced pressor responses (P < 0.01). When the two 1ARshRNA-injected rats that did not demonstrate viral infection within the NTS were excluded, a clear group difference emerged in the sympathoexcitatory effect of 8-OH-DPAT administration (Figure 21; P < 0.05).

Discussion

Transduction with a 1ARshRNA-encoding AAV produced a selective 20-50% knockdown of 5-HT₁A receptors in the commissural and mNTS and impaired the sympathoexcitatory and pressor effect of 8-OH-DPAT in unanesthetized rats subjected to hypotensive hemorrhage. Additionally, the speed at which 8-OH-
Figure 19. Relative 5-HT7 and αAR2A expression in commissural NTS and mNTS of rats injected with ScramshRNA or 1ARshRNA. Values are group means ± SEM. Unpaired Student's t-test revealed no significant effect of shRNA treatment on 5-HT7 and αAR2A receptor mRNA levels.
Figure 20. Relationship of 5-HT\textsubscript{1A} receptor mRNA levels in the commissural and mNTS to RSNA one minute after 8-OH-DPAT administration. A Pearson correlation of 5-HT\textsubscript{1A} receptor mRNA levels in the commissural (left) and mNTS (right) and RSNA 1 minute after 8-OH-DPAT administration.
Figure 21. Effect of ScramshRNA and 1ARshRNA on responses to hemorrhage after exclusion of unsuccessfully transduced rats. Mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA) during hemorrhage (shaded in gray), and subsequent recovery following 8-OH-DPAT administration (indicated by arrow) of ScramshRNA and 1ARshRNA- injected rats when data from 2 rats that showed no sign of viral infection in the NTS were excluded. Values are group means ± SEM. Number of subjects for ScramshRNA were 5 for RSNA and 11 for BP and HR recordings. The 1ARshRNA subject number were 4 for RNSA and 11 for BP and HR measurements. Data were analyzed by 2-way ANOVA with repeated measures using Time and ShRNA treatment as factors. Significant interactions were followed by Holm-Šidák post-hoc analysis. A 2-way ANOVA revealed a significant interaction between ShRNA treatment and Time due to a reduction in the initial pressor response to 8-OHDPAT in 1ARshRNA-injected rats [F (1,200) = 2.88, P < 0.01]. Similarly, a significant interaction between ShRNA treatment and Time was also seen on RSNA [F (1, 70) = 3.00, P < 0.05] due to the lack of a sympathoexcitatory effect of 8-OH-DPAT in 1ARshRNA-treated rats. There were main effects of ShRNA treatment [F (1,20) = 4.60, P < 0.05] and Time [F (10,20) = 39.24, P < 0.01] on HR. The effect of treatment was due to lower HR in 1ARshRNA-treated rats, but group differences were not significant at any one time point.
DPAT promoted BP compensation was inversely correlated with 5-HT\textsubscript{1A} receptor mRNA levels in the NTS, particularly in the commissural NTS. Moreover, the sympathoexcitatory effect of 8-OH-DPAT administration was significantly correlated with the expression of 5-HT\textsubscript{1A} receptors in the NTS. Thus, the major findings of this study are that the sympathoexcitatory and pressor effects of 8-OH-DPAT are, at least in part, mediated by 5-HT\textsubscript{1A} receptors expressed in the caudal NTS.

These findings extend our previous work by demonstrating that at least part of the effect of 8-OH-DPAT is due to a specific action of the drug on 5-HT\textsubscript{1A} receptors, most likely within the commissural NTS. In prior work we noted that the pressor effect of 8-OH-DPAT was prevented by prior administration of the α1 adrenergic receptor (αAR\textsubscript{1}) antagonist, prazosin (25μg/kg, iv) and the ganglionic blocker, hexamethonium chloride (30mg/kg, iv). Hexamethonium alone only partially blocked the 8-OH-DPAT-mediated rise in BP (Tiniakov and Scrogin, 2006). Together, these findings indicate that 8-OH-DPAT has dual effects when administered to hypovolemic rats. Specifically, 8-OH-DPAT has a direct vasoconstrictor effect on the peripheral vasculature mediated by αAR\textsubscript{1} receptors as well as a central sympathoexcitatory effect. Our findings that knockdown of 5-HT\textsubscript{1A} receptors in the NTS partially blocked the pressor response but completely blocked the immediate sympathetic response are consistent with a direct effect of 8-OH-DPAT on the vasculature. Indeed, this direct pressor effect might be expected to further inhibit sympathetic drive in the absence of a 5-HT\textsubscript{1A}-mediated sympathoexcitatory effect due to baroreceptor activation.
Prior efforts to identify the specific receptor population on which 8-OH-DPAT acts to mediate its ability to accelerate recovery from hypotensive hemorrhage have been limited by the lack of highly selective 5-HT$_{1A}$ agonists and antagonists. The most selective of the 5-HT$_{1A}$ antagonists, WAY-100635, has approximately 300 nM affinity for αAR$_{2A}$ receptors (Martin et al., 1999). Moreover, αAR$_{2A}$ receptors are also located in the NTS, and prior work suggests that 8-OH-DPAT has affinity for these receptors as well (Bonaventure et al., 2004; Kubo et al., 1990; Kubo et al., 1987). More recent data also suggests that WAY-100635 has significant affinity for dopamine receptors which are also thought to mediate BP responses within the NTS (Chemel et al., 2006; Granata, 1982; Hyde et al., 1996; Kline et al., 2002). Thus, our prior work in which WAY-100635 was found to block the effects of 8-OH-DPAT did not completely exclude the possibility that 8-OH-DPAT acted on these alternative receptor populations to mediate its effects.

Another difficulty in identifying this receptor population lies in the inability to observe an effect of 8-OH-DPAT during hemorrhage in the anesthetized animal. Thus, a pharmacological approach for identifying the precise location would involve insertion of a chronic guide cannula and subsequent injection of drug during hypotensive hemorrhage in unanesthetized animals. The NTS is located on the dorsal surface of the brainstem just above the cervical vertebrae (Berger, 1979; Ciriello, 1983; Torvik, 1956). Thus, the precise location of a guide cannula would require placement in region that is subject to significant movement during flexion of the neck and increase the potential for lesion of the brainstem.
Moreover, relatively high concentrations of antagonist are typically used for localized \textit{in vivo} brain injection in order to ensure sufficient receptor occupancy by drug. However, several alternative receptors for which 8-OH-DPAT has affinity are located within the NTS, making pharmacological methods problematic.

The use of the shRNA AAV delivery system in the current study solves both problems by ensuring that the effects of 8-OH-DPAT are mediated specifically by the 5-HT\textsubscript{1A} receptor. Moreover, this system induces chronic down regulation of receptors which enabled us to examine responses to 8-OH-DPAT in unanesthetized animals. Nevertheless, the limitation of this approach is the possibility that changes in gene expression of the target gene may interfere with the expression of other genes that may alter the cardiovascular effects during hemorrhage. While we cannot rule out the possibility that non-specific effects on other proteins contributed to our results, we can rule out off-target effects of other likely candidates. We chose to examine the 5-HT\textsubscript{7} and αAR\textsubscript{2A} receptors. 8-OH-DPAT has very high affinity for the 5-HT\textsubscript{7} receptor, and expression of the 5-HT\textsubscript{7} receptor has been identified in the dorsal midline brainstem (Bonaventure et al., 2004). Furthermore, the 5-HT\textsubscript{7} receptors expressed in the brainstem have been shown to augment cardiac vagal activity (Kellett et al., 2005). However, a role for 5-HT\textsubscript{7} receptors to modulate cardiovascular responses to hemorrhage have not been investigated. Our data illustrates that reduced 5-HT\textsubscript{1A} receptor mRNA levels did not affect 5-HT\textsubscript{7} receptor expression in the caudal NTS, suggesting that the reduced sympathoexcitatory and pressor effects of 8-OH-DPAT during
recovery from blood loss are not due to off-target effects of 1ARshRNA on 5-HT\textsubscript{7} receptors. Likewise, we investigated the effect on αAR\textsubscript{2A} receptors since antagonists of these receptors have significant sympathoexcitatory effects when injected in the RVLM, and are known to facilitate chemoreflex responses in the NTS (Allen and Guyenet, 1993; Hayar and Guyenet, 1999; Hayward, 2001). But again, our studies suggest that alterations in expression of these receptors are likely not responsible for the attenuated pressor and sympathoexcitatory effects of 8-OH-DPAT seen in 1ARshRNA-injected rats.

Our prior studies also showed that 8-OH-DPAT improved acid-base balance and reduced lactic acidosis in rats subjected to hypovolemic shock in which approximately 50% of blood volume was withdrawn (Tiniakov et al., 2007; Tiniakov et al., 2012; Tiniakov and Scrogin, 2009). However, we did not observe an effect of 5-HT\textsubscript{1A} receptor knockdown on the ability of 8-OH-DPAT to reduce lactic acidosis with the milder, 21% blood volume withdrawal used to induce hypotensive hemorrhage in this study. However, 10 minutes after the start of hemorrhage, we did note a qualitatively smaller rise in lactate in both groups given 8-OH-DPAT (3 minutes before blood sampling) than observed in animals subjected to the same blood withdrawal, but not given drug, i.e., experiments in Chapter 3. Thus, it is possible that 8-OH-DPAT was able to facilitate improved peripheral perfusion within the short time frame between drug injection and blood sampling. Nevertheless, the lack of difference in lactic acid between treatment groups observed in this study suggests that 5-HT\textsubscript{1A} receptors in the caudal NTS do not mediate this early beneficial effect of 8-OH-DPAT on acid-base status. It
should be noted that blood gases were only obtained in a small subset of animals. Unfortunately, many of the brain tissue samples obtained from animals for which we had blood gas data were used for Western blot, and thus there was no data confirming knockdown of gene expression in these animals. Therefore, it is not possible to determine if the lack of effect of 1ARshRNA treatment on lactic acidosis was due to lack of 5-HT\textsubscript{1A} receptor knockdown.

As described above, there are 2 distinct mechanisms behind the pressor effect of 8-OH-DPAT (Tiniakov and Scrogin, 2006): direct vasoconstriction through peripheral \(\alpha\text{AR}_1\) receptor activation and increased sympathetic mediated venoconstriction. It is not clear whether the direct vascular effects of 8-OH-DPAT contribute to increased venous tone and increased perfusion, but this seems quite likely given the dependence of venoconstriction on sympathetic drive (Reilly et al., 2001; Toung et al., 2000). Both \(\alpha\text{AR}_1\) and \(\alpha\text{AR}_{2A}\) receptors are expressed in the venous vasculature and give rise to increased venous tone when sympathetic drive is elevated (D’Oyley and Pang, 1990; Ito and Hirakawa, 1984; Pang and Tabrizchi, 1986). Thus, direct vasoconstrictor effects of 8-OH-DPAT on the venous vasculature may contribute to improved venous return, particularly when drug is administered early after hypotensive hemorrhage when sympathetic tone is low and venous pooling of blood is high (Guyton et al., 1954). Early mobilization of venous blood through these vascular effects of 8-OH-DPAT may have masked the beneficial effect of sympathetic-mediated vasoconstriction on lactic acidosis.
These studies further indicate that the central pressor effects of 8-OH-DPAT are mediated by activation of 5-HT\textsubscript{1A} receptors in the commissural NTS. This conclusion is based on evidence in the present study that the pressor effect of 8-OH-DPAT was robustly correlated to 5-HT\textsubscript{1A} receptor expression in the commissural NTS, and less so to receptor expression in the mNTS. Recent findings indicate that the 5-HT\textsubscript{1A} receptor is co-localized with GAD67 in cells of the rat commissural NTS (Ostrowski et al., 2014). Typically, 5-HT\textsubscript{1A} receptor activation leads to neuronal cell hyperpolarization due to inhibition of Ca\textsuperscript{2+} conductance and/or increases in K\textsuperscript{+} conductance (Albert et al., 1996; Jeong et al., 2001). Thus, hyperpolarization of GABAergic cells of the commissural NTS by 8-OH-DPAT may contribute to disinhibition of excitatory functions of the region including direct excitation of pre-motor sympathetic neurons of the RVLM.

In prior studies, others have noted that 8-OH-DPAT typically produces significant and rapid sympathoinhibition in unanesthetized euvoletic rats (Bago et al., 1999; Kubo et al., 1995; Nosjean and Guyenet, 1991; Wang and Lovick, 1992). We have also demonstrated that the sympathoexcitatory effect of 8-OH-DPAT in hypovolemic animals is dependent upon sinoarotic innervation of the NTS (Osei-Owusu and Scrogin, 2006). Thus, it is possible that the sympathoexcitatory effects of 8-OH-DPAT require activation of sinoarotic afferents during hemorrhage that, in turn, contributes to activation of GABAergic neurons in the commissural NTS. In fact, peripheral chemoreceptor afferent activation during prolonged hypoxia has been found to increase extracellular levels of GABA in the commissural NTS, and elevation of GABA with sustained
hypoxia is dependent on integrity of the carotid sinus nerve (Tabata et al., 2001). It remains to be determined if 8-OH-DPAT acts to increase sympathoexcitation by inhibiting GABA neurotransmission in the commissural NTS. Recent work suggests that 8-OH-DPAT inhibits both GABAergic and glutamatergic neurotransmission in the commissural NTS in the rat brain slice (Ostrowski et al., 2014). In these studies, the predominant effect was a decrease in glutamatergic responses under basal conditions. Our findings are consistent with the possibility that prolonged excitatory neurotransmission in the NTS during hemorrhage contributes to increased GABA cell activation, and that 8-OH-DPAT acts to reverse this effect. Thus, differences in response to 8-OH-DPAT in the presence and absence of sinoaortic nerve activation may be due to changes in the prevailing level of GABAergic tone present in the NTS. Prolonged hypoxia increases GABA release in the commissural NTS, and the ability of 8-OH-DPAT to increase blood pressure was more strongly correlated with receptor expression in the commissural NTS where chemoreceptor afferents terminate in the highest density. Moreover, we did not observe any effect of 5-HT$_{1A}$ receptor knockdown on spontaneous arterial baroreflex sensitivity. Together these findings suggest that 5-HT$_{1A}$ receptors in the commissural NTS may disinhibit sympathetic drive during prolonged activation of peripheral chemoreceptors.

In summary, our work indicates that 8-OH-DPAT acts through 5-HT$_{1A}$ receptors in the commissural NTS to produce sympathoexcitation and increased BP in the unanesthetized rat subjected to hypotensive hemorrhage. These findings suggest that 5-HT$_{1A}$ receptor agonists that penetrate the blood brain
barrier may be useful in the treatment of hypotensive hemorrhage or hemorrhagic shock.
CHAPTER V

5-HT\textsubscript{1A} RECEPTORS IN THE CAUDAL NUCLEUS TRACTUS SOLITARIUS MEDIATE THE INDICES OF AROUSAL TO HYPOXIC HYPERCAPNIA

Abstract

Prior studies in our laboratory and within this dissertation have identified a role for 5-HT\textsubscript{1A} receptors of the caudal NTS in the sympathoexcitatory response observed during recovery from hemorrhage. However, the role of serotonin and 5-HT\textsubscript{1A} receptors in chemoreflex responses, such as those elicited during hemorrhage, remain to be fully elucidated. In the current study, we tested the hypothesis that hypercapnia, sufficient to acidify arterial blood to the same extent seen in hemorrhage, would activate serotonin neurons of the caudal raphe and, in turn, augment peripheral chemoreflex responses to hypoxia through activation of 5-HT\textsubscript{1A} receptors in the NTS.

In contrast to our hypothesis, hypercapnia (in the presence of hyperoxia) sufficient to lower arterial blood pH to levels observed in hemorrhagic shock (i.e., decrease \~0.07 pH units) did not increase c-Fos expression in serotonin neurons of the caudal raphe. Instead, only hypoxic hypercapnia increased c-Fos expression in serotonin immunoreactive neurons in the RM and RO. Exposure to
hypoxic hypercapnia or hypoxic hypercapnia increased c-Fos expression in the commissural NTS to the same extent (P<0.01). Treatment with the 1ARshRNA-encoding virus did not affect overall ventilatory rate, tidal volume, or cumulative minute ventilation during hypoxic normocapnia, hyperoxic hypercapnia or hypoxic hypercapnia. However, rats treated with the ScramshRNA-encoding virus exhibited intermittent bouts of high frequency ventilation during hypoxic hypercapnia that was suppressed in rats treated with the 1ARshRNA-encoding virus. Accordingly, ventilatory rate determined over the entire course of the 45 min exposure to hypoxic hypercapnia, but not that determined during hypoxia or hypercapnia alone, was attenuated by 5-HT_{1A} knockdown (1,121 ± 99 vs 599 ± 38 bpm over 45 min, P<0.01). Group differences in intermittent high frequency ventilation were due to decreased percent of time spent sniffing in rats treated with the 1AshRNA-encoding virus compared to ScramshRNA-injected rats (4.9 ± 1.1 vs 11.2 ± 2.4 % time, P < 0.05). Together, these data support the hypothesis that the combination of hypoxia and hypercapnia stimulates serotonin neurons in the caudal raphe to release serotonin in the NTS, which in turn stimulates arousal by activation of 5-HT_{1A} receptors.

**Introduction**

Studies performed in our laboratory indicate that serotonin cells of the caudal raphe and serotonin nerve terminals in the dorsomedial medulla that include the caudal NTS are necessary for normal ventilatory and sympathetic responses following compensation from blood loss. Our work further indicates
that serotonin release improves peripheral tissue perfusion following hypotensive hemorrhage. Serotonin cells of the caudal raphe are also necessary for normal ventilatory responses to hypercapnia in unanesthetized rats (Kung et al., 2010). Together, these findings led us to hypothesize that serotonin cells of the caudal raphe are activated by the acidosis that develops during hemorrhage. The resulting release of serotonin in the NTS is further hypothesized to facilitate peripheral chemoreflex responses and thereby improve ventilation and sympathetic activity following blood loss. Our findings that the 5-HT$_{1A}$ agonist 8-OH-DPAT acts in a manner that mimics the beneficial effects of serotonin during hemorrhage led us to further speculate that serotonin acts in the NTS to facilitate peripheral chemoreflex activation by stimulating 5-HT$_{1A}$ receptors.

Controversial evidence from other laboratories has suggested a role for serotonin neurons in the caudal raphe as central chemoreceptors. Serotonin neurons located along the major blood vessels that perfuse the brainstem are ideally situated to directly assess the acid-base status of blood entering cerebral circulation (Bradley et al., 2002). Moreover, primary cultures of caudal raphe serotonin neurons were found to be directly sensitive to changes in CO$_2$ and pH (Wang et al., 1998; Wang and Richerson, 1999; Wang et al., 2001). Given our previous evidence that destruction of the same serotonin neurons resulted in impaired chemosensitivity to respiratory acidosis, we began to question whether serotonin neurons of the caudal raphe may be activated in response to the metabolic acidosis that develops during hemorrhage.
Several serotonin receptor subtypes are expressed in the caudal NTS and several of these have been implicated in the regulation of ventilatory responses to various stimuli. A recent study by Zhuang et al., showed that 8-OH-DPAT, reversed fentanyl-induced apnea in urethane anesthetized rats. This response was completely abolished by microinjection of 5HT\textsubscript{1A} receptor antagonist, WAY-100635 directly into the caudal NTS prior to 8-OH-DPAT administration, or by destruction of the vagal projections to the NTS. Together, these findings suggest that 5-HT\textsubscript{1A} receptors of the caudal NTS facilitate ventilation when an organism is challenged by central hypoxic hypoventilation (Zhuang et al., 2012).

During prolonged hypoxic exposure, attenuated ventilatory drive termed hypoxic ventilatory decline is associated with accumulation of GABA in the caudal NTS (Tabata et al., 2001). Activation of 5-HT\textsubscript{1A} receptors typically induces neuronal hyperpolarization (Fargin et al., 1989). Thus, we speculated that serotonin may activate 5-HT\textsubscript{1A} receptors in the NTS during hemorrhage and thereby facilitate peripheral chemoreflex responses that would reverse hypoxic ventilatory decline. The accumulation of GABA that mediates hypoxic ventilatory decline is dependent on the integrity of the sinoaortic nerves. The sympathoexcitatory effect of 8-OH-DPAT is also dependent on integrity of sinoaortic nerves (Osei-Owusu and Scroggin, 2006). Therefore, we hypothesized that the acidosis that develops during hemorrhage would promote activation of serotonin neurons in the caudal raphe. We further speculated that the resulting release of serotonin in the NTS would facilitate peripheral chemoreflex response to hypoxia by activating 5-HT\textsubscript{1A} receptors. Therefore, in this study, we tested
whether serotonin neurons of the caudal raphe are activated by acidosis (hypercapnia) and whether this condition further improves peripheral chemoreflex responses to hypoxia. We also tested the hypothesis that knockdown of 5-HT$_{1A}$ receptors in the NTS would attenuate peripheral chemoreflex responses to hypoxia under acidic conditions in unanesthetized rats.

Methods

Animals

Male Sprague-Dawley rats (275 to 300 g; Harlan, Indianapolis, IN) were acclimated to the institutional animal facility under standard conditions (22°C ambient temperature, 12:12-h light-dark cycle) with food and water available ad libitum for at least 1 week prior to surgery. All experimental protocols were approved by the Institutional Animal Care and Use Committee and conducted in compliance with the Principles of Laboratory Animal Care, as adopted and promulgated by the United States National Institutes of Health.

Surgical procedures

Femoral catheter implantation

In a subset of rats, blood sampling was performed during gas exposure in order to determine the extent of blood gas and acid-base balance changes elicited by the chemoreflex stimuli. Rats (n = 5) were anesthetized with isoflurane (5% for induction and 1.5-2.5% for maintenance) in 100% O$_2$ (1L/ min)
and instrumented with a femoral arterial catheter (PE-50 fused with PE-10 tubing) to enable blood sampling. The arterial catheter was externalized at the nape of the neck and secured with 3-0 silk suture. Following catheter implantation, a Vascular Access Harness™ (Instech Laboratories, Inc., USA) was attached and secured to the catheter to allow blood sampling. Rats were given ampicillin (150 mg/kg sc) and buprenorphine (0.05 mg/kg sc) and allowed to recover in their home cage. Forty-eight hours after surgery, the catheters were flushed daily with dexamethasone (0.1 mg/mL) in heparinized saline (75 U) to maintain patency (Yang et al., 2005).

**AAV injections**

Prior to surgery, AAV vectors were diluted in viral storage buffer [400nM NaCl, 20 mM Tris base, (1x10^{12} vg/ml)] and dialyzed in 0.9% saline (Slide – a – Lyzer, ThermoScientific) as described in Chapter 3. Rats (n = 28) were anesthetized with ketamine/xylazine (85/13mg/kg, im) and placed in a stereotaxic apparatus with the nose bar positioned 11 mm below a flat skull plane then AAV vectors were injected as described previously in Chapter 3 and utilizing the same injection parameters. Animals were then allowed to recover for four weeks prior to chemoreflex protocol 3.

**Experimental Protocols**

1. Unrestrained whole body plethysmography to determine caudal raphe serotonin cell activation (c-Fos expression) in response to hypoxic normocapnia, hyperoxic hypercapnia or hypoxic hypercapnia.
Naïve rats (n = 32) were habituated to a whole body plethysmograph (Buxco Research Systems) for 10 days prior to study. During habituation, rats were handled and placed in the chamber which was situated in a visually–isolated, low–ambient noise room while continuously flushed with 21% O₂ balanced with N₂ at a rate of 2 L/min. Gas flow was regulated by a custom-made mass flow controller for O₂, N₂, and CO₂ (Buxco Research Systems). Temperature within the chamber was stable across experimental groups (± 0.5° C), but varied from 23-25° C between cohorts. Ventilatory variables were captured and analyzed by iox2 software (EMKA Technologies). On the day of the experiment, rats were placed in the chamber and allowed to acclimate for at least 1 hour. Baseline ventilation variables were then recorded for 60 minutes prior to gas change. After baseline recordings, rats were randomly assigned to receive a 60 minute exposure to one of three conditions: hypoxic normocapnia (10%O₂/0.04% CO₂), hyperoxic hypercapnia (95%O₂/5%CO₂), or hypoxic hypercapnia (10%O₂/5%CO₂). Changes in gas concentration required 5-10 minutes. Once the new gas concentration had stabilized, the condition was sustained for 60 minutes during which ventilatory variables were assessed. After 60 minutes, the gas mixture was switched back to normoxic conditions and the animal recovered in the chamber for an additional 30 minutes to allow maximal c-Fos protein expression to develop. Immediately after the end of the experiment, rats were deeply anesthetized with sodium pentobarbital (100mg/kg, ip). Once anesthetized the rat was given an injection of heparinized saline (100 U/0.1 ml) into the apex of the left cardiac ventricle. The rat was then perfused
transcardially with 250 mL (25 mL/min) sodium nitrate (0.1M) in 6.7 mM phosphate buffer followed by 360 mL (18 mL/min) 4% paraformaldehyde in 0.05 M potassium phosphate buffer (KPB), pH 6.8. Subsequently, brains were removed and post-fixed with the same solution for one hour, then blocked in the same solution overnight at 4°C. The following day, brains were transferred to 30% sucrose and stored until sectioning could be performed. Brains were sectioned on a freezing microtome in 40 µm sections and collected serially into 6 wells filled with cryoprotectant (30% sucrose and 30% ethylene glycol in 0.1 M phosphate buffer) and stored at -20° until immunohistochemical analysis was performed.

2. Unrestrained whole body plethysmography for analysis of gas effects on acid-base balance and blood gases.

Two days after catheter implantation, rats were habituated for 4 days to a whole body plethysmograph (Buxco Research Systems) as described above in protocol 1, except that their catheters were connected to a tether that consisted of a spring covered catheter connected to a fluid swivel (Instech Laboratories) placed in the top of the chamber. On the day of the experiment, rats were acclimated to the chamber and attachment to the tether, after which, baseline ventilatory variables were recorded for 60 minutes during exposure to normoxic conditions. An arterial blood sample was then withdrawn through the fluid swivel. Two times the dead space of the catheter and tether system was withdrawn, after which a 150 µl sample was obtained. The fluid was returned to the animal and the catheter was flushed with isotonic saline. Rats were then randomly assigned
to receive exposure to one of the following three conditions: hypoxic normocapnia (10%O₂/0.04% CO₂), hyperoxic hypercapnia (95%O₂/5%CO₂), or hypoxic hypercapnia (10%O₂/5%CO₂). The condition was sustained for 60 minutes during which ventilatory data were measured. At the end of the 60 minute gas exposure, a second arterial blood sample was obtained in the same manner as the first. Arterial blood samples were analyzed with an i-STAT 1 analyzer (i-STAT, East Windsor, NJ). At the end of each experiment, rats were returned to their home cage and allowed to recover for 40 hours. The procedure was then repeated until blood was sampled under each condition for each rat. The order of the gas conditions was randomized across rats. After the last experiment, rats were euthanized by an overdose of sodium pentobarbital (100 mg/kg, ip) and subsequent thoracotomy.

3. Assessment of 5-HT₁A receptor knockdown in the caudal NTS on chemoreflex responses.

Four weeks following viral injections, the ventilatory function of rats was assessed in the whole body plethysmograph (Buxco Research Systems) as described above with slight modifications. Each animal was subjected to 5 acclimation sessions of escalating duration (15, 25, 35, 45, and 60 minutes) over 5 days prior to experiments. Each rat was exposed to the 3 gas conditions described above in protocol 2 on separate days, with a day of rest between each of the 3 experiments. For each experiment, rats were acclimated to the chamber and baseline ventilation variables were recorded for at least 30 minutes prior to gas change. The gas concentration was then changed over the next 5-10
minutes to one of the three conditions. Once the gas transition was complete, ventilatory data were recorded for 45 minutes. The gas was then switched back to normoxia and animals were removed and allowed to rest in their home cage for approximately 40 hours prior to the next experiment.

*Tissue homogenization*

Total RNA was extracted from a subset of micropunch samples using a homogenizing buffer that consisted of 50 mM Tris buffer (pH 7.6) with sodium chloride (0.01M) and the addition of a proteinase inhibitor cocktail (Roche). Protein concentration from the micropunch samples was determined by BCA assay (Pierce) as described in Chapter 3. Immunoblotting protocols were also performed in the same manner as described in Chapter 3.

*Tissue RNA Isolation and cDNA synthesis*

Total RNA was extracted, genomic DNA removed, and then synthesized into cDNA according to protocols described in Chapter 3.

*Quantitative Real-Time PCR*

Messenger RNA levels of the 5-HT$_{1A}$ receptor were determined from tissue obtained from the commissural NTS, mNTS, RO, hypoglossal nucleus, and DR as described in Chapter 3. The 5-HT$_{2A}$ receptor was also assessed in mNTS and commissural NTS to rule out off target, or compensatory effects of shRNA treatment. Real-time qPCR was performed on amplified cDNA using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The following general PCR protocol was used with various annealing/extension temperatures:
95°C for 30 sec, 40 cycles of 95°C for 5 sec and 56-63°C for 30 sec. Melt curves from 65°C to 95°C were included to confirm that only a single product was formed for each primer pair. Samples were prepared and analyzed as described in Chapter 3. Primer sequences used for amplification and annealing temperatures for each gene are listed in Table 11. Results are presented as relative mRNA expression normalized to relative GAPDH mRNA.

**Immunohistochemistry**

For c-Fos and serotonin (5-HT) immunolabel, one of every six 40-µM sections through the brainstem was washed 6 times for 10 minutes in 0.05 M potassium phosphate buffered saline (KBPS), incubated in 0.1% H₂O₂ for 15 minutes, washed 5 times for 5 minutes, then incubated for 48 hours at 4°C with rabbit anti-c-Fos primary antibody (1:10,000 dilution; PC#38 from EMD Sciences) in 0.4% Triton X-100 KBPS (KPBST). Sections were then washed 10 times for 6 minutes and incubated in biotinylated goat anti-rabbit IgG (1:1,000 dilution in KPBST; Vector Laboratories) for 1 hour at room temperature, washed 5 times for 10 minutes, then incubated for 1 hour in Avidin DH solution combined with biotinylated horse-radish peroxidase (5 µl each/ml prepared 30 minutes prior to use, Vectastain Elite ABC Kit Standard, Vector Laboratories) in KPBST. Sections were then rinsed 3 times for 5 minutes with KPBS and then washed 3 times for 5 minutes in 0.175M sodium acetate, pH 7.0. During washes nickel sulfate-intensified 3,3’-diaminobenzadine tetrahydrochloride (NiDAB) chromagen solution was prepared as follows: 250 mg nickel (II) sulfate hexahydrate, 2 mg DAB, 20mg D-glucose, 1.5µL glucose oxidase (Sigma) in 10 mL 0.175M sodium
Table 11. Primers for qPCR analysis of chemoreflex challenged AAV rats.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>T (°C)</th>
<th>Primer Efficiency (%)</th>
<th>Forward Primer (5’ →3’)</th>
<th>Reverse Primer (5’ →3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>56/61</td>
<td>99/96</td>
<td>GAC ATG CCG CCT GGA GAA AC</td>
<td>AGC CCA GGA TGC CCT TTA GT</td>
<td>94</td>
</tr>
<tr>
<td>5-HT1A</td>
<td>56</td>
<td>89</td>
<td>AAG AAG AGC CTG AAC GGA</td>
<td>CAG AGG AAG GTG CTC TTT GG</td>
<td>171</td>
</tr>
<tr>
<td>5-HT2A</td>
<td>56</td>
<td>102</td>
<td>TCA TCA TGG CAG TGT CCC TA</td>
<td>ATC CAG ATC GCA CAG AGC TT</td>
<td>172</td>
</tr>
</tbody>
</table>

Forward and reverse primers are shown for GAPDH, 5-HT1A (5-HT1A), and 5-HT2A receptor (5-HT2A). Annealing temperature (T), primer efficiency, and product length are also shown.
acetate buffer. Sections were incubated in NiDAB to produce a black nuclear label over the course of 7 minutes. The reaction was stopped by washing sections 3 times for 5 minutes in 0.175 M sodium. Afterwards, sections were washed 4 times for 15 minutes, and incubated with rabbit anti-serotonin primary antibody (1:400,000 dilution in KPBST; Immunostar) for 48 hrs at 4°C. Sections were then subjected to the same secondary and tertiary antibodies as described above, but this time immunoreactivity was exposed with DAB in the absence of nickel (5 minutes) to produce a brown cytoplasmic label. Omission of either primary antibody completely abolished cellular label. After the final set of washes, sections were mounted onto gelatin-coated slides and allowed to dry overnight. The next day sections were dehydrated and cleared by immersion in the following solutions: distilled water, 50% ethanol (5 minutes), 75% ethanol (5 minutes), 95% ethanol (5 minutes), 100% ethanol (10 minutes), fresh 100% ethanol (10 minutes), xylene (10 minutes), fresh xylene (10 minutes). Slides were then immediately coverslipped with Permount mounting media (Fisher Scientific).

Histology

Cells that showed immunolabel for both c-Fos and serotonin were counted in one out of every six 40 μm sections (240 μM apart) throughout the anatomical extent of each serotonin-rich caudal raphe nuclei as described previously (Kung et al., 2010). Specifically, we examined double-labeled of neurons in the RP, RM and its lateral extensions around the pyramids (referred to as the parapyramidal region), RO, cells in the subependymal layer on the ventrolateral border of the
pyramids (referred to as the subependymal region), and the RP (Steinbusch, 1981). The percent of serotonin-labeled neurons that showed c-Fos immunoreactivity were summed for the entire raphe region (Table 12). Rostro-caudal profiles of double-labelled cells were determined for each nuclei. For these determinations, the total number of double-labeled cells were combined from three sections (720 μm) and compared for each gas condition. Cell counts were pooled in this way to reduce the impact of small variations in the anatomical location of serial sections between animals.

Cells showing c-Fos immunoreactivity were also counted in one of every six sections (240 μM apart) throughout the commissural and medial NTS (-15.00 mm to -13.8 mm from Bregma).

*Statistical Analysis*

Effect of Gas Treatment on blood gases and acid-base balance was determined by 2-way repeated measures ANOVA with Newman-Kuels post hoc
Table 12. The percentage of serotonin-immunoreactive neurons that also express c-Fos immunoreactivity during chemoreflex challenges

<table>
<thead>
<tr>
<th></th>
<th>Normoxia (8)</th>
<th>Hypoxic Normocapnia (6)</th>
<th>Hyperoxic Hypercapnia (6)</th>
<th>Hypoxic Hypercapnia (7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raphe Pallidus</td>
<td>12.9 ± 2.3</td>
<td>21.3 ± 3.8</td>
<td>22.0 ± 8.0</td>
<td>22.8 ± 4.5</td>
</tr>
<tr>
<td>Raphe Obscurus</td>
<td>1.3 ± 0.3</td>
<td>2.9 ± 0.6</td>
<td>2.6 ± 0.6</td>
<td>4.7 ± 0.7**†‡</td>
</tr>
<tr>
<td>Subependymal region</td>
<td>4.0 ± 0.9</td>
<td>8.8 ± 2.0</td>
<td>7.0 ± 2.8</td>
<td>5.9 ± 1.0</td>
</tr>
<tr>
<td>Parapyramidal region</td>
<td>2.5 ± 0.6</td>
<td>5.0 ± 0.7</td>
<td>4.3 ± 2.0</td>
<td>7.2 ± 2.0</td>
</tr>
<tr>
<td>Raphe Magnus</td>
<td>2.5 ± 0.8</td>
<td>5.7 ± 0.9</td>
<td>4.5 ± 0.5</td>
<td>6.8 ± 0.6*</td>
</tr>
</tbody>
</table>

Values are group means ± SEM in 40 μm sections obtained every 240 μm throughout the anatomical extent of the indicated nucleus. A one-way ANOVA with Gas treatment as a factor revealed an effect of Gas treatment on activation of serotonin neurons within the RO [F(3) = 6.79, P < 0.01]. Post-hoc analysis with Newman-Keuls' method determined that hypoxic hypercapnia increased the percent of serotonin neurons that were activated during the gas exposure compared to all other treatment groups. A Kruskal-Wallis one-way ANOVA on Ranks also revealed an effect of Gas treatment on activation of serotonin neurons within the RM [H(3) = 13.06, P < 0.01]. Post-hoc analysis by Dunn's method determined that the difference of treatment was due to the fact that hypoxic hypercapnia increased the percent of serotonin neurons that expressed c-Fos compared to normoxia, *P < 0.05 vs Normoxia, **P < 0.01 vs Normoxia, †P < 0.05 vs Hyperoxic hypercapnia, ‡P < 0.05 vs Hypoxic Normocapnia. The raphe pallidus, subependymal region, and parapyramidal region did not show significant changes in double-label following gas treatments, P = 0.384, P = 0.257, and P = 0.101 respectively.
tests. Effect of Gas Treatment on total number of cells expressing c-Fos was determined for each raphe region by one-way ANOVA or a Kruskal-Wallis one-way ANOVA on Ranks. Effect of Gas Treatment and Distance from Bregma for RO and RM were analyzed by 2-way ANOVA with repeated measures. Newman-Keuls post-hoc tests were used to compare effects of gas exposure at each location. Effect of Viral injection over Time on ventilatory variables was assessed independently for each gas treatment by 2-Way ANOVA. Effect of Viral injection on cumulative minute volume and area under the curve of ventilatory rate for the entire 45 minute exposure was assessed by 2-way repeated measures ANOVA with Bonferroni correction for the 3 gas exposures. PCR data from rats treated with the 1ARshRNA-encoding virus were normalized to the group average of rats injected with the ScramshRNA that were in the same qPCR cohort. Typically, two animals of each shRNA group were run in the same cohort. Significance was determined by unpaired Student’s t-tests. When group data failed the Shipiro-Wilk normality test, the Mann-Whitney Rank Sum Test was used instead. All qPCR results are expressed as the group mean ± SEM.

Results

Chemoreflex- elicited c-Fos expression in caudal raphe serotonin neurons

As expected, exposure to hypoxic normocapnic gas reduced \( P_{\text{aCO}_2} \) and \( P_{\text{aO}_2} \), caused respiratory alkalosis and increased lactate accumulation.
Hyperoxic hypercapnia increased $P_aCO_2$ and $P_aO_2$, caused a primary respiratory acidosis and reduced lactate levels. The addition of hypercapnic gas to hypoxia normalized $P_aCO_2$ and $P_aO_2$ and respiratory alkalosis and also lowered lactate accumulation (Table 13).

More serotonin neurons of the RM showed c-Fos expression over the entire nucleus following hypoxic hypercapnic gas exposure compared to normoxia, hypoxic normocapnia, and hyperoxic hypercapnia (Table 12, $P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively), but not in any one specific region of the RM (Figure 22). Similarly, only hypoxic hypercapnia increased c-Fos expression in the RO compared to normoxia and hyperoxic hypercapnia (Figure 23, $P < 0.01$ and $P < 0.05$ respectively), but not in any particular region of the RO. No other caudal raphe region showed elevated c-Fos expression in serotonin neurons.

Expression of c-Fos was increased in the middle and rostral portions of the commissural NTS, as well as the caudal portions of the medial NTS by hypoxia alone and hypoxic hypercapnia, and the response was similar between gas treatments (Table 14 and Figure 24, $P < 0.01$). Hyperoxic hypercapnia only increased c-Fos expression in the caudal portions of the medial NTS.
Table 13. Arterial blood gases and acid-base status determined before and after a 60-minute exposure to chemoreflex challenges

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>pH</th>
<th>$p_aCO_2$ (mmHg)</th>
<th>$p_aO_2$ (mmHg)</th>
<th>BE (mmol/L)</th>
<th>Lactate (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypoxia Normocapnia</strong></td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td></td>
<td>7.46 ± 0.01</td>
<td>43.86 ± 0.65</td>
<td>84.40 ± 1.03</td>
<td>7.00 ± 0.63</td>
<td>0.84 ± 0.17</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>7.58 ± 0.01##</td>
<td>29.76 ± 1.01###</td>
<td>37.80 ± 0.66###</td>
<td>6.00 ± 0.89</td>
<td>1.21 ± 0.11##</td>
</tr>
<tr>
<td><strong>Hyperoxic Hypercapnia</strong></td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td></td>
<td>7.45 ± 0.01</td>
<td>45.74 ± 0.60</td>
<td>88.60 ± 1.40</td>
<td>7.4 ± 0.68</td>
<td>0.97 ± 0.08</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>7.38 ± 0.01**##</td>
<td>60.18 ± 2.12###</td>
<td>437.20 ± 21.73**##</td>
<td>10.00 ± 0.71</td>
<td>0.42 ± 0.03###</td>
</tr>
<tr>
<td><strong>Hypoxic Hypercapnia</strong></td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td></td>
<td>7.45 ± 0.01</td>
<td>43.78 ± 0.72</td>
<td>89.80 ± 3.46</td>
<td>6.2 ± 0.66</td>
<td>1.03 ± 0.12</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>7.40 ± 0.01**†##</td>
<td>49.00 ± 0.90###</td>
<td>54.00 ± 1.10†##</td>
<td>7.4 ± 1.12</td>
<td>0.62 ± 0.07##</td>
</tr>
</tbody>
</table>

Values are group means ± SEM. Significant interactions were followed up by Newman-Keuls’ post-hoc analysis. *P < 0.05, **P < 0.01 vs hypoxia normocapnia, †P < 0.05, ‡P < 0.01 vs hyperoxic hypercapnia, #P < 0.05, ##P < 0.01 vs baseline (0 minutes) within group.

Two-way ANOVA with repeated measures revealed an interaction between Gas treatment and Time on arterial blood pH [$F (4,8) = 171.07, P < 0.01$]. Post-hoc analysis showed that hypoxic normocapnia increased pH while both hyperoxic hypercapnia and hypoxic hypercapnia reduced pH.
Two-way ANOVA with repeated measures revealed a significant interaction between Gas treatment and Time on $p_aCO_2$ values [$F(4,8) = 273.59, P < 0.01$]. Post-hoc analysis showed that hypoxic normocapnia reduced $p_aCO_2$ while both hyperoxic hypercapnia and hypoxic hypercapnia increased $p_aCO_2$.

Two-way ANOVA with repeated measures revealed an interaction between Gas treatment and Time on $p_aO_2$ values [$F(4,8) = 303.96, P < 0.01$]. Post-hoc analysis showed that hypoxic normocapnia reduced $p_aO_2$ while $p_aO_2$ was reduced to a similar degree when hypercapnia was combined with hypoxia. Conversely, hyperoxic hypercapnia dramatically increased $p_aO_2$ following treatment.

Two-way ANOVA with repeated measures revealed an interaction between Gas treatment and Time on lactate levels [$F(4,8) = 96.56, P < 0.01$]. Post-hoc analysis showed that hypoxic normocapnia augmented lactate accumulation, whereas hyperoxic hypercapnia and hypoxic hypercapnia both reduced lactate levels.
Figure 22. Effect of gas challenges on c-Fos expression in serotonin neurons of the raphe magnus. A) Representative 5-HT and c-Fos immunolabel in raphe magnus (RM) of rats subjected to normoxia (left) and hypoxic hypercapnia (right). B) number of serotonin neurons within the RM that were also expressed c-Fos in rats exposed to normoxia, hypoxic normocapnia, hyperoxic hypercapnia, hypoxic hypercapnia. Total number of serotonin neurons are shown at each location for comparison. Data were combined for 3 sections (720μm) at each point along the rostro-caudal extent of the nucleus and are expressed as groups means ± SEM. A 2-way ANOVA with repeated measures with Gas treatment and Distance from Bregma as factors revealed only a main effect of Distance from Bregma in the RM [F (3) = 14.65, P < 0.01].
**Figure 23.** Effect of gas challenges on c-Fos expression in serotonin neurons of the raphe obscurus. A) Representative coronal sections of brainstem showing typical c-Fos and serotonin double immuno-label (arrows) in the Raphe Obscurus (RO) of rats exposed to normoxia (left) and hypoxic hypercapnia (right). B) Number of serotonin-immunoreactive neurons that also showed c-Fos expression in brain sections from rats exposed to normoxia, hypoxic normocapnia, hyperoxic hypercapnia or hypoxic hypercapnia and total number of serotonin positive neurons. Data are group mean of cells identified in 3 sections taken at 240 μm intervals across the rostro-caudal plane, and shown as a single value every 720 μms. A 2-way ANOVA with repeated measures with Gas treatment and Distance from Bregma as factors revealed main effects of Gas treatment \( [F (3,23) = 5.93, P < 0.01] \) and Distance \( [F (3,23) = 13.31, P < 0.01] \). Post-hoc analysis using Newman-Keuls’ method showed that the main effects were due to increased c-Fos immunoreactivity in the RO of rats exposed to hypoxic hypercapnia vs normoxia \( (P<0.01) \) and hypercapnia \( (P<0.05) \). Although not significant, there was a strong tendency for hypoxic hypercapnia to increase c-Fos expression compared to hypoxic normocapnia \( (P = 0.055) \).
Figure 24. Effect of gas challenges on c-Fos expression in the commissural and mNTS.

A) Hypoxic normocapnia (B) hyperoxic hypercapnia (C) and hypoxic hypercapnia (D).

E) Numbers of c-Fos positive cells in the commissural and mNTS shown throughout rostral-caudal extent of the nucleus for each gas condition. Data group means ± SEM. 2-way ANOVA with repeated measures using Gas treatment and Distance from Bregma as factors revealed a significant interaction between factors [F (3, 183) = 4.98, P < 0.01]. Post-hoc analysis with Newman-Keuls showed that both hypoxic normocapnia and hypoxic hypercapnia significantly increased c-Fos expression in the mid and rostral portions of the commissural NTS and all three conditions resulted in increased c-Fos expression compared to normoxia, #P < 0.05 and **P < 0.01. Both hypoxic normocapnia and hypoxic hypercapnia also showed significantly increased c-Fos expression compared to hyperoxic hypercapnia, ^P < 0.05, $P < 0.01.
Table 14. Total number of cells that expressed c-Fos in the commissural and medial NTS during exposure to chemoreflex challenges.

<table>
<thead>
<tr>
<th></th>
<th>Normoxia (8)</th>
<th>Hypoxic Normocapnia (6)</th>
<th>Hyperoxic Hypercapnia (6)</th>
<th>Hypoxic Hypercapnia (7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commissural NTS</td>
<td>79 ± 3</td>
<td>606 ± 32*</td>
<td>255 ± 14</td>
<td>590 ± 24*</td>
</tr>
<tr>
<td>Medial NTS</td>
<td>131 ± 5</td>
<td>556 ± 27*</td>
<td>369 ± 20</td>
<td>649 ± 17*</td>
</tr>
</tbody>
</table>

Total number of c-Fos positive cells in the commissural and medial NTS after exposure to normoxia, hypoxic normocapnia, hyperoxic hypercapnia and hypoxic hypercapnia. Values are group means ± SEM. A Kruskal-Wallis one-way ANOVA on Ranks with Gas treatment as the factor revealed a main effect of Gas treatment on c-Fos expression within the commissural NTS \([H (3) = 15.99, P < 0.01]\). Post-hoc analysis using Dunn’s method showed that hypoxic hypercapnia and hypoxic normocapnia increased c-Fos expression in the commissural NTS. A Kruskal-Wallis one-way ANOVA on Ranks also revealed a significant effect of Gas treatment on activation of neurons within the mNTS \([H (3) = 16.36, P < 0.01]\). Post-hoc analysis using Dunn’s method showed increased c-Fos expression during hypoxic normocapnia and hypoxic hypercapnia. *P < 0.05. Exposure to normoxia or hyperoxic hypercapnic gas did not produce significant changes in c-Fos expression in either the commissural or medial NTS.
**5-HT\textsubscript{1A} receptor activation contributes to ventilatory compensation during hypoxic hypercapnia**

Body weight did not differ between groups injected with virus encoding the ScramshRNA and 1ARshRNA (353 ± 5 and 358 ± 5 g respectively). Baseline ventilatory variables of ventilatory rate (VR), and minute ventilation (MV) did not differ between groups (Figure 25). In both treatment groups, hypoxic normocapnic gas exposure elicited a dramatic increase in VR and MV which quickly declined over the first 5 minutes of exposure and then declined more slowly over the remainder of the recording period. Hyperoxic hypercapnic gas exposure also produced an immediate and robust increase in VR and MV that declined over the first 10 minutes of exposure. Ventilation stabilized after 10-15 minutes and remained at this elevated level throughout the gas exposure period. There were no differences in ventilation between groups during either hypoxic normocapnia or hyperoxic hypercapnia alone. During hypoxic hypercapnia, both groups had an initial increase in ventilation that diminished over the first 10 minutes and stabilized, but there were obvious intermittent fluctuations in ventilatory rate in the ScramshRNA-treated group that were not observed in 1ARshRNA-treated rats. But there was no group difference in ventilatory variables at any time point during the recording period.

Examination of data from individual rats showed a clear increase in ventilatory rate fluctuations in control rats that was not apparent in rats treated with the 1ARshRNA (Figure 26A). To determine if the fluctuations were greater
Figure 25. Ventilatory rate (VR) and minute volume (MV) in rats treated with AAV encoding the scrambled shRNA (ScramshRNA) or the 1ARshRNA. Shown are values determined prior to gas change (−5-0 min) and during exposure to hypoxic normocapnia (left panel), hyperoxic hypercapnia (middle panel), and hypoxic hypercapnia (right panel). Two-way ANOVA with repeated measures using ShRNA treatment and Time as factors revealed no significant effects or interactions.
Figure 26. Ventilatory rate changes of individual rats over the course of exposure to hypoxic hypercapnia. A) Individual ventilatory rate (VR) during 45 minute exposure to hypoxic hypercapnia in all animals treated with the ScramshRNA-encoding virus (left) or the 1ARshRNA-encoding virus (right). B) Area under the curve of VR during entire exposure period hypoxic hypercapnia in ScramshRNA- and 1ARshRNA-injected rats. Data are group means ± SEM. Mann Whitney U Rank Sum test determined that 1ARshRNA-injected rats had lower overall variability in ventilatory rate during the course of hypoxic hypercapnia compared to ScramshRNA treated rats (U = 14, T = 105, P < 0.01).
To determine if intermittent fluctuations in ventilatory rate affected total ventilation, cumulative minute ventilation at the end of gas exposure was determined (Figure 27). There was a strong trend for decreased ventilation over the course of gas exposure during hyperoxic hypercapnia in rats treated with the 1ARshRNA-encoding virus. But correction for repeated measures reduced significance below the acceptable alpha level.

Inspection of the ventilatory wave form data revealed that 1ARshRNA-treated rats spent less time sniffing than ScramshRNA rats during hypoxic hypercapnia (Figure 28A and B). There was no difference in time spent sniffing between hypoxic normocapnia or hyperoxic hypercapnia alone. In a small subset of rats, ventilation was recorded during normoxic conditions prior to gas change for at least 45 minutes. Therefore, data from these animals were also analyzed for sniffing. These data are shown for comparison but were not included in the analysis. Qualitatively, animals showed little sniffing during normoxia and the percent time spent sniffing was similar between groups.

Analysis of brain tissue showed a correlation between 5-HT$_{1A}$ mRNA levels in the commissural NTS and the area under the curve of ventilatory rate during exposure to hypoxic hypercapnia ($R = 0.52$, $r^2 = 0.27$, $P < 0.05$), but there was no correlation with the mNTS 5-HT$_{1A}$ receptor expression and ($R = 0.05$, $r^2 = 0.01$, $P = 0.87$; Figure 29A and B). The 5-HT$_{2A}$ receptor gene expression in the caudal NTS was not affected by 1ARshRNA AAV injection (Figure 29C).
Figure 27. Effect of ScramshRNA and 1ARshRNA expression in NTS on cumulative minute ventilation (MV) during gas challenges. Cumulative minute ventilation (MV) over the 45 minute exposure to hypoxic normocapnia (left panel), hyperoxic hypercapnia (middle panel) and hypoxic hypercapnia (right panel) in ScramshRNA-treated and 1ARshRNA-treated rats. Values are group means ± SEM. Effects of AAV injection and gas exposure on cumulative MV at 45 minutes of gas exposure (i.e., last value at 45 minutes) were assessed by 2-way ANOVA with repeated measures of Gas treatment and ShRNA treatment as factors. Though a strong trend for decreased cumulative MV was observed (P=0.048) in rats treated with the 1ARshRNA-encoding virus, Bonferroni correction for exposure to the 2 other gas treatments reduced the significance of the effect.
Figure 28. Percent of time during each gas challenge spent sniffing. Normoxia is shown for comparison, but not included in the analysis. Rats treated with the 1ARshRNA-encoding virus showed decreased sniffing during hypoxic hypercapnia compared to ScramshRNA-injected rats. A) Time spent sniffing (determined as VR > 250 bpm) during normoxia, hypoxic normocapnia, hyperoxic hypercapnia, and hypoxic hypercapnia in ScramshRNA (blue) and 1ARshRNA (pink) injected rats. Group n for normoxia is 4 and 5 for ScramshRNA and 1ARshRNA-injected rats, and 15 and 13 for ScramshRNA and 1ARshRNA-treated rats respectively in other conditions. Bars show group means ±SEM. Data from individual animals are shown as dot plots over the appropriate bar. Mann-Whitney U Rank Sum test showed that ScramshRNA-treated rats spent more time sniffing during exposure to hypoxic-hypercapnic gas compared to 1ARshRNA-injected rats [U = 44, T = 135, P < 0.05]. The shRNA treatment during exposure to hypoxic gas alone [df = 26, t = -0.01, P = 0.996] or hypercapnia alone [U = 77, T = 168, P = 0.357] as determined by Student’s t-test and Mann-Whitney Rank Sum test respectively, failed to have any significant effects. B) Individual recording of flow in a ScramshRNA-injected rat illustrating a short bout of sniffing.
Figure 29. Relationship of 5-HT\textsubscript{1A} mRNA expression in commissural and medial NTS to area under the curve of ventilatory rate during exposure to hyperoxic hypercapnia. Pearson’s correlation between the area under the curve of ventilatory rate over the full duration of exposure to hyperoxic hypercapnia and relative 5-HT\textsubscript{1A} receptor mRNA in the commissural NTS (cNTS) A) and mNTS B) showed significant correlation only in the commissural NTS. C) Relative 5-HT\textsubscript{2A} receptor mRNA levels in the commissural and medial NTS of AAV injected rats. Values are group means ± SEM. Unpaired Student’s t-test revealed no significant effect of shRNA treatment on 5-HT\textsubscript{2A} receptor mRNA levels.
Discussion

In the current study, hypoxic hypercapnia increased c-Fos expression in caudal serotonin neurons of the RO and RM. However, both hypoxia and hypoxic hypercapnia induced c-Fos expression in the commissural and mNTS to the same extent. Reduction in 5-HT$_{1A}$ receptor gene expression reduced intermittent bouts of sniffing behavior during hypoxic hypercapnia, but did not otherwise affect ventilation during normoxia, hypoxic normocapnia, hyperoxic hypercapnia or hypoxic hypercapnia. These findings indicate that in the absence of central chemoreceptor activation, peripheral chemoreceptor activation is sufficient to activate serotonin neurons of caudal raphe regions known to innervate the commissural and mNTS. Moreover, activation of the peripheral chemoreflex sensitizes serotonin cells of the caudal raphe to activation by hypercapnia. Serotonin, released in the NTS during the combined activation of peripheral and central chemoreflexes, acts on 5-HT$_{1A}$ receptors to mediate arousal.

Our findings contradict our original hypothesis that hypercapnia per se is sufficient to activate serotonin cells of the caudal raphe. This prediction was based on evidence provided by da Silva et al., who recently showed that lesion of RO neurons using a saponin toxin targeting cells that express serotonin reuptake proteins, reduced the ventilatory response to 7% CO$_2$ in unanesthetized rats when oxygen levels were normal (da Silva et al., 2013). We also found that selective lesion of serotonin neurons in the caudal raphe using 5, 7-
Dihydroxytryptamine attenuated the ventilatory and sympathetic responses to hyperoxic hypercapnia (Kung et al., 2010). In these studies hyperoxic gas was used to suppress peripheral chemoreceptors and determine the effect of central chemoreceptor activation per se. However, recent findings have shown that the sensitivity of the ventilatory response to hypercapnia is suppressed when peripheral chemoreflex responses are inhibited (Blain et al., 2010). In our prior study, the effect of serotonin cell lesion on ventilatory response to hypercapnia was only apparent when CO$_2$ levels were raised to 8%. There was no difference in ventilatory response upon exposure to 5% CO$_2$. Thus, the inability of 5% hypercapnia to activate serotonin cells of the caudal raphe in the current study may have been due to reduced sensitivity of serotonin cells as a result of peripheral chemoreceptor suppression by hyperoxia. Thus, when peripheral chemoreceptor activation is absent, serotonin cells of the caudal raphe may require a greater CO$_2$ challenge to become activated. Indeed, we found that when peripheral chemoreceptors were activated by hypoxia, c-Fos expression in serotonin cells was evident with 5% hypercapnia.

As expected, hypoxia also induced c-Fos expression primarily in the commissural NTS where the majority of peripheral chemoreceptor afferents terminate, as has been repeatedly shown by others (Gozal et al., 1999; Song et al., 2011; Teppema et al., 1997). Hypoxic hypercapnia also produced the same degree of NTS cell activation. Thus, despite greater activation of serotonin neurons in the caudal raphe during hypoxic hypercapnia, there was no additional
activation of NTS cells, contrary to our hypothesis. However, it remains to be
determined what percentage of cells activated by hypoxia are GABAergic or
glutamatergic. We originally hypothesized that serotonin facilitates peripheral
chemoreflex responses by reversing the GABAergic cell activation that mediates
hypoxic ventilatory decline. Thus, it is possible that the same number of NTS
cells are activated during hypoxia in the presence or absence of serotonin cell
activation, but that a larger percent of activated cells are glutamatergic when
serotonin cells have been activated by hypoxic hypercapnia. This remains to be
determined.

Studies in this dissertation implicate 5-HT$_{1A}$ receptors expressed in the
NTS as the mediator of the sympathoexcitatory effects of endogenous serotonin
and 8-OH-DPAT during the second wave of compensation that develops after
syncope with blood loss. This effect appears to be dependent upon sinoatrial
nerve signaling. During the syncopal phase of hemorrhage, loss of blood flow to
the carotid bodies increases activation of chemoreceptor afferents. Thus, we
speculated that 8-OH-DPAT may act on 5-HT$_{1A}$ receptors in the NTS to facilitate
chemoreflex responses to hypoxia. However, our data did not support this
conclusion. Decreased 5-HT$_{1A}$ receptor mRNA expression did not influence
either ventilatory frequency, tidal volume or cumulative minute volume at any
time during a 45 min exposure to hypoxic hypercapnia, despite our evidence that
this same stimulus was sufficient to activate serotonin neurons. Instead, during
hypoxic hypercapnia we did notice a difference in ventilatory pattern between
groups. The ventilatory pattern of control rats was characterized by fluctuations in ventilatory frequency. This was verified by statistical evidence of increased variability in ventilatory rate over the course of hyperoxic hypercapnic gas exposure. Assessment of ventilatory wave forms showed an increased incidence of intermittent bouts of high frequency ventilation characteristic of snifff behavior. Indeed, during 5% hypercapnia, rats treated with the ScramshRNA-encoding virus tended to investigate the chamber and sniff the perimeter in what looked like an effort to find the gas inlet. In contrast, rats with successful 5-HT$_{1A}$ receptor knockdown did not show such investigative behaviors. Sniffing during chemosensory stimuli has been associated with arousal (Carnevali et al., 2013). Thus, it appears that instead of mediating chemoreflex responses, serotonin may act on 5-HT$_{1A}$ receptors in the NTS to mediate arousal to chemosensory input.

It remains to be determined if such 5-HT$_{1A}$ receptor-dependent arousal facilitates sympathetic and ventilatory responses to hemorrhage. Interestingly, a similar deficit in sniffing was reported during peripheral and central chemoreflex challenge in unanesthetized rats with lesion of the lateral parabrachial nucleus (Mizusawa et al., 1995). It was reported that control animals were restless and rambled during the initial gas change while lesioned animals remained calm. Control animals eventually calmed down with prolonged hypoxia. Thus, activity appeared to parallel changes in ventilation attributed to hypoxic ventilatory decline. It was proposed that such behavioral responses to chemosensory input require transmission of chemosensory-related signals to higher cortical or
subcortical centers that project through the parabrachial nucleus. Indeed, full ventilatory responses to hypercapnia requires intact supercollicular structures and is attenuated in sleep (Murphy et al., 1990). Interestingly, similar lesions of the parabrachial nucleus have also been found to greatly attenuate the secondary compensatory phase of compensation during hemorrhage (Blair et al., 2001; Blair and Mickelsen, 2006). Thus, our data are consistent with the hypothesis that arousal during the combined activation of peripheral and central chemoreflex responses is dependent upon 5-HT\textsubscript{1A} receptors in the NTS. It remains to be determined if the same arousal mechanism is responsible for sympathetic and ventilatory compensation following hypotensive hemorrhage.
CHAPTER VI

GENERAL DISCUSSION

The main findings from the studies conducted in this dissertation are highly novel in that they are the first to identify 5-HT$_{1A}$ receptors in the caudal NTS as a downstream target by which endogenous serotonin and exogenous 8-OH-DPAT mediate sympathoexcitatory and pressor effects in recovery from hypotensive hemorrhage. Furthermore, 8-OH-DPAT appears to augment the effects of endogenous serotonin release that occurs with blood loss. Given our evidence that 5-HT$_{1A}$ receptors in the NTS, particularly the commissural NTS, are critical in reducing lactic acidosis in the unanesthetized rat subjected to hemorrhage, the data further indicate an important role for 5-HT$_{1A}$ receptor agonists in improving oxygenation of peripheral tissue following hypotensive hemorrhage.

Based on prior work, we constructed a working hypothesis of how endogenous serotonin and 8-OH-DPAT mediate their ability to improve peripheral oxygenation following hypotensive hemorrhage (Figure 29). Evidence from other laboratories identified serotonin neurons in the caudal raphe as putative central chemoreceptors that are intrinsically sensitive to changes in pH and CO$_2$. Although this remains a controversial topic, there is some consensus that serotonin neurons somehow facilitate the chemoreflex response to
Figure 30. Original working model. During hypotensive hemorrhage, peripheral chemoreceptors in the carotid bodies are activated by reduced blood flow. Activation of second-order chemosensitive neurons in the caudal NTS to stimulate sympathetic and ventilatory drive through downstream activation of the RVLM and PreBötC respectively. During prolonged activation of the peripheral chemoreceptors, GABA interneurons become activated and reduce sympathetic and ventilatory drive. As blood loss continues, metabolic acidosis develops, which activates serotonin neurons in the caudal raphe to release serotonin in the NTS. In turn, activation of 5-HT$_{1A}$ receptors on GABAergic neurons disinhibits the peripheral chemoreflex, ultimately restoring sympathetic and ventilatory drive.
respiratory acidosis in unanesthetized animals (Bradley et al., 2002; Guyenet et al., 2010; Teran et al., 2014; Wang et al., 1998; Wang and Richerson, 1999; Wang et al., 2001). Whether serotonin neurons themselves have intrinsic chemosensitivity in vivo remains to be determined. Given their importance in mediating the response to acidosis, we predicted that serotonin neurons in the caudal raphe would be stimulated by the metabolic acidosis that develops with hypotensive hemorrhage and, as a consequence, would release serotonin into the NTS and activate 5-HT$_{1A}$ receptors to facilitate peripheral chemoreflex responses. However, 5-HT$_{1A}$ receptors are negatively coupled to signaling pathways that excite cells (Fargin et al., 1989). Therefore, in our model we predicted that 5-HT$_{1A}$ receptors involved in the response to hemorrhage would be expressed by inhibitory GABAergic interneurons that are activated during prolonged hypoxia and mediate the decrease in sympathetic activity and ventilation that accompany hypoxic ventilatory decline. As such, 5-HT$_{1A}$ receptor activation was speculated to reverse the decline in ventilation that develops with sustained peripheral chemoreceptor activation. Hypoxic ventilatory decline is accompanied by reductions in sympathetic drive as well. Therefore, reversal of this GABA-mediated response would be expected to increase ventilation and sympathetic activation.

To address this hypothesis, we examined whether acidosis stimulates serotonin neurons in the caudal raphe, and whether acidosis would further augment peripheral chemoreflex responses to hypoxia. Contrary to our working model, hyperoxic hypercapnia did not reliably activate serotonin neurons in the
caudal raphe despite causing an acidosis comparable to that seen in severe hemorrhage. Instead, we noted that hypoxic hypercapnia caused more activation of serotonin neurons of the RO and RM than did hypercapnia alone. Our results are incongruent with evidence that serotonin neurons are intrinsically sensitive to changes in arterial blood pH and PaCO₂. We utilized hyperoxic conditions in the presence of hypercapnia in order to prevent the activation of peripheral chemoreceptors which are also sensitive to pH. However, recent evidence has shown that in unanaesthetized dogs peripheral chemoreceptor input can sensitize the ventilatory response to central chemoreceptor activation (Blain et al., 2010). Conversely, denervation of the carotid sinus nerve impairs the central chemoreceptor-induced increase in ventilation. In light of the recent evidence that hyperoxia may reduce the sensitivity of central chemoreceptors, it is plausible that peripheral chemoreceptor activation may contribute to the excitability of serotonin neurons in the caudal raphe and this may contribute to the sensitization of central chemoreception. This may explain why more caudal raphe serotonergic neurons were activated during hypoxic hypercapnia than hyperoxic hypercapnia.

Nevertheless, our results indicate that acidosis did not appear to augment the chemoreflex ventilatory response to hypoxia, as indicated by the lack of consistent increase in minute ventilation over the course of the hypoxic hypercapnic challenge. Interestingly, we instead observed increased sniffing during hypoxic hypercapnia that appeared to be attenuated in rats subjected to 5-HT₁A receptor knockdown. Based on these final experiments, we deduced that
the sympathoexcitatory effect of 5-HT\textsubscript{1A} receptors in the caudal NTS was not due to augmentation of the peripheral chemoreceptor responses.

Instead, we now propose that activation of 5-HT\textsubscript{1A} receptors in the caudal NTS may be an important signaling mechanism that stimulates arousal. Several laboratories have documented sniffing as a measure of exploratory behavior as well as an arousal response (Carnevali et al., 2013). High frequency ventilation or sniffing behavior is dramatically increased in response to novel stimuli such as odors and visual cues as well as in response to rewards (Freeman et al., 1983; Waranch and Terman, 1975; Wesson et al., 2009). Therefore, increases in arousal may be demonstrated by increases in sniffing behavior such as that seen in our rats during exposure to hypercapnic gas mixtures. We noted sniffing behavior during hypoxia and hyperoxic hypercapnia in both treatment groups. However, only sniffing in response to hypoxic hypercapnia was reduced by 5-HT\textsubscript{1A} receptor knockdown. These findings suggest that multiple mechanisms contribute to sniffing behavior, but that sniffing behavior and thus arousal during hypoxic hypercapnia requires activation of 5-HT\textsubscript{1A} receptors in the caudal NTS.

Arousal responses require connections to the higher centers of the brain. One such pathway may be through the parabrachial nucleus (Kubo et al., 1998; Saper, 1982). The lateral subnucleus of the parabrachial nucleus (LPBN) has reciprocal projections with the NTS (Herbert et al., 1990). Lesion of the LPBN impairs sniffing behavior during chemoreceptor activation (Mizusawa et al., 1995). Interestingly, similar lesions impair the secondary compensatory response to hypotensive hemorrhage in rats. (Blair et al., 2001; Blair and
Mickelsen, 2006). Prior work has shown that the LPBN is activated in response to hypotensive hemorrhage, chemoreceptor activation, and peripheral tissue hypoxia (Chan and Sawchenko, 1994; Hirooka et al., 1997; Jaworski et al., 2002). Thus, it is tempting to speculate that 5-HT1A receptors in the NTS transmit arousal signals to higher-order centers through the LPBN. This could also explain the inability of 8-OH-DPAT to raise sympathetic activity in anesthetized models of hemorrhage in which behavioral arousal is not expressed.

Based on our findings from this project, we revised our working model to include a pathway through LPBN to higher-order centers. It remains to be determined how this pathway participates in sympathetic and ventilatory activation. However, it is recognized that 5-HT1A receptors expressed in the RVLM inhibit sympathetic activity (Bago et al., 1999). Therefore, it seems less likely that 8-OH-DPAT (and therefore, endogenous serotonin) is able to mediate sympathetic excitation through a pathway that incorporates RVLM.

Alternatively, it is possible that both sympathetic and ventilatory activation mediated by serotonin during hemorrhage are due to downstream activation of the paraventricular nucleus of the hypothalamus (PVN). The PVN is recognized as an integration site of higher-order sensory and central inputs with autonomic functions and provides direct excitatory projections to the intermediolateral cell column containing pre-ganglionic sympathetic fibers as well as to the RVLM (Badoer, 2001; Coote et al., 1998). Moreover, the PVN has direct projections to PreBötzC and phrenic nuclei in the spinal cord and electrical stimulation of these
projections can augment ventilation, HR and BP (Duan et al., 1997; Kc et al., 2002a).

Even more interesting, several investigators have shown that electrical or chemical activation of the PVN results in increased BP and HR as a result of increased sympathetic activation (Chen et al., 2003; Kenney et al., 2003; Martin et al., 1991). Since larger venules and veins have extensive sympathetic innervations, it is plausible that PVN-mediated increases in sympathetic drive can augment CO and venous tone (Furness and Marshall, 1974). In fact, studies performed by Martin et al. demonstrated that disinhibition of the PVN results in an increase in CO, without altering TPR in a conscious rat (Martin and Haywood, 1993). Since 8-OH-DPAT administration during hemorrhagic shock and disinhibition of the PVN both result in similar hemodynamic effects, it is tempting to speculate that PVN-mediated increases in venous tone may be disinhibited by 5-HT_{1A} receptor activation during hemorrhage, but the exact mechanisms require further investigation. Moreover, the PVN is also activated in response to hypoxia, hypercapnia, and hemorrhage (Badoer and Merolli, 1998; Kc et al., 2002b; King et al., 2012). Thus, it is plausible that PVN-mediated increases in sympathetic drive could augment venous tone and promote peripheral tissue perfusion during blood loss.

The LPBN also has direct projections to the central nucleus of the amygdala (CeA), a region also important in regulating arousal responses and cardiovascular control (Dampney, 1994; Davis and Whalen, 2001; Saha, 2005; Saha et al., 2005; Saper, 1982; Saper and Loewy, 1980). Electrical stimulation of
the CeA produces increases in HR and BP in conscious animals, but produces the opposite effect when stimulated in an anesthetized animal (Iwata et al., 1987). Furthermore, hypotension produced by hydralazine infusions causes c-Fos activation of neurons within the CeA. Electron microscopy studies confirmed that these same neurons that are activated by hypotension in the CeA, receive innervation from the LBPN (Takeuchi et al., 2004). Therefore, activation of the CeA may be contributing to the arousal responses seen during hypoxic hypercapnic gas exposure. Taken together, we revised our model to incorporate the projections from the NTS to the LPBN, CeA, and PVN which we now believe to be components of the pathways involved in compensation from hypotensive hemorrhage.

In our revised model (Figure 30) we speculate that some pathway that is initiated by peripheral chemoreceptor afferents contributes to increased excitability of serotonin neurons of the caudal raphe, such that they become more responsive to acidosis. Thus, the combination of peripheral and central chemoreflex stimulation increases serotonin release in the NTS. Subsequent activation of 5-HT$_{1A}$ receptors stimulates excitatory projections to higher-order centers, possibly through connections in the LPBN that contribute to a combination of effects that include arousal via the CeA, and parallel increases in sympathetic activity and ventilation possibly mediated by PVN. We further predict that this promotes increased tissue perfusion by stimulating increased venoconstriction.
Based on our most recent conclusions from this dissertation, we hypothesized that in our revised working model, serotonin neurons become more sensitive to metabolic acidosis during hemorrhage with concurrent activation of the peripheral chemoreceptors. In turn, serotonin activates 5-HT$_{1A}$ receptors in the caudal NTS that project directly to the lateral parabrachial nucleus (LPBN). The LPBN signals arousal to hemorrhage via projections to the amygdala and promotes sympathetic and ventilatory drive by downstream activation of the paraventricular nucleus of the hypothalamus (PVN). Once activated, the PVN promotes sympathoexcitatory activation via IML.
REFERENCE LIST


alpha-adrenergic receptors expressed in the nucleus tractus solitarii of rats. Experimental physiology 94, 773-784.


VITA

Jaime Vantrease was born on February 19th, 1986 in Chicago, IL to Dennis and Gemma Vantrease as the oldest of three children. She graduated from Woodstock High School in 2004 and began her scientific career at Augustana College (Rock Island, Illinois) that fall. She pursued her first research internship at Harbor Branch Oceanographic Institution in Fort Pierce, Florida during the summer of 2007, where she isolated an antibacterial compound from the deep sea marine sponge, *Axinella corrugata*. As a result of this experience, she became interested in the field of Pharmacology during college. Jaime graduated with a Bachelor of Arts degree in Biology with a minor in Chemistry in 2008.

In August of 2008, Jaime joined the Department of Molecular Pharmacology and Experimental Therapeutics at Loyola University Medical Center. Shortly thereafter, she joined the laboratory of Dr. Karie Scrogin, where she studied the role of central serotonin in the hemodynamic compensation that follows blood loss. While at Loyola, Jaime served as a student representative on the Graduate Student Council and mentored an undergraduate student through the Loyola Undergraduate Research Opportunity Program. After completing her Ph.D., Jaime will pursue a postdoctoral position in the field of neuroscience.