EFFECT OF CHRONIC EXERCISE ON CENTRAL OPIOID-MEDIATED RESPONSES TO HEMORRHAGE

By

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To all the rats who gave their lives in support of this body of research
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</tr>
<tr>
<td>HEM</td>
<td>Hemorrhage</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>Arterial pressure</td>
<td></td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
<td></td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus tractus solitarius</td>
<td></td>
</tr>
<tr>
<td>CVLM</td>
<td>Caudal ventrolateral medulla</td>
<td></td>
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<tr>
<td>RVLM</td>
<td>Rostral ventrolateral medulla</td>
<td></td>
</tr>
<tr>
<td>IML</td>
<td>Intermediolateral cell column</td>
<td></td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
<td></td>
</tr>
<tr>
<td>SAD</td>
<td>Sinoaortic barodenervation</td>
<td></td>
</tr>
<tr>
<td>TBV</td>
<td>Total blood volume</td>
<td></td>
</tr>
<tr>
<td>HIS</td>
<td>Hemorrhage-induced sympathoinhibition</td>
<td></td>
</tr>
<tr>
<td>AVP</td>
<td>Vasopressin, arginine vasopressin</td>
<td></td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal gray</td>
<td></td>
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<tr>
<td>vlPAG</td>
<td>Ventrolateral PAG</td>
<td></td>
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<td>LPBN</td>
<td>Lateral parabrachial nucleus</td>
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<tr>
<td>RVMM</td>
<td>Rostral ventromedial medulla</td>
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<tr>
<td>dlPAG</td>
<td>Dorsolateral PAG</td>
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</tr>
<tr>
<td>SAL</td>
<td>Saline control</td>
<td></td>
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<tr>
<td>HYDRAZ</td>
<td>Hydralazine</td>
<td></td>
</tr>
<tr>
<td>S-HEM</td>
<td>Slow rate of hemorrhage</td>
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</tr>
<tr>
<td>I-HEM</td>
<td>Intermediate rate of hemorrhage</td>
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<td>--------------</td>
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<tr>
<td>F-HEM</td>
<td>Fast rate of hemorrhage</td>
<td></td>
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<tr>
<td>FLI</td>
<td>Fos-like immunoreactivity</td>
<td></td>
</tr>
<tr>
<td>GS-PBS-TX</td>
<td>Goat serum-PBS-triton X100 solution</td>
<td></td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine hydrochloride</td>
<td></td>
</tr>
<tr>
<td>SCP</td>
<td>Superior cerebellar peduncle</td>
<td></td>
</tr>
<tr>
<td>LC</td>
<td>Locus coeruleus</td>
<td></td>
</tr>
<tr>
<td>CnF</td>
<td>Cuneiform nucleus</td>
<td></td>
</tr>
<tr>
<td>KF</td>
<td>Kolliker-Fuse nucleus</td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of measure</td>
<td></td>
</tr>
<tr>
<td>dmPAG</td>
<td>Dorsomedial PAG</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>Non-hemorrhaged control</td>
<td></td>
</tr>
<tr>
<td>FGI</td>
<td>Fluorogold immunoreactivity</td>
<td></td>
</tr>
<tr>
<td>FG</td>
<td>Fluorogold</td>
<td></td>
</tr>
<tr>
<td>EX</td>
<td>Exercise group</td>
<td></td>
</tr>
<tr>
<td>SED</td>
<td>Sedentary group</td>
<td></td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus of the hypothalamus</td>
<td></td>
</tr>
<tr>
<td>LF</td>
<td>Low frequency</td>
<td></td>
</tr>
<tr>
<td>HF</td>
<td>High frequency</td>
<td></td>
</tr>
<tr>
<td>CMM</td>
<td>Caudal midline medulla</td>
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</tr>
<tr>
<td>CVLM</td>
<td>Caudal ventrolateral medulla</td>
<td></td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin releasing hormone</td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar-Kyoto rats</td>
<td></td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
<td></td>
</tr>
<tr>
<td>CeA</td>
<td>Central nucleus of the amygdala</td>
<td></td>
</tr>
<tr>
<td>MnPO</td>
<td>Median preoptic nucleus</td>
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<tr>
<td>KOR</td>
<td>Kappa-opioid receptor</td>
<td></td>
</tr>
<tr>
<td>PRE</td>
<td>Before drug injections</td>
<td></td>
</tr>
<tr>
<td>POST</td>
<td>After drug injections</td>
<td></td>
</tr>
<tr>
<td>IOD</td>
<td>Integrated optical density</td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td>Inferior colliculus</td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
<td></td>
</tr>
<tr>
<td>DOR</td>
<td>Delta-opioid receptor</td>
<td></td>
</tr>
<tr>
<td>MOR</td>
<td>Mu-opioid receptor</td>
<td></td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
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<tr>
<td>SON</td>
<td>Supraoptic nucleus</td>
<td></td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamate decarboxylase</td>
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<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
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<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
<td></td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
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EFFECT OF CHRONIC EXERCISE ON CENTRAL OPIOID-MEDIATED RESPONSES TO HEMORRHAGE

By
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December 2009

Chair: Linda F. Hayward
Major: Veterinary Medical Sciences

Based on observations that central opioids are modulated by chronic exercise and the cardiovascular response to hemorrhage (HEM) involves opioid release in the central nervous system (CNS), the present studies were undertaken to test the hypothesis that chronic exercise protects against centrally-mediated hemorrhagic shock. These studies are particularly important in light of the paucity of information focusing on exercise-induced plasticity of CNS regions involved in cardiovascular control and health.

Study #1 tested the hypothesis that the hemodynamic responses and neural activation of various brainstem nuclei would differ when blood was withdrawn at a slow, intermediate, or fast rate. Results from this study support our hypothesis and identified that a rate of blood withdrawal of 1 ml/kg/min most reliably induces severe HEM in conscious rats. This rate of HEM also produced the most significant and least variable increases in neural activation of rostral brainstem sites, including the ventrolateral periaqueductal grey (vlPAG) and the lateral parabrachial nucleus (LPBN).

Study #2 tested the hypothesis that LPBN-projecting afferents from the vlPAG are involved in the cardiovascular response to severe HEM in the conscious rat. Results showed no difference in neural activation between LPBN-projecting and non-LPBN-
projecting vIPAG neurons. These findings do no support our initial hypothesis that this projection is actively involved in the descending control of autonomic responses to HEM.

Study #3 tested the hypothesis that exercise would modulate the cardiovascular and neural responses to severe, conscious HEM. Exercised animals displayed blunted HEM-induced bradycardia and hypotension and displayed altered neural activation in brain regions involved in cardiovascular control. Heart rate variability analysis also pointed to a possible change in basal respiratory function following exercise which may have contributed to an enhanced tolerance to HEM in the trained rats. These results suggest that exercise results in an augmented tolerance to severe HEM.

Study #4 tested the hypothesis that opioid receptors within the LPBN play a role in the exercise-induced tolerance to HEM. While there was no difference in KOR content in the dorsolateral rostral pons between EX and SED animals, exercise-induced tolerance to HEM was abolished by opioid receptor blockade in the LPBN. This study suggests that in EX animals, the inhibitory function of the LPBN may rely more heavily upon opioidergic mechanisms, perhaps due to decreased gamma-aminobutyric acid (GABA) inputs following training.

Together, these studies confirm our global hypothesis that chronic, voluntary exercise protects against the deleterious effects of acute, severe blood loss in the conscious rat. Additionally, this body of work has identified several important brain structures which may be modulated by chronic exercise that contribute to the protective effects of exercise against HEM. These studies are particularly important for future
research evaluating specific regions of the CNS which may serve as potential targets for
drug therapies and treatments for trauma victims.
CHAPTER 1
HEMORRHAGE, OPIOIDS, AND EXERCISE

Physiology of Hemorrhage

The first recorded observations regarding the arterial blood pressure response to hemorrhage (HEM) were performed by Stephen Hales in 1733 (Evans et al., 2001). Hales reported that upon initial removal of blood from a conscious horse, the blood pressure response was disproportional to the change in blood volume. It was not until 14-15 quarts of blood were removed that blood pressure significantly dropped and the animal fell into a state of shock. These foundational observations by Hales gave us a glimpse into the complex cardiovascular response to HEM. Much later, the hemodynamic response to HEM was characterized more completely by directly measuring right atrial pressure and use of the direct Fick method for calculating cardiac output (Barcroft et al., 1944; Dishman et al., 1995). In response to blood loss, two phases were identified in which the body was initially able to compensate for the blood loss with increases in heart rate (HR) and vascular resistance (phase I) followed by an inability to maintain normal blood pressure and HR (phase II), which eventually resulted in loss of consciousness.

Phase I Hemorrhage: Compensation

Prior to the late 1960’s, the “classical” textbook description of the hemodynamic response to HEM was a mono-phasic decline in blood pressure and HR (Schadt and Ludbrook, 1991). This discrepancy from previous reports and experiments performed in conscious humans and horses was determined to be the result of the use of anesthetics in research animals subjected to HEM protocols. Figure 1-1 is modified from a study that evaluated the cardiovascular response to HEM in both anesthetized and conscious
rats (Leskinen et al., 1994). Clearly, anesthesia alters the response by eliminating the “phase I” response to acute blood loss. Accordingly, more recent experiments evaluating the underlying physiological mechanisms regulating the complex hemodynamic response to HEM have largely begun utilizing conscious animals.

Figure 1-2 shows an arterial blood pressure (AP) and HR recording from a conscious rat during a 30% total blood volume (TBV) HEM. The principal mechanism regulating the initial, sympathoexcitatory, phase of hemorrhage is the withdrawal of baroreceptor input to the central nervous system (CNS) (Schadt and Ludbrook, 1991). Transient changes in blood pressure (resulting from blood volume loss) are sensed by these stretch-activated receptors in the aortic arch and carotid sinuses. In situations of increased blood pressure, baroreceptors increase neural signals to the CNS via the glossopharyngeal (from the carotid sinuses) and vagus nerves (from the aortic arch). These afferents synapse first in the nucleus tractus solitarius (NTS) located in the caudal, dorsal brainstem. Excitatory projections from the NTS are sent to the caudal ventrolateral medulla (CVLM) which, in turn, sends inhibitory projections to the rostral ventrolateral medulla (RVLM). The RVLM sends direct excitatory projections to the sympathetic preganglionic neurons of the intermediolateral cell column (IML) of the spinal cord (T1-11). Thus, decreased activation of the baroreceptors, as occurs during acute loss of blood, results in an augmented sympathetic drive—via disinhibition of the RVLM—and thus an elevated heart rate and increased peripheral vascular resistance (Li and Dampney, 1994). Baroreceptor unloading also leads to inhibition of vagal preganglionic neurons found in the nucleus ambiguus, thus resulting in decreased cardiac vagal drive and further increases in HR (Li and Dampney, 1994; Henderson et
Because of this baroreflex mechanism early in HEM, the decline in cardiac output at the onset of HEM does not elicit a decline in mean arterial pressure (MAP) (Ludbrook and Graham, 1984; Schadt et al., 1984). Figure 1-3 (modified from Dampney et al., 2002) shows a simplified diagram outlining this neural network.

The bulk of support for baroreceptor unloading being the primary contributor to the acute response to HEM comes from a large body of literature in which animals underwent sinoaortic barodenervation (SAD) prior to being subjected to some degree of HEM (Goetz et al., 1984; Ludbrook and Graham, 1984; Schadt and Gaddis, 1986; Quail et al., 1987; Meller et al., 2003). Following SAD, animals do not display the typical compensatory hemodynamics seen in baro-intact animals (tachycardia and increased vascular resistance) (Schadt and Ludbrook, 1991). There are other afferent signals, however, that may also contribute to the sympathoexcitation elicited by the onset of HEM in some species. Morita and Vatner (1985) measured renal sympathetic nerve activity in conscious dogs during a HEM that produced a MAP of 40-50 mmHg following various levels of barodenervation. The results of this study demonstrated that both arterial baroreceptors as well as cardiac receptors contributed to the sympathoexcitation recorded at the renal sympathetic nerves during the normotensive phase of HEM, suggesting that the low-pressure sensing cardiopulmonary receptors located in atrial and ventricular walls, in the coronary and pulmonary arteries, and in the inferior and superior vena cavae also play a role in the acute response to HEM (Morita and Vatner, 1985). Multiple other studies, however, argue against any major role of the cardiopulmonary receptors in the initial response to blood loss in conscious animals (e.g., dogs and rabbits) since chemical blockade or complete denervation of the cardiac
nerves does not alter the blood pressure response to non-hypotensive HEM (Burke and Dorward, 1988; Ludbrook and Graham, 1984; Morita and Vatner, 1985; Quail et al., 1987; Evans et al., 1989; Meller et al., 2003). Experiments performed on human heart transplant recipients show a role for cardiopulmonary receptors in the early rise in sympathetic nerve activity during acute HEM. Heart transplant patients who lacked atrial cardiac receptor innervation but maintained ventricular cardiac receptor innervation displayed a significant attenuation in vasoconstriction (indicative of sympathoexcitation) in response to simulated moderate HEM using lower body negative pressure (Mohanty et al., 1987). Since previous studies evaluating the role of cardiac receptors have been performed in a variety of human and animal models and have resulted in varying outcomes, the effect of species should certainly not be overlooked as a possible explanation for differing results.

**Phase II Hemorrhage: Decompensation**

When blood loss exceeds a critical value (~15-25%) of total blood volume (TBV), a decompensatory phase ensues (see Figure 1-2) (Schadt and Ludbrook, 1991). This phase is characterized by severe bradycardia and hypotension. The bradycardia is due to both a withdrawal of sympathetic nerve activity (Schadt and Ludbrook, 1991; Evans et al., 1994; Schadt and Hasser, 2004) and a rise in vagal drive (Barcroft et al., 1944; Murray and Wise, 1996; Porter et al., 2009). Decompensatory hypotension is the product of a centrally-mediated sympathoinhibition and a resultant fall in total peripheral resistance. Multiple studies in which sympathetic nerves were recorded during HEM or simulated HEM (e.g., caval occlusion) demonstrate an initial increase in nerve activity followed by a precipitous fall in activity coincident with the onset of hypotension (Morgan et al., 1988; Hasser and Schadt, 1992). Additionally, studies have shown decreased
vascular resistance in conscious rabbits and decreased blood flow in specific regional vascular beds of humans that occurs with the onset of hypotension following decreases in central blood volume (Barcroft et al., 1944; Schadt et al., 2006).

While arterial baroreceptors play a chief role in the sympathoexcitatory response to acute HEM, the mechanisms mediating the transition from compensation to decompensation are less clear. Multiple studies, however, support a role for the cardiac receptors in the hemodynamic response to blood loss. For example, Oberg and Thoren demonstrated increased nerve fiber activity in cardiac afferents during caval occlusion (Lund et al., 2002) and, later, Thoren reported that vagal cooling could entirely block the associated bradycardia (Thoren, 1979). Studies performed in conscious rabbits also support a role for cardiac afferents in the initiation of HEM-induced sympathoinhibition (HSI). In one study, cardiac afferent nerve blockade prevented the sympathoinhibition and hypotension following blood loss (Burke and Dorward, 1988). In a separate study, rabbits were subjected to caval occlusion coupled with either saline or procaine (to induce a reversible cardiac nerve blockade) applied within the cardiac space. Results from this study showed that procaine was able to prevent the decline in vascular resistance, suggesting that stimulation of cardiac receptors precedes the sympathoinhibition associated with decreased central blood volume (Evans et al., 1989).

Once HEM has advanced to this second, decompensatory phase, continued bleeding can result in massive organ failure and cardiac death. Although this suggests that the decompensatory phase of hemorrhage initiates eminent death, it may actually serve as a protective mechanism. Thoren and others have postulated that the
sympathoinhibition may act to guard against excessive heart rate during progressively declining diastolic filling and/or prevent increases in perfusion pressure from prolonging uncontrolled bleeding (Thoren, 1979; Blair and Mickelsen, 2006), slowing the flow of blood and perhaps aiding in clotting. Additionally, the severe hypotension characteristic of this phase of HEM coincides with the significant increases in the release of pressor hormones (e.g., renin, vasopressin [AVP], and catecholamines) (Korner et al., 1990; Schadt and Ludbrook, 1991; Hager et al., 2009) involved in recovery from blood loss. Although the release of all these hormones are increased during the compensatory phase of HEM—due to sympathetic stimulation, they do not reach concentrations that result in a pressor response until a fall in MAP is induced (Hall and Hodge, 1971; Darlington et al., 1986; Thrasher and Keil, 1998; Haberthur et al., 2003).

**Phase III Hemorrhage: Recompensation**

If blood loss is stopped before cardiac arrest occurs, animals display a spontaneous recovery (see Figure 1-2). During this period, both MAP and HR slowly return to baseline or near-baseline values (Ahlgren et al., 2007). This recovery process is the result of acute elevations in circulating angiotensin and AVP (Fejes-Toth et al., 1988; Schadt and Hasser, 1991; Ponchon and Elghozi, 1997) coupled with a restoration of sympathetic tone to arterial (Blair and Mickelsen, 2006) and venous (Potas et al., 2003) vascular beds. Following this initial recovery process, a more gradual course of vascular refilling takes place via a shift of fluids from the interstitium into the plasma (Blair and Mickelsen, 2006). This volume restoration is initiated by a decreased capillary hydrostatic pressure drawing protein-free fluid into the capillaries—which begins at the onset of recovery from blood loss. As fluid shifts from the interstitium to the plasma, extracellular osmolality increases. Hours after fluid restoration has begun,
proteins are slowly moved into the plasma, which facilitates even more fluid influx. This continues until blood volume has been reestablished, taking as much as 18-24 hours (Byrnes et al., 1982; Pirkle et al., 1982; Slimmer and Blair, 1996).

**Central Nervous System Control of Hemorrhage**

Multiple lines of evidence point to specific regions of the brain as well as specific modulatory neurotransmitter systems that may be involved in the complex cardiovascular response to hypotensive HEM. The focus of this part of the introduction and the following experiments will be on two brainstem nuclei, the periaqueductal gray (PAG) and the lateral parabrachial nucleus (LPBN).

In the early 1990’s, a study was performed in which mesencephalic decerebrate rabbits displayed complete abolition of decompensation to a simulated HEM (caval occlusion) (Evans et al., 1991). Authors of this study concluded that suprapontine centers provided the sympatheoinhibitory input to brainstem regions regulating blood pressure (such as the RVLM) that were necessary for eliciting the decompensation following acute central hypovolemia. Later, however, it was shown that inactivation of specific opioid receptors in the midbrain PAG could also attenuate hemorrhagic decompensation (Cavun and Millington, 2001), suggesting that the midbrain may be more important in this later phase of HEM than the forebrain. Critics of the study by Evans et al (1991) postulated that their results may have been due to accidental midbrain damage during the decerebration procedure (Troy et al., 2003). In an effort to clarify the roles of the forebrain and midbrain in decompensation, Troy et al (2003) evaluated the hemodynamic response to severe HEM in rats that had undergone either a pre-collicular or pre-trigeminal decerebration versus intact animals. In pre-collicular decerebrate rats, in which midbrain-brainstem connections were preserved, but
forebrain involvement was eliminated, there was an attenuated compensation and recovery from HEM compared to controls. In contrast, pre-trigeminal decerebrate animals, who lacked midbrain-brainstem integrity, displayed an attenuation of the decompensatory phase. The results of this study suggest a role for the forebrain in the compensatory and recovery phases of HEM while the midbrain seems to be more important for control of hemorrhagic decompensation.

The importance of midbrain structures in the full expression of the cardiovascular response to severe blood loss is further supported by studies showing that the PAG—specifically, the caudal ventrolateral PAG (vlPAG)—plays a crucial in hemorrhagic decompensation (Ward and Darlington, 1987; Cavun and Millington, 2001). As shown in Figure 1-4 (modified from (Cavun and Millington, 2001)), chemical inhibition of the caudal vlPAG (with lidocaine or cobalt chloride) results in a significant attenuation in the fall of MAP following HEM in conscious rats. The vlPAG likely contributes to the onset of HSI via direct projections to other caudal brainstem sites, like the RVLM and/or the rostral ventromedial medulla (RVMM), that have also been shown to mediate decompensation following HEM (Mason et al., 1985; Henderson et al., 1998; Henderson et al., 2000; Li et al., 2001).

The vlPAG sends efferents to a second brainstem nucleus, the LPBN, a rostral pontine region that has also been shown to affect hemorrhagic decompensation (Figure 1-5, modified from (Krout et al., 1998)). Following partial lesion of the LPBN, conscious animals showed a less severe bradycardia during HEM compared to both complete LPBN lesioned and sham lesioned animals (Blair et al., 2001). Finally, in accordance with other reports (Krukoff et al., 1995; Anselmo-Franci et al., 1998; Keay et al., 2002),
work from our lab confirms neural activation of both the vlPAG and the LPBN, as marked by increased c-Fos expression during severe conscious HEM (Ahlgren et al., 2007). Both the caudal vlPAG and the LPBN show increased Fos-positive nuclei following withdrawal of 30% of estimated TBV in conscious rats compared to non-HEM controls. While the connectivity of these two nuclei, as well as their individual influences over the cardiovascular response to HEM are well established, the specific role they play as an integrated and functional network during HEM has yet to be described.

Recent work has shown that the recovery phase following HEM is also dependent upon the structural integrity of the LPBN. Blair et al (2001) showed that complete bilateral LPBN lesions resulted in a deficient capacity for MAP and HR to recover following HEM. Additionally, infusion of kynurenic acid (a glutamate receptor antagonist) within the ventrolateral aspect of the LPBN immediately following hypotensive HEM in a conscious rat results in prolonged depression of HR and MAP. This would suggest that glutamate receptor activation within the LPBN is necessary for normal recovery from severe blood loss (Blair and Mickelsen, 2006). This same study concluded that the delayed recovery from HEM displayed by LPBN-lesioned animals was the result of an impaired sympathetic drive to the vasculature—which is not all together surprising since activation of specific subnuclei located in the ventrolateral region of the LPBN (e.g. external lateral subnucleus) can produce significant hypertension and tachycardia via activation of the RVLM (Chamberlin and Saper, 1992; Len and Chan, 1999; Blair and Mickelsen, 2006).

**Central Opioid Modulation of Hemorrhage**

In the last 20 years, opioidergic mechanisms in the CNS have been implicated in the hemodynamic response to HEM. Several lines of evidence connect opioid mechanisms
within the CNS to the onset of hemorrhagic decompensation and HSI. It is important to have a basic understanding of the structure and functions of opioid peptides and their receptors prior to discussing physiological experiments involving them.

**Opioid Peptides and Associated Receptor Function**

Opioid peptides were first described by Hans Kosterlitz et al. in 1975. Kosterlitz described an endogenous substance that behaved like morphine in the body (Chen et al., 1997). This substance was titled “enkephalin” (meaning ‘in the head’) as it was first characterized in brain and adrenal tissue (Burke and Dorward, 1988). Since the discovery of enkephalins, several other opioid peptides and their receptors have been described. Table 1-1 lists the three main classes of endogenous opioids, their precursor proteins, and the primary opioid receptor on which each ligand acts. Opioid receptors are $G_{i/o}$ protein-coupled receptors. Ligand binding of an opioid peptide to its respective opioid receptor leads to activation of a specific class of G-protein, which can inhibit intracellular cyclic AMP production, inhibit voltage-gated calcium channels, or stimulate inwardly rectifying potassium channels (Law et al., 2000)—all of which result in hyperpolarization of the cellular membrane. Accordingly, opioid peptides are associated with inhibitory-type functions and in the CNS they generally function to depress or inhibit neurotransmission.

**Opioidergic Modulation of Hemorrhage in the Brainstem**

Endogenous opioids were first associated with hemorrhagic hypotension in the late 1970’s with the work of Faden and Holaday (1979) when they showed that the opioid receptor antagonist naloxone was able to produce increases in MAP in hemorrhaged, but not normotensive, animals (Faden and Holaday, 1979). Multiple studies since then have further described the contribution of endogenous opioid
peptides and opioid receptor activation in HEM. From these studies it is clear that opioid systems within the brainstem are integrally involved in the hemodynamic response to severe HEM—particularly the onset of HSI and the resultant decompensation. Furthermore, multiple lines of evidence have isolated central opioid modulation of HEM to the rostral brainstem, including the vlPAG and the LPBN.

Both the vlPAG and LPBN contain considerable amounts of opioid peptides and their associated receptors (Atweh and Kuhar, 1977; Seeger et al., 1984; Mansour et al., 1987; Kalyuzhny and Wessendorf, 1998). The PAG has been shown to express mu-, delta-, and kappa-opioid receptors (Mansour et al., 1995). While the LPBN has an abundance of both mu- and kappa-opioid receptors (Unterwald et al., 1991; Yasuda et al., 1993; Mansour et al., 1995; Chamberlin et al., 1999), there is only one report suggesting the sparse presence of delta-opioid receptors within the parabrachial complex (Arvidsson et al., 1995). It is generally accepted that there is a functional lack of delta-opioid receptors in the LPBN (Mansour et al., 1995; Imai et al., 1996).

General lesion or blockade of either the vlPAG or the LPBN results in altered blood pressure and/or HR responses to HEM (Blair et al., 2001; Cavun and Millington, 2001). More specifically, opioid agonists injected into the vlPAG cause HEM-like symptoms. Keay et al (1997) reported decreased arterial blood pressure and bradycardia following microinjection of a delta-opioid receptor agonist into the vlPAG (Keay et al., 1997). Opposite results (hypertension and tachycardia) were seen when injections of the same drug were directed toward the dorsal aspect of the PAG—an area more closely associated with autonomic modulation of respiration (Hayward et al., 2004) and “fight or flight” responses (Dielenberg et al., 2001). In the LPBN, bilateral
administration of a mu-opioid receptor agonist results in an increased hypertonic saline consumption by sodium-depleted as well as normohydrated conscious rats (De Oliveira et al., 2008), implicating opioids within the LPBN in volume regulation.

Opioid antagonists injected into the dorsolateral pons markedly attenuate hemorrhagic decompensation. Figure 1-6 demonstrates that naloxone microinjected bilaterally into the vlPAG nearly abolishes HEM-evoked hypotension (Cavun et al., 2001). Animals from the same study that were subjected to HEM in which naloxone was microinjected into the dorsal part of the PAG did not show an attenuation of the hypotensive response to HEM. Similarly, bilateral microinjection of a delta-opioid receptor antagonist, but mu- or kappa- specific receptor antagonists, effectively prevented a fall in MAP, suggesting involvement of a fairly specific delta-opioid mechanism within the vlPAG. In a separate study by Iwasaki and colleagues (1993), the non-selective opioid antagonist diprenorphine was microinjected into the LPBN prior to acute hypovolemia (Iwasaki et al., 1993). This study reported that opioid antagonism in the LPBN caused an inhibition of vasopressin release that would normally occur in response to hypovolemia or HEM. Unfortunately, blood pressure and HR responses to low blood volume were not reported in this study.

**Exercise Training and the Central Nervous System**

A large body of research has clearly shown that exercise induces overall health outcomes and enhances general brain function (Warburton et al., 2006; Engesser-Cesar et al., 2007). With regard to cardiovascular health benefits, much of the basic and clinical research has focused primarily on peripheral effects of exercise and/or alluded to some modulation in CNS involvement, but failed to give in-depth explanations or discussions regarding such (Meredith et al., 1989; Hambrecht et al., 2000). More
recently, however, research has documented the ability of exercise to alter the “molecular machinery” (Engesser-Cesar et al., 2007) of the CNS--anatomically and functionally--in ways that promote cardiovascular health (Warburton et al., 2006) and reduce and prevent a host of cardiovascular pathologies, such as hypertension (Appel et al., 2003), heart disease (Dunn et al., 1996), and type II diabetes (Knowler et al., 2002). In addition to stimulating neurotrophic factors in the brain (Engesser-Cesar et al., 2007), exercise has been shown to modulate the synthesis, metabolism, and dynamic release of a range of neurotransmitters throughout the brain and spinal cord, many of which are involved in autonomic control and cardiovascular regulation (Brown et al., 1979; Meeusen and De Meirleir, 1995; Dunn et al., 1996; Dishman et al., 1997; Engesser-Cesar et al., 2007).

**Exercise Alters Central Opioids and Opioid Receptor Function**

Acute bouts of exercise stimulate the release of endogenous opioids (Sommers et al., 1990; Goldfarb et al., 1991; Boone et al., 1992) which can result in increased nociceptive thresholds in both humans (Janal, 1996) and rats (Smith and Yancey, 2003). The fact that this is an opioid-mediated phenomenon is supported by the observation that pre-treatment with naloxone is able to completely block the exercise-induced change in pain threshold (Janal, 1996; Smith and Yancey, 2003). Conversely, chronic exercise, and thus continued release of these endogenous opioids, has been shown to lead to a developed “tolerance” or decreased sensitivity to exogenous opioid agonists (Kanarek et al., 1998; D’Anci et al., 2000). While no study to date has actually reported a decreased number of opioid receptors in any given loci within the CNS as the result of chronic exercise training, a number of studies do support this theory. Houghten et al (1986) reported decreased hypothalamic β-endorphin binding sites in
rats given access to running wheels for as little as five weeks (Houghten et al., 1986) and proposed that the effect was modulated by a compensatory decrease in opioid receptors. Similarly, other studies have shown a down-regulation of opioid receptors following chronic administration of exogenous mu-opioids (morphine) (Malatynska et al., 1996; Chen et al., 1997). Additionally, one study evaluated the sensitivity of exercised versus sedentary rats to a number of different analgesic drugs with varying efficacy for opioid receptors. Chronic opioid exposure leads to greater degrees of tolerance when lower efficacy opioids are administered compared to more potent analgesics (Paronis and Holtzman, 1992; Smith et al., 1999; Walker and Young, 2001). This is due to the fact that lower efficacy opioid drugs require activation of a larger number of opioid receptors and therefore have a larger receptor pool from which to subtract. Therefore, authors reasoned that if chronic exercise leads to the development of opioid tolerance similar to that resulting from chronic exogenous opioid administration, then exercised rats should display less significant changes in sensitivity to opioid drugs as they increased in magnitude of relative efficacy at the mu-opioid receptor. This was, in fact, the outcome of the study (Smith and Yancey, 2003). Taken together, these studies indicate that increased mu-opioid receptor stimulation, whether by endogenous (chronic exercise) or exogenous (drug abuse) means, elicits a functional alteration in opioid (most likely mu-opioid) receptors.

**Dynorphins**

Dynorphins, a family of opioid peptides that preferentially bind to kappa-opioid receptors, have been shown to be up-regulated in response to both short-term and chronic exercise (Aravich et al., 1993; Persson et al., 1993). Studies showing a decreased kappa-opioid-dependent antinociception in rats allowed access to running
wheels for 5-6 weeks suggest that, similar to what is seen with mu-opioid receptors following exercise training or chronic morphine administration (Smith and Yancey, 2003), long-term exercise leads to either a decreased sensitivity or a down-regulation of kappa receptors (D'Anci et al., 2000).

**Specific Aims**

In summary, based on observations that central opioids are modulated by chronic exercise and the cardiovascular response to HEM involves opioid release in specific regions of the brain, the present studies were undertaken to evaluate the impact of chronic exercise on the physiological response to blood loss. These studies are particularly important in light of the paucity of information focusing on the central effects of exercise on cardiovascular health. A better understanding of the centrally-mediated effects of exercise in the response to HEM is important for the development of individual treatment plans for trauma victims. If, indeed, trained individuals respond differently to blood loss than do sedentary individuals, a difference in effective treatment may exist. There is significant translational relevance and potential for clinical application for the results of these studies in light of the large number of soldier deaths associated with trauma HEM (Alam et al., 2005). A more developed picture of how chronic exercise affects the CNS will enhance our ability to determine optimal doses of drugs targeting central sites for patients in different physical conditions (e.g., trained verses sedentary, or soldier versus civilian). This is also potentially important for the prescription of certain psychoactive and analgesic drugs—many of which target central opioid receptors.

**Specific Aim 1**

Assess the neural responses to different rates of HEM in order to develop a standard protocol that best demonstrates HSI during severe HEM in the conscious rat.
Hypothesis: We hypothesized that the fastest rate of HEM would induce the earliest and greatest level of decompensation and that this would be associated with increased levels of c-Fos immunoreactivity in the vlPAG and LPBN.

**Specific Aim 2**

Identify whether vlPAG neurons that project to the LPBN are activated in response to severe HEM in the conscious rat. **Hypothesis:** We hypothesized that a greater percentage of vlPAG area neurons projecting to the LPBN would be activated, as indicated by the cellular marker c-Fos, compared to the dorsolateral PAG (dIPAG), in response to severe HEM.

**Specific Aim 3**

Identify the effect of chronic exercise on the hemodynamic response to HEM. **Hypotheses:** Exercise trained animals will display an altered hemodynamic and neural response to hypotensive HEM compared to sedentary animals. Specifically, we hypothesized that exercise training would result in an augmented compensatory response to HEM as evidenced by either a greater increase in HR early in HEM and/or a prolonged time to decompensation. Additionally, we anticipated a blunted decompensatory response to HEM in exercised versus sedentary rats.

**Specific Aim 4**

Determine whether the effect of chronic exercise on the modulation of the hemodynamic response to HEM is due to a modification of opioid receptors in the LPBN. **Hypotheses:** Chronic exercise will cause a down-regulation of central kappa opioid receptors, specifically in the dorsolateral pons and blockade of opioid receptors in the LPBN will potentiate the protective effects of chronic exercise in response to HEM.
Summary

The overall goal of these studies was to gain a more comprehensive understanding of how voluntary exercise training alters central mechanisms involved in the hemodynamic response to HEM. Based on previous work showing that opioid receptors present in specific midbrain nuclei (vlPAG and LPBN) are involved in the cardiovascular response to HEM and that chronic exercise functionally alters opioid receptor populations, we hypothesized that exercise-trained animals would display an altered neural and cardiovascular response to HEM compared to sedentary animals. Since exercise has been shown repeatedly to be both cardio- and neuro-protective, we anticipated an enhanced tolerance to severe HEM in the trained versus sedentary conditions. We further hypothesized that a down-regulation of opioid receptors in the dorsolateral pons may underlie such a modified response. These studies will, additionally, provide novel information regarding central neuroanatomical networks mediating the cardiovascular response to HEM that may be affected by chronic exercise.
Figure 1-1. Effect of hemorrhage on mean arterial pressure and heart rate in anesthetized (left panel) versus conscious (right panel) rats. Open circles represent group averages for rats in which 1.5 ml of blood was removed every 10 minutes (arrows; n=6). Closed circles represent group averages for control animals (n=6) that underwent the same hemorrhage protocol, except the removed blood volume was immediately replaced with donor blood. Modified from Leskinen et al., 1994.
Figure 1-2. Heart rate (HR) and mean arterial pressure (MAP) recording from a conscious rat during 30% estimated total blood volume hemorrhage. Grey bar indicates time of blood withdrawal (20 minutes). Progressive phases of the cardiovascular response to hemorrhage are outlined by boxes and labeled accordingly.
Figure 1-3. Brainstem and spinal cord pathways that subserve the baroreceptor reflex control of sympathetic output to the heart and blood vessels. Open triangles indicate excitatory synapses and filled triangles indicate inhibitory synapses. CVLM = caudal ventrolateral medulla; IML = intermediolateral cell column in the spinal cord; NTS, nucleus tractus solitarius. Modified from Dampney et al., 1994.
Figure 1-4. Effect of synaptic blockade in the ventrolateral periaqueductal gray (vIPAG) on the change in mean arterial pressure (MAP) from baseline during severe hemorrhage (HEM). Cobalt chloride (top panel) or lidocaine (bottom panel) was bilaterally injected into the caudal vIPAG of conscious, chronically instrumented rats five minutes prior to HEM at a rate of ~1.25 ml/kg/min. Modified from Cavun and Millington, 2001.
Figure 1-5. Diagram of connections between the ventrolateral periaqueductal grey (vlPAG) and lateral parabrachial nucleus (LPBN) that subserve cardiovascular regulation. SCP = superior cerebellar peduncle; NTS = nucleus tractus solitarius. Modified from Krout et al., 1998.
Table 1-1. Three main classes of endogenous opioids, their precursor proteins, and the primary opioid receptor on which each ligand acts.

<table>
<thead>
<tr>
<th>Opioid Peptide</th>
<th>Precursor Protein</th>
<th>Primary Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endorphins</td>
<td>Pro-opiomelanocortin</td>
<td>Mu, µ</td>
</tr>
<tr>
<td>Enkephalins</td>
<td>Pro-enkephalin</td>
<td>Delta, δ</td>
</tr>
<tr>
<td>Dynorphins</td>
<td>Pro-dynorphin</td>
<td>Kappa, κ</td>
</tr>
</tbody>
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Figure 1-6. Effect of opioid receptor blockade in the caudal ventrolateral periaqueductal grey (vlPAG) on mean arterial pressure (MAP) during hemorrhage. Bilateral injections of naloxone (10nmol; 5nmol/cannula) or saline (0.5 µl/cannula) were placed into the caudal vlPAG of halothane-anesthetized rats five minutes prior to hemorrhage at a rate of ~1 ml/kg/min. Reproduced with permission from Cavun et al., 2001.
CHAPTER 2
HEMODYNAMIC RESPONSES AND C-FOS CHANGES ASSOCIATED WITH HYPOTENSIVE HEMORRHAGE: STANDARDIZING A PROTOCOL FOR SEVERE HEMORRHAGE IN THE CONSCIOUS RAT

Introduction

Conscious mammals subjected to severe, progressive hemorrhage (HEM) have been shown to pass through two hemodynamically distinct phases. Upon initial blood loss, an augmented sympathetic drive results in an elevated heart rate (HR) and increased peripheral resistance. In this compensatory phase, the declining cardiac output is not large enough to cause a fall in mean arterial pressure (MAP). When blood loss approaches 25-30% of total blood volume, a decompensatory phase consisting of sympathetic withdrawal, hypotension, and bradycardia ensues. Anesthesia can significantly alter the response pattern to blood loss (Evans et al., 1989; Heslop et al., 2002). Anesthetized animals, in general, show little if any compensatory response to HEM and therefore enter the decompensatory phase earlier than conscious animals following loss of much smaller volumes of blood (Heslop et al., 2002). Additionally, under anesthesia, MAP drops more quickly and is typically proportional to the amount of blood withdrawn. The duration of the decompensatory phase is often associated with increased peripheral organ damage and a reduced chance of survival (Runciman and Skowronski, 1984).

Over the last ten years there has been increased interest in understanding the central-neural mechanisms underlying hemorrhage-induced sympathoinhibition (HIS) with the hope of identifying therapies that might delay the onset of the decompensatory response and facilitate recovery. While the mechanisms underlying the initial, compensatory phase of hemorrhage are fairly well accepted (baroreceptor unloading)
(Runciman and Skowronski, 1984; Courneya et al., 1991; Evans et al., 2001), the signal that prompts the transition to a decompensatory state is less clear. There is some evidence, however, that input to the midbrain is critical for inducing HSI (Evans et al., 1991). For example, blockade of the ventrolateral periaqueductal grey (vlPAG) has been shown to both delay and attenuate hemorrhage-evoked hypotension (Cavun and Millington, 2001). Furthermore, in a recent brain transection study, it was demonstrated that pre-trigeminal decerebrate rat, lacking midbrain-brainstem communication, had a markedly attenuated HSI response to severe HEM compared to controls (Troy et al., 2003). In contrast, pre-collicular decerebrate animals (in which midbrain-brainstem connections were still present) displayed a prolonged decompensation compared to controls. These findings support the current idea that midbrain structures play a pivotal role in the onset of HSI. Interestingly, in the same study it was shown that prolongation of the decompensatory response observed following pre-collicular decerebration was only observed in animals that underwent a 30% total blood volume (TBV) HEM over 20 minutes but was not present when 30% TBV HEM occurred over 40 minutes. Thus, the rate of HEM may profoundly impact brain mechanism(s) contributing to or perhaps initiating HSI.

To our knowledge, no previous studies have combined a thorough evaluation of the cardiovascular outcome of constant-volume HEM over different withdrawal times with the identification of the associated specific central nervous system (CNS) regional sites of activation. Furthermore, an in-depth evaluation of the impact of rate of HEM on the brain mechanisms involved in HSI might be considered necessary at the present time, since a review of the literature reveals a distinct lack of consistency in HEM
protocols between studies (Shirley et al., 1991; Buller et al., 1999; Heslop et al., 2002; Scrogin, 2003; Molina et al., 2004; Schadt and Hasser, 2004; Ditting et al., 2005).

The purpose of this project was to assess the neural responses to different rates of HEM in order to develop a standard protocol that best demonstrates HSI during severe HEM in conscious rats for use in future studies. Because specific regions throughout the CNS have been shown to play integral roles in regulation of homeostasis during HEM (Buller et al., 1999; Cavun and Millington, 2001; Troy et al., 2003; Heslop et al., 2004), we felt that, in addition to hemodynamics, evaluating neural activity in response to hemorrhagic hypotension would offer a more complete picture of the natural consequences resulting from severe blood loss. Accordingly, this study aimed to evaluate hemodynamic responses and c-Fos immunoreactivity in specific regions of the brain following different rates of severe (30% TBV) HEM in the conscious rat. Two areas of particular interest were the vlPAG and the lateral parabrachial nucleus (LPBN) because of the independent and integrated roles they have been shown to play in response to cardiovascular challenge (Ward, 1989; Polson et al., 1995; Krout et al., 1998; Blair et al., 2001; Cavun and Millington, 2001; Dean and Woyach, 2004). We hypothesized that the fastest rate of hemorrhage would induce the earliest and greatest level of decompensation and that this would be associated with increased levels of c-Fos immunoreactivity in the vlPAG and LPBN.

Methods

General Preparation

All experimental procedures were approved by the Animal Care and Use Committee at the University of Florida. Male Sprague-Dawley rats (357±6 g, Harlan Industries, Minneapolis, IN) were anesthetized with an i.p. injection of
ketamine/xylazine/acepromazine (80-100/8-20/1-3 mg/kg, respectively) and then randomly placed into one of three groups: hemorrhage (HEM); saline volume control (SAL); or hydralazine pressure control (HYDRAZ).

Following assignment to a group, all rats were surgically instrumented with catheters (PE-10 connected to PE-50 tubing, Braintree Scientific, Braintree, MA) filled with heparinized saline (100 IU/ml). HEM rats were instrumented with two femoral arterial catheters. SAL and HYDRAZ rats were instrumented with a femoral venous and a femoral arterial catheter. Catheters were then tunneled subcutaneously, exteriorized at the nape of the neck, and sealed with 23-gauge obturators until the day of the experiment. Analgesics (Rimadyl, 0.01 ml/kg; Buprenorphine, 0.01 ml/kg) were administered subcutaneously following catheterization and animals were allowed 48 hours to recover. During recovery, animals were housed singly under controlled illumination (12-hour cycle) with food and water ad libitum. The day following catheter placement, animals were brought to the lab to ensure catheter patency and for acclimating purposes. Animals were weighed, lightly handled, and allowed to sit quietly for 2-3 hours in the testing chamber (9x9 inch bucket) they would be placed in for the experiment. Animals were returned to their home cages following acclimation.

**Experimental Protocol**

On the day of the experiment, animals were brought to the lab, weighed, placed in the testing chamber and a single arterial catheter was connected to a calibrated pressure transducer in-series with an amplifier (Stoelting, Wooddale, IL). The arterial and venous catheters were then attached to a swivel system (Instech, Plymouth Meeting, PA) so the animals could move unrestrained about the testing chamber. Both pulsatile and MAP were recorded on-line at 100 Hz using a Cambridge Electronics
Design computer interface and Spike2 data software. HR was derived on-line from the interval between peak systolic pressure waves in the arterial pressure (AP) trace.

After 60-90 minutes of quiet rest, the experiment began. First, baseline AP, MAP, and HR were recorded for a 30-minute baseline period. Next, animals underwent one of 5 experimental procedures: 1) slow hemorrhage (S-HEM, 0.5 ml/kg/min, n = 7); 2) intermediate hemorrhage (I-HEM, 1.0 ml/kg/min, n = 7); 3) fast hemorrhage (F-HEM, 2.0 ml/kg/min, n = 6); 4) saline control (SAL n = 5); or 5) hydralazine control (HYDRAZ, n = 5). All hemorrhaged animals underwent a 30% estimated TBV extraction through the second arterial catheter. TBV was calculated using a previously reported equation for estimation of rat blood volume: \((0.06 \text{ ml/g}) \times \text{body weight in g} + (0.77)\) (Lee and Blaufox, 1985). S-HEM, I-HEM, and F-HEM were performed over 40, 20, and 10 minutes, respectively. For HYDRAZ animals, 1 ml of hydralazine (3mg/kg) was infused over 60-seconds through the venous catheter in order to induce a level of hypotension similar to that induced by HEM (Graham et al., 1995; Pelaez et al., 2002). For the SAL animals, 1 ml of heparinized saline (0.9% NaCl, 2 IU/ml) was infused over 60-seconds through the venous catheter.

Ninety minutes after the cessation of HEM or drug infusion protocols, animals were administered a lethal dose of sodium pentobarbital (100-150 mg/kg) and transcardially perfused with heparinized saline followed by 4% paraformaldehyde 90-minutes. Brains were removed and post-fixed in 4% paraformaldehyde for 24 hours followed by 24-48 hours of immersion in cryoprotectant solution (30% sucrose) prior to cryostat sectioning.
**Fos Immunocytochemistry**

Extracted brains were cut into 40 micrometer coronal sections and processed for Fos-like immunoreactivity (FLI) as previously described (Hayward and Von Reitzenstein, 2002). Briefly, free floating sections were washed in sodium phosphate buffered saline (PBS, pH 7.4) followed by a second wash in a 3% goat serum-PBS-triton X100 solution (3% GS-PBS-TX) to prevent nonspecific binding. Sections were then incubated for 24 hours in rabbit anti-c-Fos primary antibody (1:2000 dilution, sc-52r, Santa Cruz Biotechnology). Following another wash in 1% GS-PBS-TX, sections were incubated in goat anti-rabbit biotin (Jackson ImmunoResearch Laboratories, Inc., 111-065-144) for two hours and re-washed (1% GS-PBS-TX) prior to being placed in avidin-biotin peroxidase complex (ABC Vectastain Kit, Vector, Burlingame, CA). Sections were put through a final wash (1% GS-PBS-TX) followed by visualization of the FLI with a chromagen solution (0.05% diaminobenzidine hydrochloride [DAB], 2.5% ammonium sulfate, 0.033% hydrogen peroxide in 0.05 M Tris-HCl, Vector). Sections were then mounted onto glass slides, air-dried, dehydrated in a graded alcohol and CitriSolv (Fisher Scientific) series, and coverslipped.

**Neuroanatomical Quantification of Fos-Like Immunoreactivity**

For each animal, two representative sections from each brain area of interest were imaged (Axioskop, Carl Zeiss; 5X) and analyzed for the number of FLI neurons present by a technician blinded to the experimental conditions. The software used for FLI quantification (Metamorph) allows the investigator to assign color, object size (7-10 µm), and/or density ranges specific to Fos-positive cells, as determined by the investigator. Once these ranges have been preset, the software is then able to “recognize” and
record FLI in a specified field of the image. This allows greater consistency and decreased human error in quantifying FLI between images.

Figure 2-1 shows a schematic of the rostral, middle, and caudal coronal sections of the LPBN and PAG analyzed in this study. The criteria used for selecting specific sections of the LPBN included the shape of the superior cerebellar peduncle (SCP), the width of the LPBN from the SCP to the ventral spinocerebellar column, and the width of the ventral spinocerebellar tract. The criteria for choosing specific PAG sections were based the shape of the central aqueduct, the shape and width of the dorsal and ventrolateral columns, and the presence of the oculomotor nucleus. Other areas imaged and quantified for FLI included the locus coeruleus (LC, interaural -0.68 to -0.80), the cuneiform nucleus (CnF, interaural 0.48 to 0.60), Kolliker-Fuse (KF) and A7 (imaged in the same section and at the level of the rostral LPBN) (Paxinos and Watson, 2005). Several standardized “masks” were prepared for each level of each brain nucleus using counterstained brain sections as well as guidance from previously diagramed images of the brain areas of interest (Behbehani, 1995; Henderson et al., 1998; Krout et al., 1998). These masks were superimposed over corresponding images using Adobe Photoshop 7.0 in order to outline boundaries of selected brain areas and the different subnuclei within the PAG (Behbehani, 1995) and the LPBN (Krout et al., 1998) prior to FLI analysis. Masks were prepared in such a way as to allow fitting to individual brain dimensions as well as various angles of cut, but to still maintain the integrity of the approximate shapes and proportions of relative subnuclei.

**Cardiovascular Measurements**

MAP and HR were averaged over five-minute intervals for each experiment. MAP and HR values from 5 minutes prior to the onset of HEM or drug were averaged to give
a single baseline value (0 min.). Following the onset of HEM or drug infusion, the first two 5 minute averages (5 min. and 10 min.) and then every other 5 minute average (20 min., 30 min. etc.) were used for calculation of group averages.

Statistical Analysis. A one-way analysis of variance (ANCOVA) was used to determine if there were any significant differences in baseline MAP or HR between treatment groups. A two-way ANOVA with repeated measures was used to identify the effects of treatment (i.e. HEM or HYDRAZ) on MAP and HR over time (minutes 0, 5, 10, 20, 30, 40, 50 and 60). When indicated, paired or unpaired Bonferroni t-tests were then used to isolate differences relative to baseline (minute 0) within treatment groups or between treatment groups at specific time points. The accepted P value (P<0.05) was adjusted for the number of t-tests performed (n = 7, P<0.007). To determine whether MAP or HR at the offset of HEM within each treatment group were significantly different from the HYDRAZ treatment group at the same time point, an unpaired t-test was used. Differences were considered significant when P<0.05.

FLI data from all regions except the LPBN were analyzed using a one-way ANOVA comparing the effect of treatment (i.e. HEM or HYDRAZ) on FLI levels within each specific rostral-caudal section chosen for analysis. In the LPBN, FLI data were analyzed using a two-way ANOVA comparing the effect of treatment and subnuclei on FLI levels within each specific rostral-caudal section. If a significant effect was indicated, unpaired Bonferroni t-tests were used to reveal differences between SAL versus other treatments on FLI levels with each brain region. The accepted P value (P<0.05) was adjusted for the number of t-tests performed (n = 4, P<0.012), All data are presented as mean ± SEM.
Results

Cardiovascular Response to Hypotension Versus Severe Hemorrhage

Baseline MAP and HR for all groups of animals is shown in Table 2-1. There was no significant difference in resting MAP and HR between groups at the start of the experiments. Figures 2-2 & 2-3 illustrate the average change in MAP and HR over time for all groups following treatment. For SAL animals, there was no change in MAP or HR from baseline throughout the experiment (Figure 2-2). HYDRAZ animals, on the other hand, showed a significant decrease in MAP from baseline starting at 5 min. following HYDRAZ administration and continued throughout the experiment (Figure 2-2A). Additionally, at 20 minutes and for the remainder of the measurement period, the MAP of HYDRAZ-treated animals was significantly different from SAL animals. In response to the HYDRAZ-induced hypotension, HR increased significantly above both baseline and the HR of SAL animals at 10 minutes post-injection and remained elevated throughout the experiment (Figure 2-2B).

HEM also induced a persistent hypotension, but in all groups, MAP did not drop significantly below baseline until > 15% TBV had been withdrawn (Figure 2-3A). In the S-HEM group at min. 30, when approximately 23% of the TBV had been withdrawn, MAP fell significantly below baseline (Figure 2-3A). In both the I-HEM and F-HEM groups, MAP was identified to be significantly below baseline at 20 and 10 min. post HEM onset, respectively. In all three HEM groups, the lowest MAP was recorded at the offset of HEM when 30% of TBV had been withdrawn—at which point MAP was not significantly different between HEM groups. Furthermore, a comparison of MAP at the time of 30% TBV withdrawal in all HEM groups versus HYDRAZ at a similar time point (Table 2-2) demonstrated that for both S-HEM and I-HEM, the decrease in MAP at the
time of HEM completion was similar to that induced by HYDRAZ. In contrast, at corresponding time points, the decrease in MAP for the F-HEM group was significantly different from the HYDRAZ group.

HR showed an increase from baseline during the first half of the HEM protocol for all groups. However, the peak increase in HR was only significantly different from baseline (Figure 2-3B) in the I-HEM group during this initial compensatory period. In all HEM groups the peak drop in HR occurred at the offset of HEM. However, because of a large amount of inter-individual variability, the decrease in HR for the S-HEM group was not significantly different from baseline at any time point following the onset of HEM. Furthermore, the decrease in HR for both the F-HEM and I-HEM groups was only significantly different from baseline at 20 and 30 minutes following the onset of HEM, respectively (Figure 2-3B). The HR of the F-HEM group was also significantly different from baseline at 30 min. following HEM onset. HR in the I-HEM group was significantly different from baseline at 50 and 60 minutes following the onset of HEM. HR values recorded at the offset of the HEM when 30% of TBV had been withdrawn were not significantly different between HEM groups. However, HR values at the offset of HEM for all groups, at the time of peak hypotension, were significantly lower than HYDRAZ values at the same time points (Table 2-2).

**Fos-Like Immunoreactivity in Brainstem Nuclei Following Severe Hemorrhage**

Figure 2-4 shows representative middle and caudal sections of the PAG for visual comparison of FLI labeling in a SAL and an I-HEM rat. Regions of the PAG chosen for quantification of FLI included both the dorsomedial PAG (dmPAG) and the vlPAG, based on their known physiological contributions to sympathoexcitation and sympathoinhibition, respectively (Cavun and Millington, 2001; Hayward and
Castellanos, 2003). As shown in Figure 2-4, I-HEM induced increased levels of FLI, relative to SAL, throughout the dorsal and ventral PAG. FLI labeling for the SAL group depicts basal levels of neural activation in these conscious animals, as MAP and HR were unchanged for the duration of the experiment. Accordingly, all changes in FLI following HEM or HYDRAZ were compared to FLI levels in the SAL treated animals.

Figure 2-5 illustrates average FLI levels in the PAG for all treatment groups. In general, both HEM and HYDRAZ treatment induced large changes in FLI in the vlPAG compared to SAL treatment and there was a significant effect of treatment (P<0.001) in both the caudal and middle vlPAG. In the caudal vlPAG, however, only I-HEM and F-HEM induced a significant increase in FLI compared to SAL. In the middle vlPAG, all HEM rates and HYDRAZ induced a significant increase in FLI above SAL. In contrast, in the caudal dmPAG there was no significant effect of treatment on FLI. In the middle dmPAG there was a treatment effect (P<0.002), but only I-HEM induced a significant increase in FLI compared to SAL.

Figure 2-6 shows representative rostral, middle, and caudal sections of the LPBN from a SAL-treated versus an I-HEM and an F-HEM rat. The top three panels show increased levels of FLI in the central subnucleus of the rostral LPBN in both HEM groups compared to SAL. In the middle and caudal LPBN, increases in FLI in the HEM above SAL treated animals were primarily located in the external and dorsal subnuclei.

The average increase in FLI induced within subnuclei of the rostral, middle and caudal LPBN following HEM and HYDRAZ is shown in Figure 2-7. In the rostral LPBN, a main effect of treatment (P<0.0004) and subnuclei (P<0.0001), as well as an interaction between factors (P<0.008), was observed. Comparisons within the
individual subnuclei demonstrated that within the central subnucleus of the rostral LPBN both I-HEM and F-HEM increased FLI significantly above SAL-induced levels. In contrast, in the superior lateral subnucleus of the rostral LPBN, I-HEM as well as HYDRAZ treatment induced a significant increase in FLI above SAL treatment.

In the middle and caudal LPBN, there was a significant effect of subnuclei (P<0.001 for both) and treatment (middle: P<0.0001; caudal: P<0.02), but no interaction between these two factors (middle: P<0.4; caudal: P<0.8). Thus, a comparison of significant effects of treatment on FLI within individual subnuclei was not permitted. In the middle LPBN however, irrespective of individual subnuclei within the middle LPBN (Figure 2-7B), all HEM and HYDRAZ treatments induced increased levels of FLI relative to SAL. In the caudal LPBN, only F-HEM and HYDRAZ showed significantly more FLI than SAL (combined subnuclei).

Figure 2-8 shows the average increase in FLI across treatment groups for four other rostral brainstem regions also quantified, including the CnF, LC, KF (a subnucleus of the PBN), and A7. In all four regions, there was a significant effect of treatment on FLI (P<0.01). In both the CnF and A7 nuclei, only I-HEM induced a significant increase in FLI compared to SAL. In the LC (Figure 2-8B), both I-HEM and F-HEM, but not HYDRAZ, induced a significant increase in FLI labeling compared to SAL. In contrast, both HYDRAZ and I-HEM induced significant increases in FLI compared to SAL treatment in KF (Figures 2-8B & C, respectively).

**Discussion**

It is generally accepted that the amount of blood loss necessary to induce hypovolemic decompensation in a rat is between 15-30% of the animal’s TBV (Schadt and Ludbrook, 1991). However, the rate at which this volume is lost may impact the
transition from compensation to decompensation and, thus, brain mechanism(s) recruited to meet the physiological challenge (Troy et al., 2003). In the present study, all rates of HEM induced clear compensatory and decompensatory stages. In all instances, during blood loss of up to 15%, there was a compensatory tachycardia, and MAP was well maintained. Yet, only during I-HEM was the increase in HR during the compensatory phase significantly different from baseline. Following 30% TBV withdrawal, all hemorrhaged animals had MAPs that were significantly reduced from baseline. Both F-HEM and I-HEM groups showed a corresponding drop in HR that was significantly different from baseline at 10 minutes following the offset of HEM. Furthermore, in the I-HEM group a significant reduction in HR was observed between 30 and 40 min. following the offset of HEM. These observations demonstrate that there are marked differences in autonomic regulation of MAP and HR when severe HEM occurs at different rates of blood loss.

Examination of FLI in the rostral brainstem identified several regions that might be selectively involved in autonomic regulation during severe HEM. These regions showed significant increases in FLI following I-HEM but not in response to HYDRAZ treatment; including the middle dmPAG, the caudal vlPAG, the central lateral subnucleus of the rostral LPBN, LC, A7, and the CnF. Other brain regions examined demonstrated increased levels of FLI associated with both HEM and HYDRAZ compared to SAL controls. This suggests that activation of these specific brainstem sites was more closely related to autonomic regulation in response to hypotension than the hypovolemia and HEM-associated adjustments in autonomic regulation. Interestingly, the outcome of our study did not support our original hypothesis that F-HEM would
induce the greatest change in both cardiovascular parameters and FLI. In contrast, our results suggest that utilization of a hemorrhage at an intermediate rate of 1 ml/kg/min for 30% TBV HEM may be most useful for investigating the potential role of the rostral brainstem regions in mediating hemorrhagic decompensation in conscious rats.

**Methodological Considerations**

Several methodological factors must be considered when interpreting the results of the present study. First, in the present study we chose to use HYDRAZ for our non-volume depleted hypotensive controls because of its use in previous studies (Graham et al., 1995; Pelaez et al., 2002), as well as its known and reliable hypotensive actions. However, HYDRAZ proved to be a potent and long lasting vasodilator. The induction of such a long lasting hypotension may have added additional stress to the animals. In retrospect, a shorter acting vasodilator, such as sodium nitroprusside, may have been a more suitable control and may have better mimicked the response seen with our selected method of HEM.

The second factor to consider in this study is the use of FLI to identify specific regions of the brain involved in cardiovascular control when multiple stimuli and physiological changes occur over a relatively short time period. FLI is induced following neuronal excitation and depolarization and is an indicator of changes in neuronal activation associated with a stimulus. However, the resolution of these changes to specific time points is limited. In our study, all animals were sacrificed at 90 minutes following the offset of HEM. Accordingly there should have been a good correlation between FLI levels and the maximum drop in MAP (Chan and Sawchenko, 1994). However, during severe HEM many other physiological changes occur in an attempt to survive the insult (Brown et al., 2005; Osei-Owusu and Scrogin, 2006). As a result, we
can only correlate changes in FLI with the recorded cardiovascular changes. Further studies are needed to more definitively identify the role of each region identified in the present study in mediating hemorrhagic decompensation or recovery.

**Cardiovascular Response to Hemorrhage**

The only other study that we are aware of that has previously addressed the issue of HEM rate maintaining the volume withdrawn constant was by Troy and colleagues (Troy et al., 2003). In that study, 30% TBV HEM were performed in conscious rats over 20 or 40 minutes. Blood pressure responses were similar to those reported in the present study. That is, MAP was well maintained for the first 15% of TBV loss; but by the time 30% TBV had been withdrawn there was approximately a 50% drop in MAP irrespective of HEM rate. The HR responses, however, were considerably different. In their study, the faster rate of HEM, which corresponded to our intermediate rate (I-HEM), produced a reflexive tachycardia that peaked at ~38% above baseline following 30% hemorrhage, and HR remained elevated compared to the pre-HEM baseline during recovery. The slower rate of HEM (30% TBV loss over 40 minutes corresponding to our S-HEM group) also produced a tachycardia in response to HEM, but it was much more variable and reached a peak of only ~10% above baseline. In contrast, we reported a transient tachycardia in both the S-HEM and I-HEM groups, which was only ~8-15% above baseline following 15% TBV loss. As blood loss continued, HR began to drop below baseline the peak drop in HR occurred at or just following 30% TBV loss and persisted throughout recovery. The discrepancy between studies might be explained by differences in protocol. For example, animals in our study were allowed 48 hours to recover from surgery, while the rats in the previous study were hemorrhaged between 70 and 120 minutes following catheterization, in which inhalant halothane anesthesia
was used. Since halothane can remain in the body’s tissues for at least two hours following anesthetic levels of exposure (Divakaran et al., 1981), residual anesthesia may have modified the HR response to HRM in their study. Similarities between our study and other HEM studies with longer post-surgery recovery times suggest that anesthesia, even 4 to 6 hours following withdrawal (Blair et al., 2001; Cavun and Millington, 2001; Scrogin, 2003), can markedly alter autonomic control of HR (Lee et al., 2002) and presumably brain mechanisms recruited for autonomic adjustments during HEM.

**Pattern of Fos-Like Immunoreactivity in the Rostral Brainstem Following Hemorrhage**

Brain regions previously identified to be responsible for the switch from a compensatory response to decompensation during HEM have been isolated to the rostral brainstem (Evans et al., 1991). More specifically, both the vlPAG in the midbrain (Cavun and Millington, 2001) and the LPBN in the rostral pons (Blair et al., 2001) have been shown to play important roles in hemorrhagic decompensation. In the present study, increased levels of FLI were observed in both the vlPAG and LPBN in response to HEM compared to control (SAL). However, only in the caudal vlPAG and the rostral LPBN were the HEM-induced changes in FLI distinguished from changes in FLI induced by HYDRAZ or hypotension alone. This raises the possibility that neurons in these regions are critical in mediating autonomic responses associated with hemorrhagic decompensation.

In the present study increases in FLI were consistently observed in the vlPAG in response to all three rates of HEM. This observation supports physiological data from Cavun and Millington (Cavun and Millington, 2001) demonstrating that synaptic
blockade in the vlPAG markedly attenuates both the hemorrhagic hypotension and HEM-induced changes in HR. Furthermore, in a recent study by Schadt and colleagues (Schadt et al., 2006), neurons in the vlPAG were shown to display discharge patterns indicative of mediating HSI. Our observation that HEM induced a significant increase in FLI in the vlPAG also corroborates the results of a previous study investigating FLI following HEM (Keay et al., 2002). However, because hypotension alone can also induce FLI in the vlPAG (Li and Dampney, 1994; Murphy et al., 1995), in the present study we also evaluated the effect of hypotension (HYDRAZ) on FLI in the vlPAG. Our results identified that, similar to HEM, HYDRAZ induced a significant increase in FLI in the middle vlPAG compared to control. In the caudal VvlPAG however, the response to HYDRAZ was more variable and was consequently not significant. Yet, increases in FLI in the caudal vlPAG in response to both I-HEM and F-HEM were significant. This raises the possibility that neurons in the middle vlPAG may be more important in regulating autonomic responses to hypotension, whereas caudal vlPAG neurons may be more involved in regulating cardiovascular function during severe HEM. This observation is supported by data demonstrating that both the neuroanatomical connectivity (Behbehani, 1995) and control over different vascular beds of the middle vs. caudal vlPAG are distinctive (Dielenberg et al., 2001).

In the present study we also observed a small but significant increase in FLI in the middle section of the dmPAG following I-HEM but not HYDRAZ. Since activation of the dmPAG induces sympathoexcitation (Dielenberg et al., 2001) and the I-HEM group was the only group that showed a significant increase in HR during the compensatory phase of HEM, this raises the possibility that activation of dmPAG area neurons plays an
important role in maintaining MAP during the initial compensatory phase of HEM. Accordingly, an increase in FLI was also reported, but not quantified, the middle region of the dmPAG by Keay et al. in response to 15% TBV withdrawal in conscious rats (Keay et al., 2002). On the other hand, it should be noted that chemical blockade of the dorsal PAG has been reported to have no effect on the cardiovascular response to severe HEM (Cavun and Millington, 2001). Yet, in that study severe HEM was induced 4-6 hours after isofluorane anesthesia and no compensatory change in HR was noted in the control conditions. Thus, it remains to be determined what role dorsal PAG neurons play in modulating cardiovascular responses to I-HEM in an anesthesia-free animal.

In the present study, increased levels of FLI following I-HEM compared to control were observed in all three rostral-caudal regions of the LPBN, including KF. Yet, only in the rostral LPBN was the increase in FLI during HEM separated from the effects of HYDRAZ or hypotension alone. More specifically, in the central lateral subnucleus of the rostral LPBN both I-HEM and F-HEM selectively induced increased levels of FLI. In contrast, the effect of HYDRAZ was not significantly different from SAL controls. These results complement recent observations by Blair et al. (Blair et al., 2001) that smaller lesions of the LPBN, which encompassed only the dorsolateral portion, attenuated the bradycardic response to severe HEM in conscious rats. In contrast, larger lesions involving the entire LPBN (dorsolateral and ventrolateral subnuclei, possibly including KF) had little effect on the decompensatory response to severe HEM but impaired recovery. This raises the possibility that that the central lateral subnucleus of the rostral LPBN may be involved in mediating or relaying signals associated with HSI. Indeed, this region of the rostral LPBN receives a large projection from the vlPAG (Krout et al.,
1998), though the physiological function of this interconnection has yet to be determined.

Several regions outside of the PAG and LPBN were also quantified for FLI levels in the present study. These regions included LC, the CnF nucleus, and A7. All three regions have been shown to be involved in responses to different types of stress (Korte et al., 1992; Van Bockstaele et al., 2001) and cardiovascular regulation (Murphy et al., 1994; Guyenet et al., 2001). In the present study all three regions demonstrated significant increases in FLI in response to I-HEM but not HYDRAZ. Since all three regions are interconnected with the PAG (Bajic and Proudfit, 1999; Van Bockstaele et al., 2001) all three may be well positioned to contribute to cardiovascular adjustments during the transition from compensation to decompensation during HEM. Future studies should focus on determining the role of each region in HSI. Additionally, future studies should identify regions of the CNS actively inhibited during severe conscious HEM since the use of FLI is limited to activated neurons only.

Conclusions

In summary, the results of the present study provide investigated the impact of different rates of severe HEM on the cardiovascular outcome coupled with regional CNS activation. We have confirmed previous observations that suggested there are marked differences in autonomic regulation when severe HEM occurs at different rates (Troy et al., 2003). The results of our study suggest that a constant withdrawal rate of 1 ml/kg/min until 30% TBV has been removed produces the most reliable pattern of tachycardia and compensation followed by hypotension and bradycardia for the study of experimental severe HEM in conscious rats. Associated with this rate of HEM were indicators of increased levels of excitation localized to the caudal vlPAG, the middle
dmPAG, the rostral central lateral subnucleus of the LPBN, and LC. Other brain regions newly identified to be potentially involved in mediating HEM responses include CnF and the A7 region. Together, these results provide further evidence of the potential importance of activation of the rostral brainstem in mediating the response to severe HEM.
Figure 2-1. Schematic of periaqueductal grey (PAG) and lateral parabrachial nucleus (LPBN) areas imaged for quantification of Fos-positive staining. All figures and numbers were adapted from Paxinos and Watson, 2005. Approximate middle, and caudal PAG (A) and rostral, middle, and caudal LPBN (B) areas used. Numbers displayed with each representative section indicate approximate coordinates caudal to Bregma. DMPAG, dorsal medial periaqueductal grey; VLPAG, ventrolateral periaqueductal grey; Sup, superior lateral parabrachial; Ctr, central lateral parabrachial; KF, Kolliker-Fuse nucleus; SCP, superior cerebellar peduncle; Dor, dorsal lateral parabrachial; Cres, crescent lateral parabrachial; Ext, external lateral parabrachial.

Table 2-1. Baseline mean arterial pressure (MAP) and heart rate (HR) of treatment groups. No significant different between groups (P>0.05).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SALINE (n=5)</td>
<td>109±4</td>
<td>351±14</td>
</tr>
<tr>
<td>HYDRAZ (n=5)</td>
<td>122±4</td>
<td>349±24</td>
</tr>
<tr>
<td>S-HEM (n=7)</td>
<td>120±4</td>
<td>353±5</td>
</tr>
<tr>
<td>I-HEM (n=7)</td>
<td>123±5</td>
<td>356±13</td>
</tr>
<tr>
<td>F-HEM (n=6)</td>
<td>123±4</td>
<td>366±23</td>
</tr>
</tbody>
</table>
Figure 2-2. Mean arterial pressure (MAP; A) and heart rate (HR; B) responses to saline (SAL; n = 5) or hydralazine (HYDRAZ; n = 5) infusion. Minute 0 represents baseline. * Indicates significantly different (P<0.05) from baseline within treatment group. # Indicates significantly different (P<0.05) from HYDRAZ treated group at specific time point.
Figure 2-3. Mean arterial pressure (MAP; A) and heart rate (HR; B) responses to slow (0.5 ml/kg/min; S-HEM; n=7), intermediate (1.0 ml/kg/min; I-HEM, n=7), and fast (2.0 ml/kg/min; F-HEM, n=6) rates of hemorrhage (HEM). Minute 0 represents baseline. The lowest MAP for each rate of HEM corresponds with the point at which 30% of total blood volume had been removed (i.e. cessation of blood withdrawal). @ Indicates significantly different from baseline for the F-HEM group. * Indicates significantly different from baseline for the I-HEM group. $ Indicates significantly different from baseline for the S-HEM group. # Indicates F-HEM value was significantly different from both I-HEM and S-HEM at specified time point. & Indicates S-HEM value was significantly different from both I-HEM and F-HEM at specified time point.
Table 2-2. Mean arterial pressure (MAP) and heart rate (HR) averaged at the time of the offset of hemorrhage (HEM) compared to hydralazine (HYDRAZ) at the corresponding time point. * Indicates significant difference from HYDRAZ average (P<0.01).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Time Point (min.)</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-HEM (n=7)</td>
<td>40</td>
<td>76±6</td>
<td>294±23*</td>
</tr>
<tr>
<td>HYDRAZ (n=5)</td>
<td></td>
<td>80±3</td>
<td>480±11</td>
</tr>
<tr>
<td>I-HEM (n=7)</td>
<td>20</td>
<td>73±15</td>
<td>277±34*</td>
</tr>
<tr>
<td>HYDRAZ (n=5)</td>
<td></td>
<td>84±5</td>
<td>487±14</td>
</tr>
<tr>
<td>F-HEM (n=6)</td>
<td>10</td>
<td>68±6*</td>
<td>305±35*</td>
</tr>
<tr>
<td>HYDRAZ (n=5)</td>
<td></td>
<td>89±5</td>
<td>481±20</td>
</tr>
</tbody>
</table>
Figure 2-4. Representative images of Fos-like immunoreactive neurons in the middle and caudal periaqueductal gray (PAG) of two individual animals that underwent either saline injection (SAL) or severe hemorrhage at an intermediate (I-HEM) rate. Aq, central aqueduct; DMPAG, dorsal medial PAG; DLPAG, dorsal lateral PAG; LPAG, lateral PAG; VLPAG, ventrolateral PAG. Numbers in the lower left of the ventral sections of SAL animal indicate approximate location of representative sections relative to bregma in mm (Paxinos and Watson, 2005).
Figure 2-5. Average Fos-positive cell counts from the middle and caudal dorsomedial periaqueductal grey (DMPAG; A & B) and ventrolateral periaqueductal grey (VLPAG; C & D) following different rates of severe hemorrhage (HEM) and hydrazaline (HYDRAZ)-induced hypotension. M = middle PAG (defined as -7.5 to -7.8 mm caudal from bregma); C = caudal PAG (defined as -8.0 to -8.4 mm caudal from bregma, Paxinos and Watson, 2005). SAL = saline control (n=5); S-HEM = slow rate of 30% hemorrhage (n=7); I-HEM = intermediate rate of 30% hemorrhage (n=7); F-HEM = fast rate of 30% hemorrhage (n=6); HYDRAZ = hydrazaline treated animals (n=5). * Significantly different from SAL.
Figure 2-6. Representative images of Fos-like immunoreactive neurons in the rostral, middle, and caudal lateral parabrachial nucleus (LPBN) of rats that underwent either saline injection (SAL) or severe hemorrhage at a fast (F-HEM) or intermediate (I-HEM) rate. Subnuclei of the LPBN outlined include: Sup=superior; Ctr=central; Dor=dorsal; Cres, lateral crescent; Ext, external. SCP is the superior cerebellar peduncle. Numbers in the lower left of SAL animal indicate approximate location of representative sections relative to bregma in mm (Paxinos and Watson, 2005).
Figure 2-7. Average Fos-positive cell counts from selected subnuclei the rostral (A) middle (B) and caudal (C) lateral parabrachial nucleus (LPBN) following different rates of severe hemorrhage (HEM) and hydralazine (HYDRDAZ)-induced hypotension. Rostral LPBN was defined as -8.8 to -9.0 mm caudal from bregma. Middle LPBN was defined as -9.05 to 9.3 mm caudal to bregma. Caudal LPBN was defined as -9.35 to -9.6 mm caudal from bregma (Paxinos and Watson, 2005). SAL = saline control (n=5); S-HEM = slow rate of 30% hemorrhage (n=7); I-HEM = intermediate rate of 30% hemorrhage (n=7); F-HEM = fast rate of 30% hemorrhage (n=6); HYDRAZ = hydrazaline treated animals (n=5). * Significantly different from SAL. Horizontal bars indicate that only the group of subnuclei, not individual subnuclei, was significantly different from SAL. Note the difference in y-axis scales.
Figure 2-8. Fos-positive cells quantified in the Cuneiform Nucleus (A), Locus Coeruleus (B), Kolliker-Fuse Nucleus (C), and A7 cell group (D) following different rates of severe hemorrhage and hydrazaline-induced hypotension. SAL = saline control (n=5); S-HEM = slow rate of 30% hemorrhage (n=7); I-HEM = intermediate rate of 30% hemorrhage (n=7); F-HEM = fast rate of 30% hemorrhage (n=6); HYDRAZ = hydrazaline treated animals (n=5). * Indicates significantly different from SAL.
CHAPTER 3
DESCENDING PROJECTIONS FROM PERIAQUEDUCTAL GRAY TO THE LATERAL PARABRACHIAL NUCLEUS ARE NOT ACTIVATED BY SEVERE HEMORRHAGE IN THE CONSCIOUS RAT

Introduction

The body’s compensatory response to blood loss involves a baroreflex-mediated sympathoexcitation which is necessary for maintenance of arterial pressure (AP). When blood loss is in excess of 25-30% of total blood volume (TBV), however, the compensatory response of the body change and a centrally-mediated sympathetic withdrawal leads to a precipitous fall in blood pressure and heart rate (HR). This second phase of hemorrhage (HEM), or the hemorrhage-induced sympathoinhibitory (HSI) phase, has been attributed to the activation of cardiac vagal afferents responding to reduced blood volume (Thoren, 1979) as well as activation of specific brain nuclei (Pelaez et al., 2002; Dean and Woyach, 2004; Frithiof et al., 2007). Two brain regions within the rostral brainstem have been identified as critical contributors to HSI: the caudal ventrolateral periaqueductal grey (vIPAG) and the lateral parabrachial nucleus (LPBN). Both of these regions have been shown to be activated in response to severe HEM in the conscious rat and blockade or lesion of either of these nuclei markedly alters HSI (Chan and Sawchenko, 1994; Blair et al., 2001; Cavun and Millington, 2001; Jaworski et al., 2002; Keay et al., 2002). While there is a well documented descending projection from the vIPAG to the LPBN (Kroot et al., 1998), the physiological role of this interconnection is unknown. Previous work in our lab has shown that LPBN area neurons play a significant role in mediating descending changes in sympathetic drive originating from the PAG (Hayward et al., 2004). Because the projection of the vIPAG to the LPBN is significantly more dense compared to the projection from the dorsal PAG
(Krout et al., 1998), the present study was undertaken to identify whether vlPAG neurons that project to the LPBN are activated in response to severe HEM in the conscious rat. We hypothesized that a greater percentage of vlPAG area neurons projecting to the LPBN would be activated, as indicated by the cellular marker c-Fos, compared to the dorsolateral PAG (dlPAG), in response to severe HEM.

**Methods**

Male Sprague-Dawley rats (Harlan Industries, Minneapolis, IN) weighing 355 ± 6 g were utilized for the present study. Rats were pair-housed in rooms with a 12-hour light-dark cycle and allowed standard rat chow and water ad libitum. All animal protocols were in accordance with guidelines established by the University of Florida’s Institutional Animal Care and Use Committee.

**Surgical Instrumentation**

Three days prior to experimentation, animals were deeply anesthetized with isofluorane mixed in 100% oxygen (4% → 2.0-2.5%). Each animal was instrumented with two femoral arterial catheters—one in each leg—that were tunneled subcutaneously and exteriorized between the scapulae (PE-10 connected to PE-50 tubing, Braintree Scientific, Braintree, MA). Catheters were filled with heparinized saline (100 IU/ml) and sealed with stainless steel obturators (Braintree Scientific, Braintree, MA). Immediately following catheter placement, rats were placed in a stereotaxic head holder (Kopf Instruments) and a small craniotomy directly overlying the left LPBN was performed with a high speed micro-drill. A glass micropipette filled with 1% Fluorogold (FG) solution was then lowered into the LPBN using a high speed microdrive (Model 662, Kopf Instruments). The coordinates of the LPBN were as follows: 9.3 mm caudal to bregma, 2.1 mm lateral to midline, and 5.5 mm deep to the dorsal brain surface
(Paxinos and Watson) (Paxinos and Watson, 2005). Approximately 45 nl of Fluorogold (FG) was pneumatically injected into the left LPBN. The glass pipette was allowed to remain in place for 2-5 minutes prior to removal. The skin overlying the craniotomy was sutured closed and topical antibiotics were applied to both the head and leg incisions. During recovery, animals were given subcutaneous injections of sterile 0.9% NaCl (1 ml, for rehydration) and buprenorphine (0.01 ml/kg, for pain management). Each day following surgical instrumentation, rats were brought to the lab where the experiment would take place, weighed, catheters checked for patency, and allowed to sit quietly in order to acclimate to the testing container in which experiments would be performed.

**Experimental Protocol**

On the third day following surgery, animals were brought to the lab, weighed and placed in the testing chamber in a quiet room. Animals were then randomly assigned to one of two treatment groups: hemorrhage (HEM) or non-HEM control (CON). One of the arterial catheters was connected to a calibrated pressure transducer in-series with an amplifier (Stoelting, Wooddale, IL) and both pulsatile and mean arterial pressure (MAP) were recorded on-line at 100 Hz using a Cambridge Electronics Design computer interface and Spike2 data software. HR was derived on-line from the interval between peak systolic pressure waves in the AP trace. The second arterial catheter was connected to additional tubing for blood withdrawal or sham manipulation. Next, baseline AP, MAP, and HR were recorded for a 30-minute baseline period. Next, animals assigned to the HEM group underwent a 20-minute hemorrhage in which 30% of estimated TBV was removed. TBV was calculated using a previously reported equation for estimation of rat blood volume: (0.06 ml/g)*(body weight in g)+(0.77) (Lee and Blaufox, 1985). CON animals did not undergo any blood removal, but their free
catheter was moderately stimulated to simulate catheter movements imposed on HEM animals. Following cessation of blood withdrawal (for HEM) or catheter stimulation (for CON), animals remained in the testing chamber for 90 min, after which animals were administered a lethal dose of sodium pentobarbital (100-150 mg/kg) and transcardially perfused with heparinized saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 24 hours followed by 24-48 hours of immersion in cryoprotectant solution (30% sucrose) prior to cryostat sectioning.

**Immunohistochemistry and Data Collection**

Extracted brains were cut into 30 micrometer coronal sections and processed for Fos-like immunoreactivity (FLI) and FG immunoreactivity (FGI). Briefly, free floating sections were washed in sodium phosphate buffered saline (PBS, pH 7.4) followed by a second wash in a 1% goat serum-PBS-triton X100 solution (1% GS-PBS-TX) to prevent nonspecific binding. Sections were then incubated for 24 hours in rabbit anti-c-Fos primary antibody (1:2000 dilution, sc-52r, Santa Cruz Biotechnology) at 4°C. Following another wash in 1% GS-PBS-TX, sections were incubated in goat anti-rabbit biotin (Jackson ImmunoResearch Laboratories, Inc., 111-065-144) for two hours and re-washed (1% GS-PBS-TX) prior to being placed in avidin-biotin peroxidase complex (ABC Vectastain Kit, Vector, Burlingame, CA). Sections were put through a final wash (1% GS-PBS-TX) followed by visualization of the FLI with a chromagen solution (0.05% diaminobenzidine hydrochloride [DAB], 2.5% ammonium sulfate, 0.033% hydrogen peroxide in 0.05 M Tris-HCl, and nickel to produce black nuclear stain, Vector). FGI staining was performed on the same tissue slices immediately following FLI staining utilizing the same protocol with rabbit anti-FG primary antibody (1:50:000) except the chromagen solution used did not contain nickel (0.05% diaminobenzidine hydrochloride...
[DAB], 2.5% ammonium sulfate, 0.033% hydrogen peroxide in 0.05 M Tris-HCl, to produce brown cytosolic staining, Vector). Sections were then mounted onto glass slides, air-dried, dehydrated in a graded alcohol and CitriSolv (Fisher Scientific) series, and coverslipped.

FG injection sites were identified using light microscopy, imaged, and rostral-caudal extent of FG spread was recorded. For each animal, two representative sections from the caudal vlPAG (ipsilateral and contralateral to the FG injection site) were imaged (Axioskop, Carl Zeiss) and analyzed for the number of FLI-positive, FGI-positive, and FLI+FGI co-labeled neurons present by a technician blinded to the experimental conditions (Figure 3-1). A FLI-positive cell was identified with black nuclear staining; a FGI-positive cell was identified with brown cytosolic staining with dendritic/axonal processes present; and co-labeling was identified as cells containing both FLI and FGI staining (Figure 3-1C). Other regions analyzed include the dorsomedial PAG (dmPAG) and dlPAG (Figure 3-1A). Because the dmPAG is a midline nucleus, it was not analyzed bilaterally (ipsi- vs. contralateral).

Data Analysis

MAP and HR were averaged over one-minute intervals, at times -5, 0, 5, 10, 15, 20, 25, 30 and 60 minutes relative to the onset of the treatment (HEM or CON). A two-way analysis of variance (ANOVA) with repeated measures was used to identify main effects of treatment (i.e. HEM vs. CON) on MAP and HR across time. Immunohistochemical data were analyzed using a one-way ANOVA comparing the effect of treatment (i.e. HEM or CON) on cellular staining levels within the caudal vlPAG, dmPAG, and dlPAG. When indicated, Student-Newman-Keuls multiple
comparison analyses were used to isolate differences within or between treatment groups. All data are presented as mean ± SEM.

**Results**

**Brain Injection Sites**

FG injections were centered in what we have previously defined as the “middle” LPBN (Ahlgren et al., 2007) (Bregma -9.16 mm) for all animals with the exception of one HEM animal whose injection site was centered more in the caudal LPBN (Bregma – 9.30 mm; Fig 1C). Figure 3-2A shows a typical rostro-caudal distribution of injected FG. Reconstruction of the injection site identified that a 45 nl-bolus of FG spread less than 0.5 mm rostral and .75 mm caudal from the injection site center for all brains.

**Heart Rate and Mean Arterial Pressure in Hemorrhage Versus Control**

Baseline HR and MAP were not different between treatments (HEM-HR: 379 ± 9 bpm; CON-HR: 383 ± 15 bpm; HEM-MAP: 120 ± 4 mmHg; CON-MAP: 115 ± 4 mmHg). CON animals did not display any deviation of HR or MAP from baseline at any time during the experiment. Animals that underwent HEM displayed a significant rise in both MAP and HR at 5 minutes following the onset of HEM compared to CON. At 10 min, however, there was no significant difference between groups. Following 10 min. of HEM or blood loss greater than 15% both HR and MAP dropped significantly below both baseline and CON values (Figure 3-3). HR reached its lowest point (261 ± 9 bpm) at minute 20 of HEM—the very end of blood withdrawal. HR in the HEM group remained significantly lower than CON animals and baseline measures for the remainder of the experiment. MAP reached its lowest (62 ± 6 mmHg) in the HEM group at minute 15—when approximately 23% of TBV had been removed and was significantly different from CON at minutes 15 and 20. Following the offset of HEM, MAP in the HEM group
remained significantly lower than the CON animals through minute 25 (5 minutes post
HEM offset) and was statistically less than HEM baseline for the remainder of the
experiment.

**Neural Activation/Co-Labeled Neurons**

In both HEM and CON animals, a greater abundance of LPBN-projecting neurons
in the left vlPAG (ipsilateral to the FG injection) relative to the right vlPAG were
identified (Figure 3-4). Thus, while the vlPAG does project bilaterally to the LPBN, there
is a much denser ipsilateral projection (approximately 72% greater). This observation is
in agreement with previous work (Krout et al., 1998).

Similar to reports from our lab and others (Ahlgren et al., 2007; Vagg et al., 2008),
HEM animals displayed a significantly greater amount of Fos-positive neurons within the
vlPAG compared to CON animals. This relationship was true both ipsi- and
contralateral to the FG injection site (Figure 3-4). However, only a small number of
FLI+FGI co-labeled neurons were identified in the vlPAG (either in the ipsi- or
contralateral vlPAG) following HEM (Figure 3-4A).

For comparison, the dorsomedial and dorsolateral subnuclei of the caudal PAG
were also evaluated for FLI, FGI, and FLI+FGI colabeled neurons (Figures 3-4B & C).
Similar to the vlPAG, the dorsal PAG showed heavier ipsilateral versus contralateral
projections to the LPBN; however, both subnuclei of the dorsal PAG showed noticeably
fewer neurons with projections to the LPBN compared to the vlPAG (roughly 50% less).
Similar patterns were seen for FLI immunoreactivity and FLI+FGI co-labeling: no
significant difference in the amount of FLI seen between ipsi- and contralateral dlPAG;
substantially less FOS-positive neurons in both the dm- and dlPAG compared to vPAG;
and little to no colabeled neurons in either dorsal PAG subnucleus.
Discussion

Anatomically, the LPBN is ideally positioned to act as a relay center for descending projections from the vlPAG to the hindbrain (Blair et al., 2001). This study investigated potential physiological stimuli that might activate descending projections from the vlPAG to the LPBN. In accordance with other neuroanatomical studies (Krout et al., 1998), we identified a large number of retrogradely labeled neurons throughout the PAG following FG microinjections into the LPBN. Furthermore, we confirmed previous findings from both our laboratory (Ahlgren et al., 2007), as well as others (Vagg et al., 2008), that severe HEM induces a significant increase in FLI labeled vlPAG neurons compared to control animals. Quantification of FLI-FGI co-labeled neurons in vlPAG however identified that relatively few FGI-labeled neurons also stained for FLI following severe HEM. Moreover, there were equal amounts of both FLI-FGI labeled neurons in the vlPAG of HEM and CON animals. Taken together, these data do not support the proposed hypothesis that vlPAG activation of the LPBN is important in relay to the hindbrain mediating autonomic or other responses during severe conscious hemorrhage in the rat. Consequently, the stimulus responsible for activating this relatively dense projection, between these two important autonomic nuclei, remains to be identified.

It is well documented that the vlPAG plays a significant role in blood pressure regulation during hemorrhage (Ward and Darlington, 1987; Evans et al., 1991; Cavun and Millington, 2001; Troy et al., 2003). At present, the cardiovascular and behavioral adjustments associated with vlPAG excitation have been shown to be mediated, in part, through descending activation of neurons in the RVMM (Lovick, 1993; Schenberg and Lovick, 1995; Hermann et al., 1997; Odeh and Antal, 2001). Furthermore, the release
of serotonin from this region of the hindbrain has been shown to participate in the blood pressure and heart rate responses to HEM (Scrogin et al., 1998; Henderson et al., 2000; Scrogin et al., 2000; Dean and Bago, 2002; Pelaez et al., 2002; Scrogin, 2003). The LPBN is situated dorsolaterally in the caudal mesencephalon/rostral pons, immediately lateral to the brachium conjunctivum (Paxinos and Watson, 2005). In addition to being reciprocally connected to both the dorsal and vlPAG, the LPBN has been shown to be interconnected with a number of other central nuclei involved in mediating autonomic control including the nucleus tractus solitarius (NTS), the amygdala, the hypothalamus, the rostral ventrolateral medulla (RVLM), and the rostral ventromedial medulla (RVMM; Fulwiler and Saper, 1984; Herbert et al., 1990). Furthermore, LPBN connections with the RVMM have been established (Henderson et al., 1998; Heslop et al., 2004; Vagg et al., 2008). Based on these studies and others showing the importance of the LPBN in modulating descending sympathetic drive to the heart from the dlPAG (Hayward et al., 2004), we hypothesized that vlPAG activation of lower brainstem nuclei may be mediated through a polysynaptic pathway involving the LPBN. The results of the present study however support more recent findings by Vagg et. al. (Vagg et al., 2008) that vlPAG activation of ventral medullary regions arise from a direct descending projection from the vlPAG to the RVMM.

In the present study, we also quantified the response of descending projections from the dorsal PAG to the LPBN. Activation of the dorsal PAG induces an increase in MAP, HR and respiratory rate and these changes are known to mediated, in part, through activation of LPBN area neurons (Hayward et al., 2004). However, to date there is evidence that dorsal PAG neurons do not respond to changes in venous return
during simulated HEM in conscious rabbits (Schadt et al., 2006). Furthermore, chemical blockade of the dorsal PAG does not alter the time course of HSI (Cavun and Millington, 2001). In a previous study however we did identify a small but significant increase in FLI labeling in the dorsal PAG in response to severe HEM (Ahlgren et al., 2007). Similarly, in the present study, we also identified a significant increase in the number of FLI-positive cells in the dIPAG following hemorrhage compared to CON. Yet, we did not identify a significant number of FLI labeled neurons with projections to the LPBN. This observation supports previous work suggesting the dorsal PAG neurons probably play little role in mediating cardiovascular responses to HEM.

In summary, the present study was undertaken to test the hypothesis that descending projections from the vIPAG to the LPBN are activated in response to severe HEM and potentially play a role in mediating HSI. The results of this study confirmed previous observations that a large number of vIPAG neurons send projections to the LPBN. However, our results suggest that this pathway is not activated in response to severe hemorrhage and therefore must be involved in mediating other types of responses associated with activation of the vIPAG (Wang and Lovick, 1993; Snowball et al., 2000; Lumb, 2004).
Figure 3-1. Periaqueductal grey (PAG) area analyzed and examples of immunohistochemical staining. (A) Adapted image of the specific midbrain level selected for Fos-like and Fluorogold immunoreactivity (FLI, FGI, respectively) quantification within the caudal PAG. Number in the bottom right-hand corner represents the distance in mm caudal to Bregma. Image adjusted from Paxinos and Watson, 2005. (B) Light microscope image (1.25x) of the PAG. (C) Light microscope image (20x) of FLI-positive (black arrows), FGI-positive (white arrows), and FLI-FGI co-labeled (red arrows) neurons in the caudal vlPAG. DMPAG = dorsomedial PAG, DLPAG = dorsolateral PAG, VLPAG = ventrolateral PAG, Aq = cerebral aqueduct.
Figure 3-2. Fluorogold (FG) injection sites. (A) Reconstruction of the rostro-caudal spread of a typical 45 nl FG injection. (B) Reconstruction of locations of focal point of all injection sites (red dots). For illustration purposes, injection sites recovered from control (CON) animals are shown on the left and hemorrhage (HEM) animals on the right. (C) Light microscope image of an actual injection site. Numbers beside reconstructed brain sections indicate distance in mm caudal to Bregma. Images adjusted from Paxinos and Watson, 2005).
Figure 3-3. Heart rate (HR; A) and mean arterial pressure (MAP; B) responses to 30% total blood volume removal over 20 minutes (HEM) or no treatment (CON). * indicates significant difference from CON at indicated time point (P < 0.05). # indicates significant difference from baseline within group (P < 0.05).
Figure 3-4. Quantification of immunohistochemical staining in the caudal periaqueductal grey (PAG). Fluorogold immunoreactivity (FGI)-positive, Fos-like immunoreactivity (FLI)-positive, and FLI-FGI colabeled neurons were counted in the ventrolateral PAG (vPAG; A) and dorsolateral PAG (dPAG; B) ipsilateral (IPSI) and contralateral (CONTRA) to the lateral parabrachial nucleus (LPBN) injection site. The dorsomedial PAG (dmPAG; C) was counted as a single nucleus because it is a midline structure. * indicates significant difference from control (CON; P < 0.05).
CHAPTER 4
VOLUNTARY WHEEL RUNNING ALTERS THE AUTONOMIC RESPONSE TO HEMORRHAGE IN CONSCIOUS MALE RATS

Introduction

The hemodynamic response to hemorrhage (HEM) follows a tri-phasic pattern. At the onset of HEM, the initial loss of blood volume, and the associated deviation in arterial pressure (AP), is sensed by the arterial baroreceptors which stimulate a reflex increase in heart rate (HR) and sympathetic drive to the vasculature to maintain a normotensive state. This first “compensatory” phase of HEM is typically sustained until blood loss reaches ~15-30% of TBV (Schadt and Ludbrook, 1991). When blood loss exceeds this critical value, the body’s initial compensatory response quickly transitions into a “decompensatory” or sympathoinhibitory phase which triggers a sudden decline in both HR and AP (Schadt and Ludbrook, 1991; Hasser and Schadt, 1992; Evans et al., 2001). These changes in autonomic drive trigger a HEM-induced hypotension that is paralleled by increasing plasma levels of renin, vasopressin, and epinephrine (Schadt and Ludbrook, 1991). These neurohumoral factors aid in the third phase of HEM, or the “recovery” phase, which follows the offset of blood loss. During the recovery phase, sympathetic tone is restored to the vasculature (Scrogin, 2003). The longer the body is exposed to reduced perfusion pressures during the sympathoinhibitory phase, the chance of recovery is decreased (Kauvar et al., 2006). Thus, interventions that can modulate central or peripheral mechanisms activated during HEM to delay the onset and limit the magnitude of the sympathoinhibitory phase and/or facilitate the recovery phase would be beneficial to survival outcomes.

Exercise has been shown to ameliorate a host of cardiovascular pathologies including hypertension (Grassi et al., 1992; Sutoo and Akiyama, 2003), heart failure
(Coats et al., 1992; Bensimhon et al., 2007), and a number of other diseases in which there is marked autonomic dysregulation (Warburton et al., 2006; Souza et al., 2007; Felber Dietrich et al., 2008). While the peripheral effects of exercise are well documented (Blomqvist, 1983; Long et al., 2004; Bensimhon et al., 2007) and constitute a large portion of exercise research, recent work has begun identifying centrally-mediated adaptations that also contribute to enhanced health outcomes associated with chronic exercise (Zhu et al., 2004; Nelson et al., 2005; Mueller and Hasser, 2006; Bakos et al., 2007; Kleiber et al., 2008). In particular, recent data suggest that some of the benefits of exercise may be mediated through increased inhibition of central sympathoexcitatory circuits (Mueller, 2007).

Current evidence suggests that chronic exercise can affect many areas of the brain involved in autonomic control of AP and HR (Zhu et al., 2004; Nelson et al., 2005; Mueller and Hasser, 2006; Bakos et al., 2007; Mueller, 2007; Kleiber et al., 2008). Interestingly, some of these same central sites have been identified to be involved in mediating autonomic changes during the different phases of severe HEM (Ward and Darlington, 1987; Krukoff et al., 1995; Krukoff et al., 1997; Chan and Sawchenko, 1998; Jhamandas et al., 1998; Buller et al., 1999; Kakiya et al., 2000; Pelaez et al., 2002). This raises the possibility that exercise training may impact the body’s ability to withstand severe hemorrhage via a change in neuronal responsiveness of specific brain nuclei known to play a role in HEM. The present study was undertaken to test the hypothesis that wheel-exercised rats would display altered autonomic responses to severe HEM and they would be better able to tolerate a significant loss of blood compared to sedentary rats. Specifically, we hypothesized that chronic voluntary
exercise would result in an enhanced compensatory response (Brum et al., 2000) and/or an attenuated decompensatory phase in response to severe HEM. Accordingly, we also hypothesized that voluntary exercise training would alter the pattern of neuronal activation (as marked by c-Fos immunoreactivity) in rostral brainstem regions identified to be involved in autonomic control during HEM including the Locus Coeruleus (LC; compensation), ventrolateral periaqueductal grey (vPAG; decompensation) and the lateral parabrachial nucleus (LPBN; recovery).

Methods

General Preparation

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida. Male Sprague-Dawley rats (175-200 g, Harlan Industries, Minneapolis, IN) were randomly placed into one of two groups: exercise (EX) or sedentary (SED). Following a preliminary study on single-housed EX rats, all subsequent animals were pair-housed for six weeks in cages that did (EX) or did not (SED) contain a running wheel. Animals were lightly handled and weighed weekly and were maintained in a 12 hour lights on: 12 hour lights off, temperature controlled environment with food and water ad libitum.

Following six weeks of "training," each animal was surgically instrumented with bilateral femoral arterial catheters (PE-10 connected to PE-50 tubing, Braintree Scientific, Braintree, MA) under isofluorane anesthesia (4% → 2-2.5%). Catheters were subcutaneously routed and exteriorized between the scapulae, filled with heparinized saline (100 IU/ml), and plugged with stainless steel obturators (23-gauge, Braintree Scientific, Braintree, MA). Analgesics (Rimadyl, 0.01 ml/kg; Buprenorphine, 0.01 ml/kg) were administered subcutaneously following catheterization and animals were allowed
48 hours to recover. During recovery, animals were housed singly. The day following catheter placement, animals were brought to the lab to ensure catheter patency and for acclimation to the testing chamber. Animals were weighed, lightly handled, and allowed to sit quietly for 2-3 hours in the testing chamber (9x9 inch bucket) to be used on the day of the experiment. Animals were then returned to their home cages for another 24 hours of recovery.

Experimental Protocol

On the day of the experiment, animals were brought to the lab, weighed, and both arterial catheters were connected to additional heparinized saline-filled tubing (10-50 IU/ml; PE-50). The animal was then placed in the testing chamber and the catheters were routed through a hole in the lid of the testing chamber in such a way that the animal could move freely within the testing chamber but not excessively twist the catheters. One of the arterial catheters was connected to a calibrated pressure transducer in-series with an amplifier (Stoelting, Wooddale, IL). Both pulsatile and mean arterial pressure (MAP) were recorded on-line at 100 Hz using a Cambridge Electronics Design computer interface and Spike2 data software. HR was derived on-line from the interval between peak systolic pressure waves in the AP trace.

AP, MAP, and HR were collected for 30-60 minutes during which the animal was undisturbed in order to ensure a stable baseline measurement. Animals were randomly assigned to one of two groups: hemorrhage (HEM) or no treatment (time control; CON). Animals assigned to the HEM group were then subjected to a 30% TBV HEM over 15 minutes (EX-HEM, SED-HEM, n=8 per group) followed by 90 minutes of recovery. Animals assigned to the CON group were allowed to sit quietly for 105 minutes (EX-CON, n=5; SED-CON, n=4). For the HEM groups TBV was estimated using a
previously reported equation: \((0.06 \text{ ml/g}) \times \text{ (body weight in g)} + (0.77)\) (Lee and Blaufox, 1985). Ninety minutes following HEM offset or after 105 minutes of quiet sitting in the CON groups, animals were administered an overdose of sodium pentobarbital (100-150 mg/kg) and transcardially perfused with heparinized saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 24 hours followed by 24-48 hours of immersion in cryoprotectant solution (30% sucrose) prior to cryostat sectioning.

**Fos Immunocytochemistry**

Extracted brains were cut into 30-micrometer coronal sections and processed for Fos-like immunoreactivity (FLI) as previously described (Hayward and Von Reitzenstein, 2002). Briefly, free floating sections were washed in sodium phosphate buffered saline (PBS, pH 7.4) followed by a second wash in a 3% goat serum-PBS-triton X100 solution (3% GS-PBS-TX) to prevent nonspecific binding. Sections were then incubated for 24 hours in rabbit anti-c-Fos primary antibody (1:2000 dilution, sc-52r, Santa Cruz Biotechnology) at 4°C. Following another wash in 1% GS-PBS-TX, sections were incubated in goat anti-rabbit biotin (Jackson ImmunoResearch Laboratories, Inc., 111-065-144) for two hours and re-washed (1% GS-PBS-TX) prior to being placed in avidin-biotin peroxidase complex (ABC Vectastain Kit, Vector, Burlingame, CA). Sections were put through a final 1% GS-PBS-TX wash followed by visualization of the FLI with a chromagen solution (0.05% diaminobenzidine hydrochloride [DAB], 2.5% ammonium sulfate, 0.033% hydrogen peroxide in 0.05 M Tris-HCl, Vector). Sections were then mounted onto glass slides, air-dried, dehydrated in a graded alcohol and CitriSolv (Fisher Scientific) series, and coverslipped.
Neuroanatomical Identification and Quantification of Fos-Like Immunoreactivity

For each animal, two representative sections from each brain area of interest were imaged (Axioskop, Carl Zeiss; 5-40X) and analyzed for the number of FLI neurons present by a technician blinded to experimental conditions. A stereotaxic rat brain atlas was referenced for identification of all areas imaged and quantified (Paxinos and Watson, 2005). Rostral, middle, and caudal aspects of the LPBN were imaged at approximately 9mm, 9.16mm, and 9.3mm caudal to bregma, respectively. Criteria used for selecting specific sections of the LPBN included the shape of the superior cerebellar peduncle (SCP), the width of the LPBN from the SCP to the ventral spinocerebellar column, and the width of the ventral spinocerebellar tract. Caudal vlPAG sections were imaged at approximately 8mm caudal to bregma. Other areas imaged and quantified for FLI included the Kolliker Fuse (KF; imaged in the same section and at the level of the rostral LPBN), LC (rostral: 9.3-9.68mm caudal to bregma; caudal: 9.8mm caudal to bregma), and the main body of the paraventricular nucleus of the hypothalamus (PVN; 1.8mm caudal to bregma).

Cardiovascular Measurements

MAP and HR values averaged over 60-second intervals beginning one minute prior to, and ending 30 minutes following the onset of HEM, as well as at 60 minutes following the onset of HEM were used for determining group averages. For non-HEM control experiments, MAP and HR values averaged over 60-second intervals for a time period that corresponded to the minute prior to blood withdrawal (baseline), 15 minutes of blood withdrawal, and 90 minutes of recovery in HEM experiments were used for determining group averages.
Changes in the autonomic control of HR during HEM was also evaluated using heart rate variability (HRV) analysis (Porter et al., 2009). HRV was analyzed using 4-5 minute time segments taken from three different time points during HEM. The three segments chosen for HRV analysis included: baseline, (within 10 minutes prior to the onset of HEM); peak (the 4-5 minute interval just preceding the drop in AP and HR associated with hemorrhagic sympathoinhibition); and nadir (the last 4-5 minutes immediately preceding the offset of HEM). Within each segment, HR was converted into a tachogram—a record of the time interval between heart beats (RRI). Next, the filtered tachogram was analyzed in the frequency domain using HRV software (Biosignal Analysis Group; University of Kuopio, Finland) (Niskanen et al., 2004). In the software used, the tachogram was interpolated at 10 Hz and detrended via the smoothness priors formulation (alpha=1000) (Tarvainen et al., 2002; Niskanen et al., 2004). The autoregressive model was set to the 40th order. The Welch’s Periodogram window width was designated to 512 points with an overlap of 256 points in the Hanning window. In the rat, the frequency components of HRV are designated by the following frequency ranges: 0.16-0.6 Hz (Low Frequency, LF), and 0.6-3.0 Hz (High Frequency, HF) (Japundzic et al., 1990). Frequency domain characteristics analyzed included the power of the LF and HF components, the ratio of LF/HF power, and the frequency associated with the HF peak.

**Statistical Analysis**

Within treatment groups, data were averaged and reported as the mean ± SEM. A one-way analysis of variance (ANOVA) was used to determine if there were any significant differences in baseline MAP or HR between treatment groups. A two-way ANOVA with repeated measures was used to identify the effects of experimental
treatment (HEM) and group (EX vs. SED) on MAP and HR across time (every minute: -1 through 30, and 60). When indicated, Student-Newman-Kuels post-hoc analysis or multiple paired t-tests with a Bonferonni adjustment for number of comparisons were used to isolate differences relative to baseline (minute -1) within treatment groups or between treatment groups at specific time points. HRV data were also analyzed by a two-way ANOVA with repeated measures comparing treatment groups against the designated time points. When indicated, a one-way ANOVA with a Scheffe Post Hoc or multiple paired t-tests with a Bonferonni adjustment for the number of comparisons (time points within groups) was utilized to determine significance. FLI data from all brain regions evaluated were analyzed using a one-way ANOVA to compare differences between experimental groups (EX vs. SED) within and between treatments (HEM vs. CON). Differences were considered significant when P<0.05 for all statistical analyses performed.

Results

Exercise Training Effect

A preliminary study was performed in a small group (n=4) of single-housed animals to compare average daily running distances and weight gain of single- versus pair-housed (n=13) animals (Figure 4-1). Two-way ANOVA with repeated measures comparison of average daily distances run by single-housed rats versus estimated daily distances run by pair-housed rats (Figure 4-1A) showed neither a treatment effect (P=0.99) nor an interaction (P=0.12). Furthermore, the weight gained over six weeks of voluntary running was also not significantly different between pair- and single-housed EX animals (P=0.5; see Figure 4-1B). Based on the similarities between groups, pair-housing was chosen as the preferred method of housing for all experiments described.
below to eliminate the potential confounding stress of solitary housing in these social animals.

Figure 4-1B shows the average weight gain over six weeks for all pair-housed animals. Two-way ANOVA with repeated measures demonstrated no significant difference in body weight between EX versus SED animals before wheel running began (193±7g versus 189.1±4g, respectively; P=0.43). From week three through the end of the training period, however, the EX rats weighed significantly less compared to their SED counterparts. At week six, EX animals displayed ~60% increase in body weight compared to ~88% gain in SED rats. In addition to weighing less, there was subjective evidence of less body fat in EX compared to SED rats (observation made by the technician during surgical instrumentation).

**Effect of Exercise on the Hemodynamic Response to Hemorrhage**

Despite significant body weight differences, there was no evidence of a training-induced bradycardia at rest in the EX animals compared to the SED group (Figure 4-2A). After the onset of HEM, HR increased in both EX and SED groups. In the SED animals, between minutes seven through ten during HEM, the increase in HR was significantly different from baseline. In contrast, the increase in HR in the EX group was only significantly different from baseline at minutes eight and nine during HEM. In both groups, a noticeable decline in HR began around minute eleven and within a minute of HEM offset (minute 15), HR reached its lowest value in both groups. The absolute drop in HR from baseline at the offset of HEM was greatest in the SED group. The mean HR at minute 15 in the SED group was statistically lower than baseline and remained statistically below baseline through minute 19. Conversely, while EX animals did display a drop in HR during the latter half of blood withdrawal, at no time point during or
following the offset of HEM did HR fall statistically below baseline. At minute 16 (one minute following the offset of HEM), the average HR in EX animals was 50 bpm higher than that observed in the SED animals (325±26 vs. 275±18 bpm, respectively; P=0.04). SED animals displayed a significantly lower HR compared to EX animals from the offset of HEM through minute 30. At 60 minutes following the onset of HEM, HR for SED animals (327±5 bpm) was not significantly different from baseline (330±5 bpm). In contrast, at minute 60, HR in the EX group (368±bpm) was elevated compared to baseline (339±9 bpm). This slight tachycardia failed to reach statistical significance following the Bonferroni correction (P=0.045). At this time point, however, HR in the EX animals (368±bpm) was significantly greater than HR in the SED animals (327±5 bpm, P=0.02).

Figure 4-2B shows the corresponding changes in MAP response to HEM in EX and SED animals. Similar to the HR results, prior to the onset of HEM, MAP was not significantly different between groups (SED: 123±4, EX: 120±3; P=0.38). Moreover, from the beginning of blood withdrawal, both EX and SED groups maintained MAP through minute nine, after which both groups displayed a drop in MAP until the offset of HEM. MAP for SED animals fell significantly below baseline starting at minute 13 and this HEM-induced hypotension lasted through minute 30 and at minute 60, MAP (105±2 mmHg) remained significantly below baseline (P=0.003). For EX animals, however, MAP was not significantly different from baseline at any time point and at minute 60, values for MAP in the EX group (110±4 mmHg) were not different from baseline (120±3mmHg, P=0.14). Both groups displayed the lowest MAP values immediately at the offset of HEM (minute 15). At this time point, MAP in EX animals was significantly
greater than MAP in the SED rats (93.7±6 mmHg compared to 58.5±5 mmHg, respectively; \( P=0.0002 \)). Overall, EX animals displayed an attenuated drop in MAP in response to HEM compared to SED animals and MAP in the EX group was significantly higher than the SED group from minutes 14 to 25.

**Heart Rate Variability During Hemorrhage**

Figure 4-3A illustrates the typical HRV frequency profile of an EX rat at rest. In both groups of animals, the area under the curve or power of the HF component was greater than the power of the LF curve at baseline. As a consequence, prior to the onset of HEM, the LF/HF ratio was less than 1.0 for all animals (Figure 4-3B, Base). During the baseline period, there was no significant difference between the EX and SED animals in the LF/HF ratio (Figure 4-3B), the HF power (Figure 4-3C), or peak frequency of the HF curve (Figure 4-3D).

Analysis of HRV during the 4-5 minute period prior to the onset of the drop in MAP during HEM indicated a significant increase in the LF/HF ratio (Figure 4-3B, Peak) relative to baseline in both EX and SED animals. There was no significant change in HF in either group at the same time point.

Analysis of HRV during the 4-5 minute period just prior to the offset of HEM (Figure 4-3B, Nadir) demonstrated that the LF/HF ratio remained elevated relative to baseline when both EX and SED groups were combined (\( P<0.003 \)). HF power at the nadir was also elevated relative to baseline (Figure 4-3C) but was significantly different from baseline only when both groups (EX and SED) were combined (\( P<0.016 \)). Finally, analysis of the frequency of the HF peak—which can be indicative of respiratory rate (Yang and Kuo, 1999)—identified a significant shift to a lower peak frequency during the nadir time point compared to baseline when the SED and EX groups were combined.
(P=0.01; Figure 4-3D). Additionally, the HF peak in the SED group was identified to be located at a significantly (P<0.006) higher frequency (1.4±0.08 Hz) compared to the EX group (1.16±0.06 Hz) when all time points were combined.

**Effect of Exercise on the Fos-Like Immunoreactivity Response to Hemorrhage**

To further evaluate the impact of exercise on autonomic control during HEM, three regions of the rostral brainstem and the PVN were evaluated for changes in FLI following HEM in EX versus SED groups. Two groups of control animals were added for this analysis: SED-CON (n=4) and EX-CON (n=5). These animals underwent the same treatments as their corresponding experimental groups but did not undergo the HEM protocol. At no point during the control experiments did HR and MAP values deviate from baseline for either SED-CON (352±3 bpm and 127±5 mmHg) or EX-CON (355±11 bpm and 132±5 mmHg) animals.

Figure 4-4 illustrates the typical FLI pattern observed throughout the LPBN from a SED and an EX animal following HEM. In general, HEM induced a greater amount of FLI in the SED versus EX animals. Based on previous work in our lab on FLI in the LPBN following HEM (Ahlgren et al., 2007), the LPBN was divided into three rostro-caudal divisions (rostral, middle, and caudal). Figure 4-5 shows the average FLI for the subnuclei of the LPBN evaluated. In the rostral division (Figure 4-5A), four subnuclei were evaluated for changes in FLI: superior, central, external and KF. Following HEM, a difference in FLI compared to CON within groups (SED and EX) was identified in two of the four subnuclei. In the central subnucleus, there was a significant increase in FLI in the EX-HEM compared to EX-CON. There was also a trend for FLI to increase in the SED-HEM animals versus SED-CON animals in the central subnucleus, but this comparison failed to reach statistical significance following the Bonferonni adjustment.
In the external subnucleus, SED-HEM, but not EX-HEM, animals displayed a significant increase in FLI compared to SED-CON. Comparisons of SED-HEM versus EX-HEM and SED-CON versus EX-CON did not reveal significant differences in the subnuclei of the rostral LPBN.

In the middle LPBN, four separate subnuclei were evaluated: dorsal, central, external, and crescent subnuclei (see Figure 4-5B). In both the dorsal and external subnuclei, SED-HEM animals had a significantly greater number of FLI-positive cells compared to SED-CON. EX-HEM animals also had greater FLI compared to EX-CON in both of these subnuclei; however, statistical comparisons failed to reach significance following Bonferonni correction (P=0.06 and P=0.02, respectively). FLI in the external subnucleus of the SED-HEM was significantly greater than FLI in the external subnucleus of the EX-HEM animals. There were no significant differences in FLI between groups in either the central and crescent subnuclei.

The same four subnuclei evaluated in the middle LPBN were evaluated in the caudal LPBN. Figure 4-5C shows that--similar to what was seen in the middle LPBN--only SED-HEM animals had significantly greater FLI compared to controls in the dorsal and external subnuclei alone. Additionally, EX-HEM animals had significantly less FLI compared to SED-HEM animals in the external subnucleus.

The average FLI counted in the caudal VLPAG, LC, and the PVN is shown in Figure 4-6. In the caudal vIPAG (Figure 4-6A) both SED-HEM and EX-HEM animals showed significant increases in FLI compared to controls, but, there was no difference between FLI in SED-HEM versus EX-HEM animals (P=0.64).
The LC was divided into rostral and caudal regions based on previous work showing functional differences along the rostro-caudal extent of this nucleus (Figure 4-6B) (Bajic et al., 2000). In the rostral LC, only SED animals displayed increased FLI following HEM compared to controls; however, there was not a difference in the amount of FLI present in SED-HEM versus EX-HEM animals (P=0.15). In the caudal LC, both SED-HEM and EX-HEM animals displayed significantly greater FLI compared to controls. A comparison between FLI in SED-HEM and EX-HEM animals, however, failed to reveal any significant differences (P=0.22).

Finally, the PVN was evaluated in three parts: the magnocellular, parvocellular, and dorsal cap regions. Shown in Figure 4-6C, both SED-HEM and EX-HEM animals displayed a significant increase in FLI in all PVN subnuclei assessed compared to controls. In the dorsal cap region alone, however, there was a significant difference between SED-HEM and EX-HEM animals with EX-HEM animals displaying an attenuated increase in FLI compared to SED-HEM animals (P=0.01).

**Discussion**

This study evaluated the effect of voluntary exercise training on the cardiovascular and neural responses to 30% TBV HEM in conscious rats. The EX animals in this study did not “train” at a high enough intensity to induce a resting bradycardia—an effect typically seen in more rigorously trained individuals. Nonetheless, following six weeks of voluntary wheel running, the EX rats displayed an attenuated cardiovascular decline during severe blood loss. These results suggest that even modest daily activity may help attenuate cardiovascular consequences associated with hypovolemic shock and thus potentially improve survival outcome in trauma situations.
During HEM, the initial response or compensatory phase is primarily mediated by baroreflex adjustments in sympathetic and parasympathetic drive, and there is evidence that exercise training can enhance baroreflex function (Brum et al., 2000). Based on these observations, we initially hypothesized that EX animals would display an enhanced compensatory response to blood loss compared to SED animals. This, however, was only partially confirmed. During the initial period of blood withdrawal, both EX and SED rats displayed the typical compensatory increase in HR and maintenance of MAP. Yet, although the EX animals did produce a slightly greater tachycardic compensatory response compared to SED animals, these differences were not statistically significant. HRV analysis, however, did identify a significant increase the LF/HF ratio at the peak time point in the EX group, potentially indicative of elevated sympathetic activity. The SED animals also showed an increase in LF/HF ratio at the peak time point, but the increase was not significantly different from baseline. Conversely, the increase in HR in EX animals appeared to reach a peak value earlier (at minute 8) and then began declining slightly sooner compared to SED animals (albeit at a slower rate). At the time of transition from compensation to a decompensatory or sympathoinhibitory response, MAP was not significantly different between the two groups. These results suggest that modest exercise training may augment the peak compensatory response to hemorrhage but the ability to sustain a peak response may be blunted compared to SED animals.

Baroreflex-mediated compensatory responses to HEM involve a decrease in baroreceptor afferent input into the brain. The primary central termination site of baroreceptor afferents is in the dorsal medulla, in the NTS. Baroreceptor-related
information integrated within the NTS is then relayed to other central nervous system (CNS) nuclei which either directly or indirectly activate sympathetic preganglionic neurons. Within the brainstem, the pontine noradrenergic cell group A6, LC, has been shown to play a pressor role in response to blood withdrawal (Anselmo-Franci et al., 1998). Anselmo-Franci et al. (1998) showed that lesioning the caudal, but not the rostral, component of the LC, resulted in a greater drop in MAP in response to 20% TBV HEM compared to sham-lesioned animals. The same study also showed an enhanced c-Fos expression following HEM in the caudal versus rostral LC (Anselmo-Franci et al., 1998). In the present study, c-Fos expression in the LC was also evaluated. Consistent with other investigators (Anselmo-Franci et al., 1998), we did observe a greater increase in c-Fos in the caudal versus rostral LC following HEM compared to CON groups. However, there was no difference in FLI following HEM between EX and SED animals. Thus, the modest effect we observed in the compensatory response following exercise training (as evidenced by the increase in the LF/HF ratio and the slightly higher HR) was not sufficient to be detected by evaluating c-Fos expression in the LC. Since a change in the reflex response of HR and MAP were seen with HEM in EX vs. SED animals, we can conclude that some central neural system was modulated by the voluntary exercise. Quantification of HEM-activated neurons (FLI) did not, however, identify this region as one modified by exercise. Furthermore, peripheral changes in hormone release, potentially from the adrenal gland, which in turn may augment compensatory responses (Erdem et al., 2002; Fediuc et al., 2006), should be evaluated in future studies as possibly contributing to the protective effect of exercise training in situations of hypovolemia.
At the point during HEM corresponding to approximately 20% TBV loss, both EX and SED animals began a sympathoinhibitory or decompensatory response, including bradycardia and declining MAP. For SED animals, MAP dropped below baseline levels at minute 13 and both MAP and HR reached levels that were significantly lower than baseline at the time of 30% TBV withdrawal. In contrast, although MAP began to decline at the same time point, in the EX group MAP and HR did not significantly deviate from basal values at any time point during or after the onset of decompensatory phase (minute 10). Moreover, from minute 14-15 during HEM and for 15 minutes following the offset of HEM, MAP and HR were elevated above the SED animals.

Multiple studies have implicated the vlPAG in the sympathoinhibitory phase that occurs following severe blood loss (Cavun and Millington, 2001; Cavun et al., 2004; Dean, 2004; Schadt et al., 2006). Cavun and Millington (2001) showed a significant attenuation of HEM-induced hypotension by blocking neural activity within the caudal vlPAG and it has been suggested that the descending innervation of brainstem depressor regions, such as the caudal midline medulla (CMM) and caudal ventrolateral medulla (CVLM), by the vlPAG may mediate the sympathoinhibitory phase of HEM (Cavun and Millington, 2001). In the present study, EX animals displayed an attenuated drop in MAP when equal amounts of blood were removed compared to SED animals. Based on this result, one might expect to see less neural activity (i.e., less FLI) within the vlPAG of EX versus SED rats following HEM (Vagg et al., 2008). Interestingly, this result was not observed. HEM induced a significant increase in FLI within the caudal vlPAG that was essentially the same between EX and SED animals. This lack of treatment effect within the vlPAG could mean that the attenuated depressor response
seen in EX compared to SED animals during HEM was not the result of differences in activation of the vlPAG, but may have been downstream. Alternatively, Mueller (2007) recently showed that exercise training leads to an increase in baseline inhibition of sympathetic activity initiated by activation of the RVLM, an area that projects directly to sympathetic preganglionic motor neurons in the IML of the spinal cord and has been shown to cause sympathoexcitation (Dampney et al., 2003). This work supports the idea that the rostral ventrolateral medulla (RVLM) is less sensitive to activation by excitatory inputs in exercise trained subjects. The same may be true for depressor regions such as the caudal midline medulla (CMM) and/or caudal ventrolateral medulla (CVLM)—a hypothesis yet to be investigated. Thus, it may be the case that HEM elicits equal levels of output from the vlPAG to downstream depressor regions in both EX and SED populations, but the overall excitability of these depressor regions may be blunted following exercise training.

In the present study, HRV analysis identified that the drop in HR during the sympathoinhibitory phase was mediated in part by an increase in vagal drive, as indicated by a significant increase in HF power during the last five minutes of HEM. Although there was no significant difference in total power between groups, the average HF power in the EX group at the nadir time point was ~50% less than that observed in the SED group. This raises the possibility that, in addition to modulating central sympathoinhibitory circuits, exercise training may also modulate central circuits involved in parasympathetic control. Indeed, analysis of HF peak location demonstrated that the peak of the EX group was located at a lower frequency compared to the SED group at all time points. Since the location of the HF peak is reported to reflect respiratory rate
(Baekey et al., 2008), this observation raises the possibility that some of the difference between EX and SED groups in response to hemorrhage may have also resulted from a training-induced change in respiratory control.

Although the respiratory response to severe hemorrhage has not been extensively studied in conscious rats, there is recent evidence from Strittmatter and Schadt (2007) that respiratory rate is unchanged during the initial phase of HEM followed by an increase in rate during the sympathoinhibitory phase of HEM in conscious male in rabbits (Strittmatter and Schadt, 2007). Our results indirectly confirm that respiratory rate may not have changed during the initial compensatory phase (i.e., no change in HF peak frequency location). During the sympathoinhibitory phase of HEM however, there was a decrease in the HF peak frequency, suggesting a drop in the respiratory rate. This raises the possibility that the respiratory response to hemorrhage may be species specific and in rodents may involve a decrease in respiratory rate possibly coupled with an increase in tidal volume. Since respiratory pattern (changes in tidal volume versus frequency) can influence venous return, and there was a trend for the HF peak of the EX-HEM animals to be located at a lower frequency, one of the central adaptations to exercise training may have been an overall increase in tidal volume in response to HEM which facilitated venous return. This observation supports our hypothesis that EX animals would better tolerate the decompensatory phase of blood loss and raises the possibility that some of the exercise training effect may be mediated by alterations in central respiratory control (Pellegrino et al., 1999; Eastwood et al., 2001).

At the offset of HEM (minute 15), both EX and SED animals showed immediate signs of recovery with spontaneous increases in HR and MAP. At 30 minutes following
the onset of HEM, SED rats were still significantly hypotensive compared to baseline and MAP was significantly lower compared to the EX group at all time points. In contrast, the EX group recovered quickly and appeared to return to baseline levels within seven minutes. One brain region known to be important for recovery from HEM is the LPBN (Ward, 1989)--more specifically, the ventrolateral region of the LPBN, including the external subnucleus has been implicated in HEM recovery. For example, Blair et al (2002) showed that lesions of the ventrolateral LPBN prevented the normal recovery response following HEM (Blair et al., 2001). In another study, these investigators showed that activation of glutamate receptors within the LPBN contributes to the recovery phase following HEM (Blair and Mickelsen, 2006). In the present study, FLI was significantly increased compared to non-HEM controls in the external subnucleus of the LPBN for SED but not EX animals throughout the rostrocaudal extent of the LPBN. Furthermore, FLI following HEM was significantly less in EX versus SED rats in the middle and caudal regions of the LPBN. These FLI data support previous findings that correlate neural activation within the external LPBN with spontaneous recovery following HEM. Greater activation of this subnucleus fits with our cardiovascular measures demonstrating that SED animals displayed a greater hypotension and therefore required greater activation of this group of neurons for recovery. Alternatively, only one subnucleus of the LPBN showed a trend for greater increase in FLI compared to controls in the EX-HEM versus SED-HEM animals. This nucleus, the central lateral nucleus of the rostral LPBN has been previously identified by our lab to be activated by central sympathoexcitatory pathways (Hayward and Castellanos, 2003), raising the possibility that EX training mediates some of its effects
through modulation of cardiorespiratory control circuits in the rostral central subnucleus of the LPBN (Baekey et al., 2008).

Finally, the hypothalamic PVN is another particularly interesting neural site that mediates cardiovascular responses to changes in blood volume. In the present study, all subnuclei of the PVN displayed an increased FLI following HEM in EX animals compared to controls; however, there was no difference seen in HEM-induced FLI within the parvocellular PVN between EX and SED groups. In addition to producing corticotropin-releasing hormone (CRH), this group of neurons produces arginine vasopressin (AVP) that acts as a neurotransmitter within the CNS (Swanson and Sawchenko, 1983; Badoer, 2001). Central blockade of AVP V1 receptors during HEM results in complete abolition of bradycardia and an attenuated hypotension in Wistar-Kyoto (WKY) rats (Budzikowski et al., 1996). Other studies also support a role for central release of AVP in HEM-induced bradycardia and hypotension—part of the decompensatory phase (Johnson et al., 1988; Evans et al., 1991; Shoji et al., 1993; Imai et al., 1996). Since EX animals displayed an attenuation of both the bradycardia and hypotension during HEM, it might be expected that these animals would express less FLI in the parvocellular PVN compared to SED animals. This, however, was not observed. Similarly, the FLI quantified in the magnocellular PVN—which contains neurons that produce peripherally-acting AVP—did not show any differences between the EX and SED animals in response to HEM. Again, one might have anticipated a greater neural activation in this region in SED animals since they produced a greater degree of hypotension compared to trained animals. These data suggest that six weeks of voluntary exercise training may not impact the role of either the centrally- or
peripherally-projecting AVP-neurons within the PVN during HEM. The dorsal cap region of the PVN, however, did show significantly less FLI in EX versus SED animals following HEM. This response was unexpected as this region of the PVN projects directly to the intermediolateral cell column (IML) and RVLM. Thus a decreased activation of the dorsal PVN would presumably translate into a decreased sympathoexcitatory response to HEM in the EX animals. Such a finding is intriguing and paradoxical as EX animals displayed higher mean values for HR and MAP compared to SED animals in the current set of experiments. It is well documented that exercise training leads to a down-regulation of sympathetic activity in pathological states such as congestive heart failure (Zucker et al., 2004) and in healthy animals as well (Mueller, 2007). The present results may simply reflect this training-induced decrease in sympathetic drive. Alternately, a decreased activation of neurons in this region of the PVN may reflect the fact that the stimulus triggering activation of this area was less intense for EX versus SED rats. Indeed, EX rats displayed a blunted hypotension, which may have resulted in a weaker signal to the dorsal PVN to activate sympathetic preganglionic motor neurons in the IML or sympathetic premotor neurons in the RVLM.

**Methodological Considerations**

There are four main methodological constraints to consider in the context of the present study. The first methodological consideration in this study is that we chose to utilize a voluntary wheel-based model of exercise training rather than forced exercise training. Forced exercise regimens typically result in training adaptations indicative of high intensity and/or high endurance exercise rather than the more moderate exercise routines prescribed to cardiovascular patients and the general population. In addition to the clinical limitations of forced exercise studies, forced treadmill and swimming has
been shown to cause alterations in the hypothalamic-pituitary-adrenal (HPA) axis that mimic chronic stress (Noble et al., 1999; Moraska et al., 2000). Such an impact on the HPA axis may further limit the clinical applicability of studies utilizing forced models of exercise training. While voluntary exercise (e.g., wheel running) has not been shown to induce the level or degree of training adaptations seen in forced exercisers, it does lead to both peripheral (Sexton, 1995) and central (van Praag et al., 1999; Mabandla et al., 2004) adaptations that benefit the animal. Furthermore, voluntary wheel running does not appear to cause deviations in the HPA axis that would imply any stress was induced in the animal (Dishman et al., 1995; Droste et al., 2003). This is important for the present study because differences in basal HPA axis function—as is the case with stressed animals—significantly alters the response to HEM (Darlington et al., 1989; Graessler et al., 1989).

A second methodological consideration in the present study is that rats were pair-housed in order to eliminate the stress of social isolation shown to occur in naturally social creatures, like rats (Gavrilovic et al., 2008). A preliminary evaluation of singly-housed wheel-exercised rats proved extremely difficult. Individually-housed runners were exceptionally anxious, appeared to be more defensive to experimenter handling, and baseline HR for these rats was higher and less stable (data not utilized in the present study) compared to the pair-housed rats used in this study. It has been reported that exposure to repeated physical exercise can offset the stress of isolated living conditions in rats (Filipovic et al., 2007). A majority of studies, however, show contrary results—that social isolation blunts some of the positive effects of chronic exercise (Stranahan et al., 2006; Leasure and Decker, 2009). Although the exact
distances run by each animal could not be discriminated in the present study, other studies have used similar methods for estimating individual running distances in group-housed rats (Stranahan et al., 2006) and the estimated distances run that we have reported for our pair-housed runners coincide very well with data collected from other investigators and from the singly-housed runners used in our preliminary study (Figure 1A). Finally, the average weight gain in the pair-housed and single-housed EX rats were not different (Figure 1B) and both were less than SED animals at weeks three through six, showing that running, and therefore at least a moderate training effect, did still occur.

A third consideration is the use of c-Fos to quantify the exercise training effects on central neural circuitry involved the autonomic response to HEM. It is generally accepted that c-Fos immunohistochemistry is an accurate indication of neuronal activation in response to sustained stimuli. In the present study, animals were sacrificed 90 minutes after MAP had reached its lowest value (minute 15, Figure 4-2) coincident with the cessation of blood withdrawal (or an equivalent time period in CON animals). Because FLI typically shows maximal expression 90-120 minutes following a stimulus (Chan and Sawchenko, 1994), the quantification of Fos-positive neurons in the present study may not accurately reflect changes in neural responsiveness to one specific phase of the HEM. Rather, the data presented here are intended to represent an overall look at the altered neuronal activation in response to the complete experience of 30% TBV HEM. Another limitation associated with the use of changes in FLI to identify central circuits involved in mediating a response is the inability of this technique to identify those neurons inhibited by a stimulus. Since changes in sympathoinhibition
have been proposed to mediate the effect of exercise training on cardiovascular control, these circuits would not necessarily be isolated by this technique.

Finally, the peripheral effects of chronic exercise, although not evaluated in the current study, cannot be excluded from the possible explanations of the enhanced tolerance to HEM seen in EX rats. Of particular relevance to the present study, is the effect of chronic exercise on blood plasma volume. Hypervolemia is a well documented outcome of long-term exercise training. If, indeed, EX animals in the present study developed an increased blood plasma volume as a result of six weeks of voluntary wheel running, the use of the same equation for TBV estimation based on body weight would be inappropriate. While blood volume was not directly measured in the present study, blood volume was not likely changed significantly by the modest amount of exercise performed. Other studies using a similar wheel-based model of exercise have reported no difference in plasma protein concentration and, presumably, therefore, no functional change in plasma volume between exercised and sedentary rats (Stranahan et al., 2006). Additionally, had blood volume been greater in the EX animals in this study, a delay in the onset of the decompensatory stage of HEM might have been expected. That is, had EX animals started off with more blood, the average HR for that group should have began declining later compared to the SED group. Yet, as Figure 4-2A shows, the peak HR for the EX group occurred slightly prior to and began decreasing slightly sooner compared to the SED group. Taken together, these findings contest the likelihood that the attenuated cardiovascular response to HEM in EX animals was the result of a greater initial blood volume.
Conclusions

The results of this study demonstrate that six weeks of voluntary exercise leads to an enhanced ability to tolerate severe blood loss in conscious male rats. While this study does not pinpoint an exact mechanism or mechanisms of action directly resulting in this protection against hypovolemic shock, this study evaluated specific brain regions involved in modulating the hemodynamic response to HEM that may be altered by chronic voluntary exercise. While alterations within such brain regions, including the dorsal cap region of the hypothalamic PVN, the external subnucleus, or central lateral subnucleus of the LPBN, may contribute to the enhanced compensation, attenuated decompensation, and faster rate of recovery following HEM, it is unclear whether these changes are specific to the response to hypovolemic hemorrhage or if there are neurochemical changes induced by exercise that have resulted in adjusted function of these regions. Further studies are needed to fully elucidate the specific role of each of these central nuclei in the exercise-induced prevention of hypovolemic shock reported in this set of experiments. Additionally, more studies are needed to further identify any peripheral adaptations that may also be critical for the altered response to HEM seen in trained subjects. Finally, more investigation into the respiratory response to HEM in EX versus SED animals is required to further elucidate whether such a change is significantly contributing to a training benefit during blood loss and whether or not such a change is central or peripheral in nature.
Figure 4-1. Evidence of exercise training. (A) Average distance run per day over six weeks of wheel access in pair- and single-housed rats. Data points for the pair-housed rats represent estimated distance run per day for individual animals (see results section for further explanation). (B) Average body weights over six weeks for pair-housed sedentary (SED) and exercised (EX) rats as well as single-housed EX rats. * Significant difference from Pair-EX rats.
Figure 4-2. Hemodynamic response to 30% total blood volume hemorrhage (HEM) in exercised (EX) vs. sedentary (SED) conscious rats. Heart rate (HR; A) and mean arterial pressure (MAP; B) response prior to (minute -1), during (minutes 0-15), and after (minutes 16-30) HEM. Grey box indicates time of blood withdrawal. * Significant difference from SED. # Significant difference from baseline (minute -1).
Figure 4-3. Heart rate variability (HRV) analysis of exercised (EX) and sedentary (SED) group response to hemorrhage (HEM). (A) Typical frequency spectrum of a heart rate (HR) interval in a conscious Sprague-Dawley EX rat at rest prior to HEM (Base). Low frequency (LF) and high frequency (HF) peak components are shown in (B). Ratio of power in the LF and HF ranges before HEM (Base), during the peak increase in HR (Peak), and just prior to the offset of HEM (Nadir). (C) HF power before and during different phases of HEM. (D) Frequency of the peak power in the HF range. * Significant difference from pre values for EX-HEM group. # Significant difference from pre values for EX-and SED-groups combined. Δ Significant difference between SED and EX groups across all time points combined. P<0.016.
Figure 4-4. Representative images of Fos-like immunoreactivity throughout the rostro-caudal extent of the lateral parabrachial nucleus (LPBN) in a sedentary (SED) and exercised (EX) animal following 30% total blood volume hemorrhage. SCP = superior cerebellar peduncle; CTR = central subnucleus; SUP = superior subnucleus; EXT = external subnucleus.
Figure 4-5. Average FLI and schematic representations for the rostral (A), middle (B) and caudal (C) lateral parabrachial nucleus (LPBN) in exercised (EX) versus sedentary (SED) rats following either 30% total blood volume hemorrhage (EX-HEM and SED-HEM) or quiet rest (EX-CON and SED-CON). Numbers accompanying the schematics represent distance in millimeters caudal to bregma (adapted from Paxinos and Watson, 2005). Ctr = central subnucleus; Sup = superior subnucleus; Ext = external subnucleus; KF = Kölliker Fuse nucleus; SCP = superior cerebellar peduncle. # Significant difference from control. * Significant difference from SED-HEM.
Figure 4-6. Average Fos-like immunoreactivity and schematic representations for the caudal ventrolateral periaqueductal grey (vlPAG; A), Locus Coeruleus (LC; B), and hypothalamic paraventricular nucleus (PVN; C) in exercised (EX) versus sedentary (SED) rats following either 30% total blood volume hemorrhage (EX-HEM and SED-HEM) or quiet rest (EX-CON and SED-CON). Numbers accompanying the schematics represent distance in millimeters caudal to bregma (adapted from Paxinos and Watson, 2005).

Magno = magnocellular; Parvo = parvocellular; Dorsal = dorsal cap. # Significant difference from control. * Significant difference from SED-HEM.
CHAPTER 5
OPIOID RECEPTOR BLOCKADE IN THE LATERAL PARABRACHIAL NUCLEUS
PREVENTS EXERCISE-INDUCED TOLERANCE TO HEMORRHAGE

Introduction

At the onset of blood loss, slight decreases in baroreceptor activation trigger an increase in sympathetic drive, which acts to speed heart rate (HR) and maintain mean arterial pressure (MAP). If bleeding continues, the body changes from a strategy involving the recruitment of compensatory mechanisms to maintain a sympathoexcitatory state to a strategy of sympathetic withdrawal. The sympathoinhibitory phase in response to severe blood loss leads to a drop in both HR and MAP. Upon cessation of blood loss, prior to cardiac arrest, HR and MAP spontaneously recover to baseline or near baseline values.

Multiple sites within the central nervous system (CNS) have been implicated in the modulation of each of these “phases” of hemorrhage (HEM). Of particular interest to our laboratory is the lateral parabrachial nucleus (LPBN), located dorsolateral to the superior cerebellar peduncles (SCP) in the rostral pons (Paxinos and Watson, 2005). This nucleus is interconnected with forebrain regions that regulate volume control and electrolyte balance (e.g., hypothalamic paraventricular nucleus [PVN], central nucleus of the amygdala [CeA], and median preoptic nucleus [MnPO] (Ciriello et al., 1984; Krukoff et al., 1993; Bianchi et al., 1998; Krout et al., 1998)) as well as hindbrain regions that receive and relay baroreceptor and blood volume information (e.g., nucleus tractus solitaries [NTS] (Herbert et al., 1990)). Thus, the LPBN is ideally situated in the CNS to act as an integration site for monitoring and influencing blood volume regulation.

De Oliveira and colleagues (2008) recently reported that opioid activation within the LPBN induces salt and water intake in both sodium-depleted and normohydrated
rats, demonstrating an opioid-mediated mechanism of osmo- or volume regulation at the LPBN. This effect was blocked by co-application of naloxone (De Oliveira et al., 2008), a predominantly mu-opioid receptor antagonist that is also able to antagonize delta- and kappa-opioid receptors when applied at higher concentrations. Furthermore, Blair and Mickelson (2006) showed that activation of the LPBN via application of excitatory amino acid resulted in augmented recovery following HEM—an effect blocked by chemical lesions of the same region (Blair and Mickelsen, 2006). Combined with the observation that the LPBN displays significantly increased FLI-positive staining following hypotensive HEM (Ahlgren et al., 2007), it is clear that the LPBN plays some role in the complex cardiovascular response to perturbations in blood volume, such as occurs during HEM. Since the LPBN contains large amounts of opioid receptors (Unterwald et al., 1991; Mansour et al., 1995) and manipulation of these receptors with agonists or antagonists can effect volume regulation (De Oliveira et al., 2008), it seems likely that the role of the LPBN in HEM may also involve an opioid-mediated component.

Physical exercise is beneficial for both healthy and unhealthy individuals and has been shown to positively impact both the systemic and central nervous systems (Ma, 2008; Harrington et al., 2009). During an acute bout of exercise, opioid peptides are released both peripherally and centrally (Shyu et al., 1982; Carmody and Cooper, 1987; Tierney et al., 1991). Chronic exercise, and coinciding chronic opioid release, has repeatedly been shown to mimic the response of opioid abuse, resulting in increased tolerance to opioid drugs (Kanarek et al., 1998; Mathes and Kanarek, 2001) and a probable down-regulation of opioid receptors (Houghten et al., 1986). Previous work from our lab has demonstrated that as little as six weeks of running wheel activity can
protect against HEM-induced hypotension and bradycardia (see Chapter 4), suggesting that physical activity confers an increased ability to buffer the cardiovascular decline associated with severe perturbations in blood volume.

Several studies have shown beneficial effects (increased MAP and survival) of kappa-opioid antagonists delivered centrally or peripherally following HEM (Ang et al., 1999; Henderson et al., 2002; Liu et al., 2005). The dorsal lateral subnucleus of the LPBN has been shown to play a role in the complex response to hypovolemic stimuli (Iwasaki et al., 1993). This area contains the vast majority of dynorphinergic neurons and kappa-opioid receptors (KORs) in the LPBN and neurons in this area send afferents directly to rostral brain nuclei involved in homeostatic responses to changes in blood volume (Moga et al., 1990; Wolinsky et al., 1996; Hermanson et al., 1998).

The present study was performed to test the hypothesis that exercise-induced tolerance to severe HEM involves an opioidergic mechanism within the LPBN—possibly resulting from an exercise-induced decrease in KORs in the LPBN. Accordingly, we hypothesized that application of an opioid receptor antagonist into the LPBN of sedentary rats would result in an increased tolerance to HEM, as is observed with voluntary exercise-trained rats and that Western blot analysis of rostral pontine brain tissue from exercise-trained rats would display less KOR protein content compared to sedentary controls.

**Methods**

**General Preparation**

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida. Male Sprague-Dawley rats (Harlan Industries, Minneapolis, IN) were randomly placed into one of two groups: exercise (EX,
n=17) or sedentary (SED, n=17). All animals were pair housed in cages that did (EX) or did not (SED) contain a single running wheel. EX animals were introduced to the running wheel at seven to eight weeks of age and allowed six weeks of access to the running wheel. Because voluntary exercise on a running wheel slows weight gain over a six-week period compared to non-exercised controls (see Chapter 4), SED animals were age-matched rather than weight-matched to EX animals. EX animals were lightly handled and weighed weekly and all animals were maintained in a 12 hour lights on: 12 hour lights off, temperature controlled environment with food and water ad libitum.

**Cranial Cannulation**

Twelve animals from each group were instrumented with bilateral brain cannulas to allow injection of drug or vehicle into the LPBN. Seven days before the day of the experiment, animals were deeply anesthetized with isofluorane gas in pure oxygen (4→2-2.5%) and placed into a stereotaxic head holder (Kopf Instruments, Tejunja, CA, USA). A mid-sagittal incision was then made in the skin overlying the skull and connective tissue was carefully scraped away from the surface of the skull using a scalpel blade. The edges of the incision were held out of the surgical field with 4-O monofilament suture weighted with a surgical hemostat instrument. Excess bleeding on the skull surface was controlled with a sterile cauterizing device. The skull was leveled between bregma and lambda. A bilateral craniotomy directly overlying the right and left LPBN was performed with a high-speed micro-drill. Following the craniotomy procedure, dura mater was carefully removed from the surface of the brain. Stainless steel, 23-gauge guide cannulas (Plastics One, Roanoke, VA, USA) were bilaterally implanted into the brain using the following coordinates: 9.3mm caudal to bregma, 2.1mm lateral to midline. Guide cannulas had a 5.5mm projection such that the tip of
the cannula was positioned approximately 1.5mm dorsal to the LPBN. Cannulas were fixed to the cranium using dental acrylic resin and a single watch screw that was drilled into a region of the skull rostral to cannula placement. Stainless steel, 30-gauge dummy cannulas were inserted into and screwed onto the guide cannulas (Plastics One). The skin incision was closed using 4-O monofilament suture and a topical antibiotic was applied. Following removal from the head holder, animals received subcutaneous injections of sterile saline (1ml 0.09% NaCl, for rehydration) and analgesics (Buprenorphine, 0.1ml/kg; Rimadyl, 0.1ml/kg). Following cranial cannulation, animals were singly housed and allowed five to seven days to recover. EX animals were allowed access to a running wheel during this recovery time.

Arterial Cannulation

One to two days prior to the experiment, animals were re-anesthetized with isofluorane (4→2-2.5%) and surgically instrumented with bilateral femoral arterial catheters (PE-10 connected to PE-50 tubing, Braintree Scientific, Braintree, MA). Catheters were subcutaneously routed and exteriorized between the scapulae, filled with heparinized saline (100 IU/ml), and plugged with stainless steel obturators (23-gauge, Braintree Scientific, Braintree, MA). Analgesics (Rimadyl, 0.01 ml/kg; Buprenorphine, 0.01 ml/kg) were administered subcutaneously following catheterization. Following arterial cannulation, EX animals were housed in cages without a running wheel in order to ensure than on the day of the experiment any effects of an acute bout of exercise did not confound the results of the study.

Experimental Protocol

On the day of the experiment, animals were brought to the lab, weighed, and both arterial catheters were connected to additional heparinized saline-filled tubing (10-50
IU/ml; PE-50). Dummy cannulas were carefully unscrewed and removed from the implanted guide cannulas. Each animal was then placed in the testing chamber and the catheters were routed through a hole in the lid of the testing chamber in such a way that the animal could move freely within the testing chamber but not excessively twist the catheters. One of the arterial catheters was connected to a calibrated pressure transducer in-series with an amplifier (Stoelting, Wooddale, IL). Both pulsatile and MAP were recorded on-line at 100 Hz using a Cambridge Electronics Design computer interface and Spike2 data software. HR was derived on-line from the interval between peak systolic pressure waves in the AP trace.

Baseline recordings of AP, MAP, and HR were collected for 30-60 minutes during which the animal was undisturbed in order to ensure a stable baseline measurement. Next, bilateral injections into the LPBN were made using a 10µl Hamilton syringe connected to internal cannulas (1.5mm longer than the guide cannulas) with polyethylene tubing (50-PE→10-PE). The internal cannula was first carefully advanced into the right guide cannula. Approximately 60 seconds later, a bolus (200-500 nL) of either naloxone (5 or 20µM in 0.09% NaCl) or vehicle (0.09% NaCl) was administered. Another 60 seconds was allowed before removing the cannula and placing it into the contralateral guide, after which a 60-second time period was permitted prior to injecting the same type and volume of drug. Following another 60 seconds, the internal cannula was removed. The animal was then allowed 10 minutes without handling.

Ten minutes after the final removal of the internal cannula, animals underwent a 30% estimated TBV HEM over 15 minutes followed by 45 minutes of recovery. AP, MAP and HR were recording continuously during the hemorrhage and recovery periods.
TBV was estimated using a previously reported equation: (0.06ml/gram)*(body weight in grams)+(0.77) (Lee and Blaufox, 1985). Animals were then administered a lethal dose of sodium pentobarbital (100-150ml/kg). The right soleus muscle was immediately dissected and removed from the hindlimb and weighed. The same internal cannula used to administer drugs during the experiment was used to inject a fluorescent marker (Fluorogold) into the brain for verification of cannula placement. Brains were then removed and allowed to sit in 4% paraformaldehyde for at least 24 hours prior to being sectioned with a cryostat, slide-mounted, and evaluated for accuracy of injections.

**Western Blot Analysis of Kappa Opioid Receptor Content**

Western blot analysis was performed on rostral pontine brain tissue from EX and SED groups of animals (n=5 per groups) for relative KOR protein density as previously described (Tanaka et al., 2005). Animals were brought to the lab and allowed to sit quietly for 2-3 hours followed by brief isofluorane anesthesia (4%) and decapitation. Brains were immediately harvested, snap-frozen in methyl-butane at -40°C, and stored at -80°C. A 200µm section of brain tissue from the rostral pons of each animal (to include the LPBN) was collected on a freezer microtome. Each section was divided into quadrants. The right dorsal quadrant from each animal was pooled for each group and homogenized in lysis buffer (20mM Tris HCl [pH 7.4], 5mM EDTA [pH 8.0], 10mM EGTA [ph 7.0], 2mM DTT, 1mM Na3VO4, 0.1mg/mL PMSF, 0.01mg/mL leupeptin, 1% triton x-100 solution in dH2O, 2.5mM Na+ pyrophosphate, 1mM β-glycerophosphate). Extracted protein samples were boiled for 5 minutes after being diluted 1:2 (v/v) with sample-loading buffer (ph 6.8, 62.5 mM Tris HCl, 20% glycerol, 4% SDS, 0.2% bromphenol blue). Equal amounts of protein (25 µg/35µL) per sample were separated by SDS-PAGE (NuPAGE Novex 4-12% BisTris Gel) at 200 volts for 60 minutes followed
by Electrophoretic transfer to a nitrocellulose membrane (Invitrogen, LC 2001, 0.45µm pores) at 200 volts for 60 minutes. A biotinylated protein latter detection kit (Cell Signaling, 7727) was utilized to identify molecular weights of the targeted protein (KOR, ~46 kDa). All samples were run in duplicate. Immunoblots were blocked with 5% nonfat milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T) for 60 minutes at room temperature followed by overnight incubation with a rabbit polyclonal antibody raised against the internal region of the rat KOR (diluted 1:1000 with 5% nonfat milk in TBS-T, Biosource, 44-302G). Immunoblots were then incubated for 1 hour at room temperature with secondary antibody (goat anti-rabbit HRP, diluted 1:500 with 5% nonfat milk in TBS-T, BioRad, 170-6515). Targeted proteins were visualized with enhanced chemiluminescence (ECL, Pierce) and band intensities were quantified using Quantity One software (BioRad). Relative protein abundance is expressed as integrated optical density (IOD) of KOR factored for Ponceau red stain (total protein loaded). Whole brain lysate was used for control comparison.

**Statistical Analysis**

For animals that underwent the HEM protocol, paired t-tests were employed to evaluate differences in average body weight at week six and soleus/body weight ratios between EX and SED rats. One-minute averages of HR and MAP values were calculated prior to the first brain injection (PRE), within one minute following the second brain injection (POST), and every five minutes from 60 seconds prior to the onset of HEM (minute 0) though the end of recovery (minute 60). Within treatment groups, HR and MAP data were averaged and reported as mean ± SEM. A two-way analysis of variance (ANOVA) with repeated measures was used to compare HR and MAP between treatment groups across time. When significant main effects and/or
interactions were present, Student-Newman-Keuls post-hoc analyses were performed to isolate differences between groups at certain time points, within groups across time, and/or across time irrespective of treatment or group. For animals used in Western blot analysis of pontine KOR density, a paired t-test was used to compare KOR integrated optical density (IOD) between groups. Significance was determined as \( P<0.05 \) for all statistical analyses.

Results

Measures of Exercise Training

Upon initial exposure to running wheels, EX rats weighed 192 ± 4 grams. Following six weeks of running wheel access, EX animals weighed 313 ± 5 grams—significantly less compared to age-matched SED rats (346 ± 6 grams; \( P<0.001 \)). EX animals were pair-housed rather than singly-housed, therefore daily running distances were collected per cage and divided by two. These data are displayed in Figure 5-1A and show an increase in average distance run per animal per day from week one to week five, after which daily running distances reached a plateau. By week six, the estimated average distance run per day per rat was 6.2 ± 0.5 km. Figure 5-1B shows no difference in soleus muscle/body weight ratio (g/kg) for EX versus SED animals (\( P=0.2837 \)).

Injection Site Verification

Figure 5-2 shows photomicrograph images of brain slices taken at the level of injection sites. The cardiovascular response to HEM was not different between bilateral (Figure 5-2A) and unilateral (Figure 5-2B) injection of either vehicle or naloxone; therefore, all brains in which at least one injection was correctly placed into the LPBN were included for statistical analyses. The primary reason for unilateral injections was
incorrect placement of one of the two implanted guide cannulas. Usually, one of the guide cannulas was not implanted deeply enough such that injections were placed into fiber tracts or nuclei located dorsal to the LPBN (see Figure 5-2B). In a couple of cases, however, one of the guide cannulas became clogged during the post-surgical recovery period, preventing advancement of the internal injection cannula during the experiment. For the SED vehicle group, two animals in which injections were placed into the inferior colliculus (IC) were included in the statistical analyses since cardiovascular responses to HEM were not different from those in which vehicle injections were correctly placed within the LPBN (see top panel of Figure 5-2D). Additionally, the cardiovascular response to HEM was not different between vehicle-injected rats and rats in which both injections of naloxone were placed outside the LPBN (n=1 per group; see Figure 5-2C & D); therefore, animals in which both injections were misplaced (not in the LPBN and not in the cerebral spinal fluid [CSF]) were included with the vehicle-treated group for statistical comparisons.

**Cardiovascular Response.**

Figure 5-3 shows the hemodynamic response to vehicle or naloxone injections prior to severe HEM in SED rats. For HR, a repeated measures ANOVA identified a main effect of time (P<0.0001), but no main effect of drug treatment (P=0.9145) and no interaction (0.6020) between factors. Injection of either drug resulted in a non-significant increase in HR that fell slightly prior to the onset of HEM. A typical response to HEM was recorded for vehicle- and naloxone-injected rats: HR rose initially, followed by a precipitous fall by minute 15, which remained significantly lower than minute 0 (just prior to HEM onset, i.e., baseline) though minute 20, irrespective of the drug injected. HR was also found to be significantly lower than baseline towards the end of recording.
at minutes 55 and 60. Similar to what was recorded for HR, analysis of MAP data with a repeated measures ANOVA identified a main effect only for time (P<0.0001). There was not a main effect of drug treatment (P=0.8101) or an interaction between factors (P=0.1184). Irrespective of drug, there was a non-significant rise in MAP immediately following injection, which dropped slightly by minute 0. Once HEM was initiated, MAP was not different from baseline for either group until minute 15. When data for both groups were combined, MAP was significantly lower than baseline from minute 15 through the end of recording, minute 60. Naloxone-injected rats appear to have a lower value for both HR and MAP at minute ten compared to vehicle-injected rats, however, these values are not statistically different.

Figure 5-4 shows the hemodynamic response to vehicle or naloxone injections prior to severe HEM in the EX rats. For HR, a repeated measures ANOVA identified a main effect across time (P<0.0001) but not treatment group (P=0.0611). There was however a significant interaction between these factors (P=0.0289). Similar to what was seen for SED rats, HR increased slightly (non-significant) following injection of either vehicle or naloxone and then fell slightly by minute 0. HR rose during the early part of HEM, and fell to below-baseline values during minutes 15-20 for the naloxone-treated rats only. Vehicle-injected rats also displayed a decline in HR by minute 15, but this value was not different from baseline. Furthermore, at no point during the experiment were HR values for vehicle-injected EX rats different from baseline. Naloxone-treated rats experienced a delayed recovery from HEM compared to vehicle rats as these animals had significantly lower HR from minute 15-30 relative to vehicle controls. For MAP data, a repeated measures ANOVA identified a main effect of time (P<0.0001) but
not treatment group (P=0.1581) and an interaction was identified between factors (P=0.0254). In naloxone-treated EX rats, MAP was not lower than baseline until minute 15 of HEM and this hypotension lasted through minute 30. MAP fell below baseline for vehicle-treated rats as well, but only for minute 15. By minute 20, vehicle controls had already recovered to near-baseline values while naloxone rats remained hypotensive. Although vehicle-treated rats displayed significant hypotension by minute 15, MAP values were significantly greater in vehicle-treated rats compared to naloxone-treated rats at both minute 15 and minute 20.

Figure 5-5 shows the hemodynamic response to naloxone injections prior to severe HEM in SED versus EX rats. Statistically and functionally speaking, these groups were not different in their cardiovascular response to HEM. For HR data, a repeated measures ANOVA isolated a main effect for time only (P<0.0001). There was no main effect of exercise training (P=0.4048) and no interaction between time and training factors (P=0.8336). Thus, when the two groups were combined, HR was maintained during HEM through minute 10, after which it fell significantly from baseline through minute 20. Similarly, MAP data subjected to a repeated measures ANOVA revealed only a main effect of time (P<0.0001), but not for exercise training (P=0.4827) and no interaction was present (P=0.8921). Irrespective of whether rats were sedentary or trained, MAP fell significantly lower than baseline at minute 10 and this hypotension lasted through the end of recovery (minute 60).

**Western Analysis of Kappa Opioid Receptor Content**

Figure 5-6B shows a chemiluminescent image of an immunoblot of pooled brain tissue from the rostral pons of EX and SED rats along with the relative KOR abundance.
Data are displayed as averages for each group of the duplicate samples. There was no difference seen in KOR IOD between EX and SED rats (P=0.57).

**Discussion**

There are three main findings from the present set of experiments. First, exercise-induced tolerance to severe conscious HEM is mediated centrally, since the effect can be completely blocked by central administration of an opioid receptor blockade in the LPBN. Second, six weeks of voluntary wheel running results in central alterations that has a greater inhibitory effect on opioidergic mechanisms within the LPBN. This is evidenced by the fact that opioid receptor blockade within the LPBN caused a significant change in the hemodynamic response to severe conscious HEM in EX rats and no change in SED rats. Finally, six weeks of voluntary wheel running does not result in a decreased KOR content in the brain region including the LPBN, suggesting that the exercise-induced tolerance to HEM is not likely the result of a down-regulation of KORs in this brain region.

Previous literature has shown that opioids in the CNS are involved in the onset of HEM-induced decompensation. In the periaqueductal grey, delta-opioid receptor (DOR) antagonism prevents the decompensatory phase of HEM in conscious rabbits (Ludbrook and Ventura, 1994). Administration of mu- (Evans et al., 1989) and kappa- (Evans et al., 1989) opioid receptor antagonists into the 4th ventricle also prevent the onset of decompensated hypotension in conscious rabbits, suggesting the possibility that more than a single type of opioid receptor is involved in mediating the reflex bradycardia and hypotension during severe HEM. In rats, blockade of both DORs and mu-opioid receptors (MORs) within the brainstem have been shown to modulate the decompensatory response to HEM (Ang et al., 1999).
Previous work from our lab demonstrates that six weeks of voluntary wheel exercise is able to protect conscious rats from the decompensation that typically occurs with 30% TBV loss (see Chapter 4). Results from the present study confirm a voluntary EX-induced tolerance to HEM. At the end of blood withdrawal (minute 15), when hypotension and bradycardia were maximal, vehicle-injected EX rats displayed a 12% drop in HR while vehicle-injected SED animals dropped 27% from baseline (P=0.04). In the same vehicle-treated group of animals, MAP in EX animals dropped 41% from baseline, while SED animals dropped 54% (P=0.002). Chronically exercised individuals exhibit decreased sensitivity to opioids (Smith and Yancey, 2003)—perhaps due to an overall down-regulation of central opioid receptors. Thus, it is possible that exercise-induced tolerance to severe HEM could, in part, be explained by decreased opioid-triggered decompensation at one or multiple central loci. The fact that naloxone injections into the LPBN were able to completely block this effect of exercise argues that this is, indeed, a centrally-regulated phenomenon.

In addition to the well-established role of the LPBN in the relay of cardiovascular afferent information to more rostral brain sites (Fulwiler and Saper, 1984; Herbert et al., 1990; Krukoff et al., 1993; Jhamandas et al., 1996) and its ability to alter autonomic tone via anatomical connections with both forebrain and brainstem nuclei (Saper and Loewy, 1980; Hubbard et al., 1987; Saleh et al., 1997), an opioidergic mechanism specifically within the LPBN has been identified to have a modulatory function in the response to perturbations in blood volume. Iwasaki et al (2001) showed that bilateral injection of naloxone into the LPBN prior to simulated HEM prevented hypovolemia-induced release of AVP (Iwasaki et al., 1993), suggesting that opioid receptor activation within the LPBN
is critical for the humoral response to hypovolemia, as occurs with severe HEM. Since
opioid receptor activation in the LPBN (as well as other CNS sites) acts to inhibit
neuronal activity (Milner et al., 1984; Xia and Haddad, 1991), it seems likely that opioid
receptor activation within the LPBN leads to arginine vasopressin (AVP) release from
forebrain regions such as the magnocellular paraventricular nucleus (PVN) via inhibition
of tonically inhibitory neurons involved in relaying osmo- or volume information. De
Oliveira et al (2008) showed that opioid receptor blockade in the LPBN of conscious rats
with naloxone resulted in significantly increased sodium intake in both normohydrated
and salt-deprived groups of animals (De Oliveira et al., 2008), which could functionally
translate to an enhanced hypotensive signal. This study also proposed a disinhibitory
mechanism of opioid receptor activation within the LPBN.

We originally hypothesized that naloxone would have a greater effect in SED
compared to EX rats based on previous literature supporting a chronic opioid exposure-
induced alteration in central opioid receptor systems in EX rats (Smith and Yancey,
2003). By blocking opioid receptors, we expected naloxone in the LPBN to prevent the
severe fall in HR and MAP normally seen during severe HEM in the SED but not EX
rats. However, SED rats displayed no differences in HR or MAP response to HEM
following unilateral or bilateral injection of naloxone or saline-vehicle. On the other
hand, the training-induced tolerance to HEM was completely blocked by naloxone
injected either unilaterally or bilaterally into the LPBN of EX rats. In fact, naloxone-
injected EX rats actually had a greater fall in HR (though not significantly different)
compared to naloxone-treated SED rats. This is likely due to the high concentration of
naloxone used. It should be noted that this effect of naloxone could not be the result of
drug leakage into the 4th ventricle as naloxone injections into the cerebrospinal fluid (CSF) have been shown to postpone decompensatory phase of HEM in both conscious sheep (Frithiof and Rundgren, 2006; Frithiof et al., 2007) and rabbits (Evans et al., 1989). Additionally, in the conscious rat, systemic administration of naloxone is able to reverse HEM-induced hypotension (Faden and Holaday, 1979) and intrathecal administration of naloxone prior to the onset of HEM can totally block HEM-induced decompensation (Ang et al., 1999). Indeed, when naloxone injections were misplaced into the 4th ventricle in the present study (n=2, data not shown or used in group averages for statistical analysis), there was no deviation in HR or MAP from baseline values during HEM. A large amount and high concentration of naloxone was intentionally utilized in these experiments in order to identify a clear effect, if any. While injections in the present study were placed into the LPBN (see Figure 5-1), the impact of naloxone at other, nearby, locations cannot be excluded from consideration as alternative sites of action.

The fact that naloxone impacted the EX rats but had no effect at all on SED rats contradicts our hypothesis that a decreased opioid tone within the LPBN may underlie the tolerance to HEM seen following exercise training or at a minimum, is an over-simplified view of how EX-induced alterations in central opioid systems may impact the cardiovascular response to severe blood loss. An alternate explanation for the present results may be a decreased gamma-aminobutyric acid (GABA) tone in EX versus SED rats rather than a down-regulation of opioid receptors. Although the reported effects of exercise on central GABA are somewhat mixed, there is general agreement that exercise can decrease GABAergic inhibition in the CNS. It is well-documented that
overactivity of the GABAergic system within the NTS occurs with the development of hypertension (Catelli and Sved, 1988; Tsukamoto and Sved, 1993; Vitela and Mifflin, 2001) and it is postulated that a decreased GABA release within the NTS is involved in the post-exercise hypotension consistently seen in hypertensive patients and less consistently seen in normotensive individuals (Chen et al., 2009). A study performed in spontaneously hypertensive rats (SHRs) demonstrated decreased GABA-mediated inhibitory post-synaptic potentials in NTS neurons immediately following an acute bout of exercise and concluded that dynamic exercise caused a decreased GABAergic synaptic input to the NTS (Chen et al., 2009). Dishman (1997) reported an elevated GABA concentration in rats given eight weeks of free access to running wheels relative to sedentary controls, results from the same study also showed a concomitant decrease in GABA-A receptor density (as measured by 3H-bicuculine binding) in EX versus SED rats (Dishman et al., 1997). Finally, Mueller and Hassar (2006) showed that exercise training blunts the bradycardic effect of bilateral microinjection of bicuculline, a GABA-A receptor antagonist, into the NTS, suggesting that exercise training decreases tonic GABA-A receptor mediated inhibition of NTS neurons involved in modulating HR (Mueller and Hasser, 2006). While these studies do not give conclusive evidence that exercise training results in a suppression of GABAergic mechanisms within the CNS, they do point to the plasticity of the GABA inhibitory system as a function of exercise training.

Activation of either GABA-A or opioid receptors within the LPBN results in a reduced overall inhibition of this nucleus (Kobashi and Bradley, 1998; De Oliveira et al., 2008), indicating that these neurotransmitters act on inhibitory neurons. While these
neurotransmitter systems work via different cellular mechanisms, they may represent individual, parallel systems which function as an inhibitory influence over other inhibitory neurons important for the relay of volume information or salt appetite (De Oliveira et al., 2008). If GABAergic tone is dampened as a result of EX training, the effect of opioid receptor blockade in the LPBN of trained rats could represent a near-complete loss of inhibitory influence within this nucleus and a significant alteration in the relay of osmo- or volume information. In SED rats, however, the effect of opioid blockade would be less significant because of the remaining inhibitory presence of GABAergic mechanisms. This may explain the severe decompensation seen in the present study in EX but not SED rats following naloxone injection into the LPBN prior to HEM.

In summary, the results of this study reveal a centrally mediated exercise-induced tolerance to severe blood loss in the conscious rat that involves an alteration of an opioidergic mechanism within the LPBN. The present study was unable to identify a difference in KOR protein content between EX and SED rats. Combined with the generally accepted lack of DORs present in this particular brain locus, these data point to an exercise-induced alteration of MORs in the LPBN. Naloxone was used in the present study to antagonize opioid receptors within the LPBN. This drug preferentially binds to MORs; but, when used at higher concentrations—such as was utilized in the present study, naloxone will also block KORs and DORs. Thus, further studies are needed to fully elucidate the exact group (or groups) of opioid receptors that may be altered following chronic exercise. Furthermore, results from this study were contrary to our original hypothesis that opioid receptor blockade within the LPBN would replicate the exercise-induced tolerance to HEM in SED rats. Because the opposite was
observed, it seems implausible to attribute the observed exercise-induced tolerance to blood loss to a down-regulation of central opioid receptors, at least within the LPBN. In light of these unexpected results, more studies are warranted to elucidate the exact central mechanisms underlying the protection against hypovolemic shock afforded by modest amounts of physical exercise. Finally, the trained rats in the present study did not display a resting bradycardia or a difference in soleus muscle/body weight ratio compared with sedentary rats. These data lend support to the notion that even modest physical activity can lead changes within the brain that may benefit the overall health of the individual.
Figure 5-1. Measures of exercise training. A) Estimated average distance run per animal per day (see results section for detailed description). B) Soleus/body weight ratio (g/kg) for exercised (EX) and sedentary (SED) rats. EX rats did not display an increased soleus/body weight ratio relative to SED rats following six weeks of voluntary wheel running (P=0.2837).
Figure 5-2. Injection sites. Photomicrograph images (1.25X) of brain slices showing typical patterns and locations of internal cannula placement. A) Bilateral placement of tip of injection cannula into the lateral parabrachial nucleus (LPBN). B) Unilateral placement of tip of injection cannula into the LPBN. Tip of the contralateral injection cannula was located dorsal to the LPBN. C) Bilateral misplacement of internal cannulas. D) Schematic composite of injection sites used in group analyses (modified from Paxinos and Watson). Injections for exercised (EX) animals are displayed on the left and injections for sedentary (SED) animals are displayed on the right. For simplification, only one injection site is displayed for animals that received bilateral injections. In two SED animals, vehicle was bilaterally injected into the inferior colliculus (IC), represented by red squares labeled “V”. Data from these animals was included in the group averages for the vehicle-treated SED group. Red boxes labeled “N” represent bilateral injections of naloxone outside the LPBN. Data from these animals was included in the group averages for vehicle-treated rats within their respective groups. SCP = superior cerebellar peduncle.
Figure 5-3. Effect of naloxone versus vehicle injection into the lateral parabrachial nucleus (LPBN) on the heart rate (HR) and mean arterial pressure (MAP) responses to severe conscious hemorrhage (HEM) in sedentary (SED) rats. Grey box indicates time of blood withdrawal (minute 0-15). # indicates significant difference from pre-HEM baseline (minute 0) irrespective of drug treatment.
Figure 5-4. Effect of naloxone versus vehicle injection into the lateral parabrachial nucleus (LPBN) on the heart rate (HR) and mean arterial pressure (MAP) responses to severe conscious hemorrhage (HEM) in exercise (EX) rats. Grey box indicates time of blood withdrawal (minute 0-15). $ indicates significant difference from pre-HEM baseline (minute 0) within treatment group. * indicates significant difference from vehicle at the indicated time point.
Figure 5-5. Effect of naloxone injection into the lateral parabrachial nucleus (LPBN) on the heart rate (HR) and mean arterial pressure (MAP) responses to severe conscious hemorrhage (HEM) in exercised (EX) versus sedentary (SED) rats. Grey box indicates time of blood withdrawal (minute 0-15). # indicates significant differences from pre-HEM baseline (minute 0) irrespective of treatment group.
Figure 5-6. Western blot analysis of relative kappa opioid receptor (KOR) content in the rostral pons of exercised (EX) and sedentary (SED) rats. A) Schematic demonstrating the way in which brain slices from the rostral pons were separated into quadrants for Western blot analysis of the dorsolateral region only. Dashed lines indicate where each section of brain tissue was sectioned. B) Chemiluminescent photograph of an immunoblot showing KOR protein expression between 40 and 50 kDa for EX and SED animals and averages of the quantified KOR integrated optical density (IOD) for both groups. There was not a statistical difference in KOR IOD between groups (P=0.57).
CHAPTER 6
SUMMARIES AND CONCLUSIONS

Summaries of the Study Findings

Study #1 Summary

This study evaluated the hemodynamic responses and neural activation of various rostral brainstem nuclei (as marked by c-Fos immunoreactivity) during three different rates of hemorrhage (HEM) in conscious rats. This study was important for identifying a reliable HEM protocol that would best simulate the bi-phasic hemodynamic response to severe blood loss and induce distinct changes in neural activation within nuclei previously shown to be involved in regulating the cardiovascular and autonomic responses to blood loss. Previous studies have utilized a wide range of HEM protocols which makes interpreting results across studies somewhat difficult. The results of study #1 demonstrated that while the fastest rate of HEM (2.0 ml/kg/min) produced a significant bradycardia and hypotension at the end of HEM, there was not a clear compensatory tachycardia at the onset of blood withdrawal. Animals that underwent the slowest rate of HEM (0.5 ml/kg/min) showed neither a compensatory tachycardia nor a decompensatory bradycardia or hypotension. Only animals from the intermediate rate of blood withdrawal (1.0 ml/kg/min) displayed clear compensatory and decompensatory phases that resulted in deviations in heart rate and mean arterial pressure that were significantly different from pre-HEM baseline values. Additionally, the intermediate rate of HEM produced the most significant and least variable increases in neural activation of rostral brainstem sites (including, but not limited to, the caudal ventrolateral periaqueductal grey [vlPAG] and the central subnucleus of the rostral lateral parabrachial nucleus [LPBN]) when compared to hypotension alone. Results from this
study further substantiate the integral role of the rostral brainstem in the autonomic
response to severe HEM and provide a dependable protocol for future HEM
experiments in this lab as well as for other investigators.

**Study #2 Summary**

This study evaluated whether or not LPBN-projecting afferents from the vlPAG are
involved in some component of the cardiovascular response to severe HEM in the
conscious in the rat. Results from this study corroborated previous work which showed
a dense axonal projection from the vlPAG to the LPBN. However, these data do not
support our initial hypothesis that this projection is actively involved in the descending
control of autonomic responses to HEM. This was evidenced by a lack of difference in
Fos-Fluorogold (FG) co-labeling within the vlPAG neurons of HEM verses non-HEM
animals which had received a unilateral FG injection into the LPBN. Since the
neuroanatomical networks underlying the transition from hemorrhagic compensation to
decompensation are not well understood, the results of this study will allow investigators
interested in elucidating these neural pathways to redirect and focus their attention and
resources on other anatomical connections involved in hemorrhage-induced
sympathoinhibition (HIS).

**Study #3 Summary**

This study evaluated the effect of chronic, voluntary exercise on the hemodynamic
and neural responses to severe HEM in conscious rats. This study identified an
exercise-induced tolerance to severe HEM. Rats that were allowed access to running
wheels for six weeks displayed a significantly blunted bradycardia and hypotension
associated with the decompensatory phase of HEM compared to sedentary rats. While
exercised rats displayed a fall in heart rate (HR) and mean arterial pressure (MAP) that
corresponded with the time point—and therefore the same percentage of blood volume removed—at which decompensation occurred in sedentary animals, at no point did the fall in HR or MAP for exercised animals differ from pre-HEM baseline values. Additionally, exercised animals displayed significantly less neural activation (as marked by Fos-like immunoreactivity [FLI]) in sub-regions of the LPBN and hypothalamic paraventricular nucleus (PVN), suggesting that exercise-induced tolerance to HEM may result from the suppression of central circuits involved in autonomic control during blood loss. Heart rate variability (HRV) analysis of data collected before, during, and after HEM demonstrated a difference in heart rate regulation between exercised and sedentary rats which may correspond to differences in basal respiratory function that may have contributed to an enhanced ability to cope with the internal stress of severe blood loss in the exercised group. Finally, it is noteworthy that exercised (EX) animals in this study did not demonstrate a lower resting HR compared to sedentary (SED) rats, which is commonly used to indicate a training effect. Therefore, even moderate physical activity seems to be sufficient to induce central changes that may contribute to an exercise-induced protection against the cardiovascular decline associated with severe HEM.

**Study #4 Summary**

This study evaluated whether opioid receptors in the LPBN play a role in the exercise-induced tolerance to HEM. One part of this study compared the protein expression of kappa opioid receptors (KORs) within the dorsolateral rostral pons of EX and SED rats. Although we expected to see significantly less KOR protein expression in this region of the brain in EX versus SED rats, no difference was seen between groups. Since the LPBN contains mainly KORs and mu opioid receptors (MORs), these
data point to a possible MOR-mediated mechanism. Accordingly, either saline or a MOR antagonist (naloxone) was administered bilaterally to the LPBN of EX and SED conscious rats prior to HEM. Interestingly, the cardiovascular response to HEM was not different in SED rats given saline or naloxone. On the other hand, while saline had no effect on the HR and MAP of exercised rats in response to HEM, naloxone in the LPBN completely blocked the exercise-induced tolerance to HEM. In fact, there was no difference in the cardiovascular response of naloxone-treated EX and SED rats following HEM. The combined results of this study indicate that although there is an opioidergic mechanism within the LPBN that is involved in the exercise-induced protection against hypovolemic decompensation, it is not likely due to a down-regulation of opioid receptors. Rather, chronic exercise may decrease other inhibitory mechanisms within the LPBN (such as gamma-aminobutyric acid [GABA]) that leads to an increased influence of opioidergic inhibition within the LPBN.

**Discussion**

The peripheral effects of exercise have been studied more thoroughly than those in the central nervous system (CNS). A number of studies, however, have identified both anatomical and neurochemical changes in the brain and spinal cord that occur following chronic exercise (Nelson et al., 2005; Vaynman and Gomez-Pinilla, 2005). While the effect of exercise training on brain opioidergic systems has been studied to some extent, there is still little known about the exact locations in the brain at which exercise affects opioids and opioid receptors—particularly those that contribute to cardiovascular control. Some investigators have postulated that chronic exercise, like chronic morphine administration, leads to the down-regulation of opioid receptors in the brain (Smith and Yancey, 2003). Partly based on these studies, we also initially
hypothesized that exercised animals would display less opioid receptors in the LPBN and that there would be less of an opioid-mediated influence on the response to HEM in exercised versus sedentary rats. Surprisingly, the results from the present studies indicate something quite different. No difference in KOR protein expression was identified between groups. Additionally, exercise-trained animals responded in such a way as to indicate that this group of animals possessed a greater opioidergic influence within the LPBN compared to their sedentary counterparts.

In addition to its influence on autonomic reflexes, such as the arterial baroreflex (Hayward and Felder, 1998), the LPBN also plays a significant role in salt appetite (Johnson and Thunhorst, 1997), which implicates this group of neurons in volume or osmo-regulation, as ingestion of salt and water are stimulated by circumstances such as cellular dehydration or low plasma volumes (De Castro e Silva et al., 2006). It is possible that such stimuli are interpreted by the brain in a similar way as hypotensive signals as salt-induced water retention and, later, increases in plasma volume may act long-term to rectify decreases in mean arterial pressure. A number of forebrain regions (like the PVN, median preoptic nucleus, supraoptic nucleus, and the central nucleus of the amygdala) (Ciriello et al., 1984; Krukoff et al., 1993; Bianchi et al., 1998; Krout et al., 1998) act to stimulate salt ingestion. The LPBN is interconnected with all of these forebrain regions and its overall influence in salt appetite is to inhibit the afore-mentioned forebrain regions (Johnson and Thunhorst, 1997), thereby dampening afferent signals which would drive salt appetite (see Figure 6-1). Activation of a number of neurotransmitters, such as serotonin and corticotrophin releasing hormone (CRH) within the LPBN results in decreased sodium ingestion in rats exposed to blood volume
expansion or peripheral angiotensin II, both of which produce an elevation in blood pressure (De Castro e Silva et al., 2006; Margatho et al., 2008). On the other hand, activation of GABA-A and opioid receptors within the LPBN result in inhibition of neurons within this nucleus and withdrawal of inhibitory drive to forebrain regions, which leads to increased sodium and water intake (De Oliveira et al., 2008), suggesting that this may be more closely associated with hypotensive-like input to the brain.

As mentioned in the discussion of study #4 (chapter 5), a possible mechanism of enhanced opioidergic tone within the LPBN following exercise training may result from a decreased GABAergic tone within the LPBN. While some studies have identified increases in GABA levels following wheel exercise in spontaneously hypertensive rats (SHRs) (Kramer et al., 2000), others have shown decreases in GABAergic synaptic input and GABA-A receptors following similar exercise regimens in SHRs (Chen et al., 2009). Furthermore, in normotensive rats, as little as 28 days of activity wheel exposure has been shown to decrease the gene expression of both GABA-A receptor and glutamate decarboxylase (GAD) in the brain (Molteni et al., 2002). Thus, exercise may result in decreased inhibitory GABAergic tone in the LPBN—placing a greater proportion of inhibitory influence on opioid receptors in this region (irrespective of any possible change in opioid receptor density or function induced by the exercise training). In Figure 6-1, this is depicted by the tipped scale balancing GABAergic and opioidergic inputs to the LPBN. It should be noted, however, that this figure is not intended to imply any change (increase or decrease) in the density of opioidergic input to the LPBN following exercise training. Alternatively, based on a greater abundance of evidence that GABA-mediated inhibitory systems display some degree of neural plasticity as a
function of exercise training, we have focused our revised model of the influence of exercise training on neurotransmission in the LPBN to reflect decreased GABAergic control during HEM.

The HRV analysis from study #3, which evaluated alterations in HRV before, during, and after HEM in EX versus SED rats, did not show any difference in the way these two groups responded to severe blood loss. However, the location of the high frequency (HF) peak of EX rats was significantly lower across all time points compared to SED rats. Since this HF peak supposedly reflects respiratory rate (Baekey et al., 2008), these data suggest an exercise-induced plasticity within the respiratory control system. If exercise results in an altered respiratory pattern that facilitates increased venous return (increased tidal volume), this may contribute to the enhanced tolerance of these animals to severe HEM. A number of investigators have evaluated the role of the LPBN in respiratory control and have concluded that activation of LPBN neurons results in an increased respiratory rate (Miura and Takayama, 1991; Chamberlin and Saper, 1994; Lara et al., 1994). Recently, Hayward and colleagues (2004) reported a significant change in baseline respiratory pattern following bilateral blockade of LPBN neurons with muscimol (a GABA-A receptor agonist), clearly demonstrating an inhibitory role of GABA on LPBN neurons which project to and activate respiratory control centers (Hayward and Castellanos, 2004). Thus, a decreased GABAergic tone within the LPBN may feasibly contribute to an exercise-induced alteration in the central control and modulation of respiratory timing which may, in turn, underlie a training-induced tolerance to HEM.
Study Limitations and Directions for Future Studies

While the present set of experiments have contributed meaningful information to what is currently known about the central effects of exercise and the central control of HEM, there are limitations to these studies which make the reported results less comprehensive. Accordingly, the following is a brief discussion of methodological considerations which may have enhanced the present studies along with some proposals for experiments that could potentially broaden this body of work.

Rate of Hemorrhage.

Multiple studies have evaluated the cardiovascular responses to HEM. These previous studies have utilized a number of animal models, including sheep (Frithiof and Rundgren, 2006), dogs (Thrasher and Keil, 1998), rats (Ahlgren et al., 2007), pigs (Salerno et al., 1981), mice (Liaudet et al., 2000), cats (Hall and Hodge, 1971), and rabbits (Schadt and Ludbrook, 1991). It is beneficial to investigators to compare results from experiments performed on such a wide range of species because it allows interspecies differences to be teased out and permits investigators to select a species which they feel may be best-suited for their own experiments in terms of time management, cost, translation, etc. What is less beneficial about the current large body of literature regarding HEM is the variety of protocols used. For example, much of the earlier HEM experiments were performed on anesthetized animals, which (as mentioned earlier in the introduction) has been shown to significantly alter the phasic response to severe blood loss. Accordingly, more recent studies have implemented a conscious model of HEM. Even still, the actual method of blood removal remains inconsistent between investigators, making results difficult to translate and repeat. Since the focus of this series of studies was to evaluate the complex hemodynamic and neural responses to
HEM, it seemed warranted to determine the rate of blood removal that produced the most reliable, phasic, hemodynamic and neural responses to blood loss. It was determined from study #1 that removal of blood at a rate of 1 ml/kg/min over 20 minutes was ideal for inducing a clear compensatory tachycardia, decompensatory bradycardia and hypotension, and a recompensatory recovery to baseline or near-baseline values. Although this specific protocol was found to be reliable and repeatable, blood removal over a 20 minute time period was sometimes infeasible. In our hands, several experiments had to be stopped because blood clots developed at the tip of the catheter—a problem that was not encountered when blood was removed faster (2.0 ml/kg/min over 10 minutes). When blood was removed too quickly, however, a clear compensatory phase was not present. Consequently, hemorrhages performed in studies #2, #3, and #4 utilized a blood withdrawal rate of approximately 1.2-1.3 ml/kg/min over 15 minutes. This rate was sufficient to produce all three phases of HEM as well as neural activation similar to that seen at the slightly slower rate.

**Indices of Exercise Training**

The indices used in the present studies to document an exercise training effect, though commonly used, are relatively primitive and somewhat inconclusive. Evidence of a resting bradycardia and blunted weight gain over time are not consistently seen following running wheel activity under 8-12 weeks duration and often times require a much greater training stimulus, such as forced treadmill exercise. Additionally, soleus muscle/body weight ratios were not different between exercised and sedentary rats in study #4. Evaluation of soleus and/or cardiac muscle citrate synthase activity would have greatly enhanced our conclusions about the level of exercise performed on the rats utilized in these experiments, especially considering the fact that the rats were
paired rather than individually housed with running wheels. We observed a significant difference in the HR and MAP response to HEM seen in exercised versus sedentary rats, thus a more critical means of determining the level of exercise training was not all together necessary. However, future studies comparing the effect of voluntary versus forced exercise would need to implement such measures. It would also be of interest to evaluate the time course over which moderate levels of activity confer HEM tolerance to conscious animals. In other words, how long after one starts/stops exercising will a benefit be present in the face of blood loss?

**Effect of Exercise on Plasma Arginine Vasopressin Response to Hemorrhage**

The role of AVP in hypotensive hemorrhage has been well established. Release of this hormone corresponds with the decompensatory drop in mean arterial pressure observed late in HEM (Schadt and Hasser, 1991) and is important for blood pressure recovery following HEM (Korner et al., 1990). Additionally, there is evidence for a central effect of endogenous opioids on AVP release during hypotensive HEM (Korner et al., 1990). Naloxone pretreated rabbits display significantly greater plasma AVP content following conscious HEM compared to saline pretreated controls—an occurrence which could not be explained simply a pressor affect of naloxone (Schadt and Hasser, 1991). Authors of this study concluded that naloxone was augmenting the AVP response to HEM through blockade of the opioid-mediated suppression of AVP release, which supported previous work showing that KOR agonists inhibit AVP release in situations of hypovolemia (Oiso et al., 1988). Comparison of plasma AVP concentrations in exercised versus sedentary rats during and following HEM would have added an interesting component to the present studies and certainly would have been manageable in terms of blood collection during and following HEM experiments since
animals were chronically instrumented with vascular catheters. Additionally, evaluation of brain tissue co-labeled for AVP and c-FOS following HEM might have further elucidated differences in central control mechanisms regulating the humoral response to HEM in EX versus SED rats which may involve a central opioidergic component.

**Effect of Exercise on Opioidergic Influence in the Lateral Parabrachial Nucleus**

Although study #4 evaluated the KOR content within the dorsolateral quadrant of the rostral pons, more molecular studies are needed to better and more accurately quantify any changes in opioid receptors within the LPBN. Mastery of the micropunch technique specific to the LPBN would have greatly enhanced the specificity of the Western analysis of KOR protein content in study #4. Additionally, future studies are warranted to evaluate whether exercise training alters MOR protein expression and/or opioid peptides release within the LPBN. When opioid ligands bind their receptor, the receptor is phosphorylated, resulting in internalization of the receptor (Gaudriault et al., 1997). Literature supports the idea that opioid phosphorylation reflects level of opioid peptide release (Wang et al., 1996) as well as opioid receptor desensitization (Pan, 2003). Throughout the body, the opioidergic system is very plastic, showing signs of receptor up- and down-regulation and even functional shifts from overall inhibitory to excitatory influences in response to varying degrees of opioid peptide exposure (Wang et al., 1996). Thus, measures of opioid phosphorylation within the LPBN would seemingly be an appropriate means to evaluate the effect of exercise on opioidergic control and modulation within this (or any) brain locus.
Effect of Exercise on Gamma-Aminobutyric Acid Influence in the Lateral Parabrachial Nucleus

As suggested earlier, the GABAergic inhibitory influence within the LPBN may potentially be blunted following chronic exercise. Although examination of this hypothesis was not within the realm of the current set of studies, it is, nonetheless an interesting and testable hypothesis. Presumably, application of a GABA-A agonist, such as muscimol, within the LPBN, would prevent the naloxone-induced augmentation of the hemorrhagic decompensation observed in exercised rats (study #4). Another brain region of interest regarding GABAergic input to the LPBN and GABA/opioid interaction within the CNS is the arcuate nucleus of the hypothalamus (ARC). This nucleus, situated next to the third ventricle and the median eminence [Paxinos], is one of only two groups of neurons in the brain containing pro-opioimelanocortin (POMC) neurons (which produce melanocortin and endorphins) (Palkovits et al., 1987) and is integrally involved in the regulation of energy balance and appetite (Minor et al., 2009). The ARC provides both GABAergic and melanocortin (via POMC neurons) input to the LPBN (Cone, 2005). Since POMC neurons produce both melanocortin and endorphins, there is a possibility that endorphins acting in the LPBN may also, at least in part, originate from the ARC. This notion is supported by the results from study #2 which demonstrated that another possible source of opioid release in the LPBN during HEM, the vIPAG, is not activated.

In addition to their inhibitory role within the LPBN, ARC-originating GABAergic neurons inhibit local POMC neurons with efferent connections to regions of the brain involved in fluid and electrolyte balance, such as the PVN (Dietrich and Horvath, 2009). GABA-A receptor blockade within the LPBN and decreased GABA-mediated inhibition
of POMC neurons within the ARC both, independently, suppress the drive to eat (Cone, 2005; Wu et al., 2009), indicating that both GABAergic and POMC projections from this region significantly alter afferent visceral signals (such as hunger) via connections with the LPBN and other hypothalamic nuclei. Combined with the fact that lidocaine inactivation of ARC neurons is able to dramatically attenuate the fall in arterial pressure during severe HEM (Goktalay et al., 2006), future studies should focus on this nucleus as an interesting modulator of the autonomic response to HEM via its projections to the LPBN.

**Effect of Exercise on Serotonergic Influence in the Lateral Parabrachial Nucleus**

Finally, serotonin (5-HT) is another neurotransmitter system that has been identified to play an important role both in the sensory processing within the LPBN and modulation of the cardiorespiratory response to HEM. Specifically within the LPBN, 5-HT has been shown to modulate sodium and water ingestion (Menani et al., 2000) via projections from areas such as the NTS and dorsal raphe nucleus (Margatho et al., 2008). In addition, activation of 5-HT 1A receptors in the hindbrain has been shown to reverse the hypotensive and sympathoinhibitory responses to severe blood loss in conscious rats (Scrogin, 2003; Osei-Owusu and Scrogin, 2004). Moreover, some of the influence of 5-HT 1A receptor activation on modulating the response to HEM was shown to be mediated by a change in respiration. This was identified by a significant increase in the HF or respiratory-related component of spectral analysis of renal sympathetic nerve activity (Osei-Owusu and Scrogin, 2004).

Chronic exercise has been shown to increase central 5-HT synthesis and release (Hong et al., 2003; Min et al., 2003) and decrease the sensitivity of 5-HT 1A autoreceptors (Dwyer and Browning, 2000) which act to slow 5-HT release.
Additionally, as little as 3 days of activity wheel running results in increased serotonergic neuron fiber length (Engesser-Cesar et al., 2007), further supporting the possibility of an exercise-induced plasticity of the serotonergic systems of the CNS. Although such exercise-induced changes in 5-HT mechanisms within the CNS have been proposed to underlie mood enhancement in depressed patients (Chaoulloff, 1997), whether these changes can or do affect modulation of the autonomic responses to changes in blood volume remains to be seen. Future studies should evaluate whether or not exercise induces changes in the way 5-HT acts within the LPBN to alter the cardiovascular or respiratory responses to HEM.

**Conclusions**

Results from all four studies presented support previous work showing that the LPBN is an integral site for the complex cardiovascular and respiratory response to severe HEM in the conscious rat. Additionally, this body of work supports the idea that even modest amounts of physical exercise can induce changes within the central nervous system that are beneficial for autonomic regulation and general health. Specifically, chronic exercise may induce changes within the LPBN which result in greater dependence on opioidergic mechanisms supporting the overall inhibitory role of this nucleus in body fluid regulation.
Figure 6-1. Schematic of revised research hypothesis regarding the impact of chronic exercise on the balance of gamma-aminobutyric acid (GABA) and opioid contributions to the overall inhibitory influence of the lateral parabrachial nucleus (LPBN) on salt appetite. Exercise training may increase the opioidergic component of the inhibitory mechanisms acting within the LPBN.


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BIOGRAPHICAL SKETCH

Joslyn K. Ahlgren was born and raised in Wichita, Kansas. After graduating Suma Cum Laude with a Bachelor of Science degree in kinesiology from Kansas State University in May 2002, Joslyn began pursuing a graduate education in the field of autonomic neurophysiology. Joslyn joined the laboratory of Dr. Linda Hayward in June of 2004 and has spent the last five years studying the central effects of exercise training. During her graduate studies, Joslyn was awarded a Pre-doctoral Fellowship from the Florida/Puerto Rico affiliate of the American Heart Association. She received her Doctor of Philosophy degree in veterinary medical sciences from the Department of Physiological Sciences in the College of Veterinary Medicine in December 2009 and immediately began a teaching career in the Department of Applied Physiology and Kinesiology in the University of Florida’s College of Health and Human Performance.