SENSORY INTEGRATION DURING BLOOD LOSS IN CONSCIOUS RABBITS

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by

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SENSORY INTEGRATION AND CARDIOVASCULAR CONTROL IN CONSCIOUS RABBITS

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A candidate for the degree of Doctor of Philosophy

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DEDICATION

I received an incredible amount of support and encouragement

along the journey from point A to point PhD.

Many thanks to

my husband, Nick,

my father and mother,

friends and family, near and far.

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ABSTRACT

Central nervous system integration of sensory inputs is critical for orchestrating physiological responses to stress. The focus of my doctoral research was investigating the physiological consequences of sensory stimulation to begin to answer the question of how the body integrates information about stress, pain and other sensory inputs. Conscious, chronically instrumented rabbits were used to minimize the influence of anesthesia and surgery. Two studies explored the role of the midbrain periaqueductal gray in modulating the cardiovascular response to hemorrhage and sensory stimulation. Other studies describe the assessment and use of a model of visceral pain, colorectal distension, to quantify the effect of concurrent stress and pain on the cardiovascular and respiratory response to blood loss. The results from these studies provide insight into 1) a central nervous system site for integration of multiple sensory inputs; and 2) potential sex differences in the response to concurrent stress, pain and hemorrhage.

CHAPTER 1

Introduction

Homeostasis is the active and ongoing process of maintaining optimal physiologic function in dynamic internal and external environments. Interoceptors and exteroceptors are sensory receptors that detect changes in the internal and external environments, respectively. Afferent sensory input is relayed from these receptors to the central nervous system where integration of sensory inputs leads to a coordinated physiological and behavioral response with the goal of maintaining homeostasis. Alerting and noxious stimuli, as well as internal stressors such as blood loss can all signal a potential threat to survival and, thus, trigger autonomic and behavioral changes. Therefore, it is not surprising that anatomical and functional overlap exists between central nervous system circuits that process internal and external stressful sensory stimuli.

A stressful stimulus is one that signals a potential threat to homeostasis. This signal should promote autonomic and behavioral changes to remove or minimize the threat to the internal environment. For example, preserving homeostasis in the face of a menacing predator may involve changes in energy balance and skeletal muscle blood flow to facilitate active escape behavior. Similarly, increased vigilance involving the alteration in central processing of, or

sensitivity to intero- and exteroceptive stimuli may prove beneficial for the maintenance of homeostasis in a stressful situation.

Sensory integration, at the whole organism level, is a common thread connecting the chapters to follow. Because I was interested in how the body integrates information about stress, pain and other sensory stimuli, it was critical that these experiments be carried out in conscious animals. In addition, animals were chronically instrumented to minimize the influence of anesthesia and surgery.

Preview of chapter sequence. The first project I was involved with evaluated the potential role of the midbrain periaqueductal gray in mediating the response to the progressive hypotensive stimulus, hemorrhage (Chapter 2). An incidental finding during these experiments was the fact that some neurons in the periaqueductal gray responded to auditory and tactile stimuli. Despite evidence for distinct anatomical and functional columns of neurons within the periaqueductal gray, we were unable to define unique characteristics of spontaneously active neurons that would aid in identifying their locations antemortem. A second project described in Chapter 3, recorded the response of neurons in the dorsolateral and ventrolateral periaqueductal gray to a variety of internal and external sensory stimuli in an effort to functionally identify the location of spontaneously active neurons. I had hoped to evaluate the response of periaqueductal gray neurons to a noxious stimulus for two reasons: 1) the periaqueductal gray mediates endogenous analgesia; and 2) response to noxious stimulation has been used to identify neuronal cell types in the

periaqueductal gray and other brain regions. However, use of a noxious stimulus in conscious animals without the concurrent use of anesthetics or analgesics required us to first address ethical and experimental design concerns.

Chapter 5 details the process of evaluating, selecting and validating a model of pain for use in conscious, chronically instrumented rabbits. The effect of the opioid analgesic, buprenorphine, on the physiological response to noxious stimulation was evaluated during the pain model validation process. Data gathered from these experiments are presented in Chapters 6 and 7.

During the process of evaluating colorectal distension in conscious rabbits, I had to choose which project to pursue first: the response of periaqueductal gray neurons to noxious and non-noxious stimuli, or the effect of pain on the response to blood loss. This laboratory has been gathering data on the cardiovascular and central nervous system responses to hemorrhage in conscious rabbits in an effort to improve the understanding of, and therapies for, blood loss in all animals. How concurrent stress and pain might alter the response to blood loss was a clinically compelling question that I committed to exploring first, with the expectation that neuronal recording would follow.

The electrodes described in Chapter 4 were important for evaluating the respiratory response to sensory stimuli (i.e. air jet stress, colorectal distension, etc) in the presence and absence of hemorrhage. The data presented in Chapters 8 and 9 represent the culmination of the process to evaluate the effect of concurrent stress and pain on the response to blood loss in conscious, chronically instrumented rabbits. Evaluating the role of the periaqueductal gray

in modulating the multiple internal and external sensory stimuli remains to be investigated.

Chapter format and publication status. With the exception of Chapters 4, 5 and 6, chapter format follows the manuscript guidelines for the *American Journal of Physiology*. The figures and tables associated with each chapter follow the chapter text.

Chapter 2 (Periaqueductal gray neurons and simulated hemorrhage) is the draft of the manuscript I submitted to the *American Journal of Physiology*. At the request of reviewers, the data were re-analyzed and the text was extensively revised prior to acceptance for publication. Dr. Jim Schadt is the primary author of the accepted manuscript (120) because he did the bulk of the re-analysis and revisions. Chapter 4 (Novel electrode design) was published in the *Journal of Neuroscience Methods* with Drs. Rachel Strittmatter and Jim Schadt as coauthors (126). Chapter 8 (Noxious visceral stimulation and the response to hemorrhage) has been submitted to the *American Journal of Physiology* with Dr. Jim Schadt as coauthor. I anticipate submitting the manuscripts presented in Chapters 3, 6, 7 and 9 for publication, but do not plan to submit Chapter 5 for publication.

CHAPTER 2

Neuronal activity within the ventrolateral periaqueductal gray during simulated hemorrhage in conscious rabbits

ABSTRACT

The rapid fall in blood pressure during hemorrhage in conscious animals is an active process. In conscious rats, inactivation of the ventrolateral periaqueductal gray (vlPAG) alters this active transition to hypotension during blood loss (18). We characterized the response of vlPAG neurons to simulated hemorrhage in conscious rabbits to test the hypothesis that vlPAG neurons change their firing frequency prior to the onset of hypotension during simulated hemorrhage. The response of dorsolateral PAG neurons was used as an anatomical control. Ten rabbits were instrumented with arterial and venous catheters and an intrathoracic vena caval occluder. Microelectrodes on a microdrive were implanted in the midbrain. During simulated hemorrhage the occluder was inflated until arterial pressure $\leq$ 40 mmHg. We evaluated neuronal activity during simulated hemorrhage and defined a change in firing frequency as $\geq$ 20% of control. While 41 of 62 vlPAG neurons changed firing frequency during simulated hemorrhage, most (56%) did so after the onset of hypotension. Only 2 vlPAG neurons changed firing frequency at a time consistent with triggering the

fall in arterial pressure. The response of recording sites to electrical stimulation was assessed to test the hypothesis that activation of the vlPAG would result in hypotension. Electrical stimulation increased arterial pressure at 48 of 51 vlPAG sites. No hypotension was seen in response to electrical activation of the vlPAG. Thus, in conscious rabbits, it appears unlikely that the vlPAG triggers the drop in arterial pressure during hemorrhage.

INTRODUCTION

The cardiovascular response to blood loss in conscious animals is biphasic (118). Phase 1 is characterized by maintenance of arterial pressure primarily through sympathetic activation. At a critical point during continued blood loss, arterial pressure drops precipitously, initiating phase 2. This phase 2 hypotension is due, at least in part, to a profound sympathoinhibition. An important question from a clinical and physiological point of view is what triggers the rapid fall in blood pressure during hemorrhage. The transition to phase 2 appears to be an active process, rather than simply a result of threshold volume depletion or sympathetic exhaustion. For example, exposure of conscious animals to stressful sensory stimuli during hemorrhage can prolong phase 1, thus extending maintenance of arterial pressure in the face of a larger volume loss (115;116). In addition, the phase 2 sympathoinhibition can be reversed by a variety of endogenous and exogenous compounds (e.g. opioid antagonists) (36) indicating that, indeed, the animal has sympathetic reserve at the time pressure drops (45).

Active modulation of sympathetic activity and ultimately blood flow and arterial pressure during hypotensive hemorrhage has to occur within the central nervous system. Evidence suggests that the midbrain periaqueductal gray (PAG) may be involved in this modulation. For example, chemical inactivation of the ventrolateral PAG (vlPAG) in conscious and anesthetized rats appears to delay or prevent phase 2 hypotension and sympathoinhibition during hemorrhage (18;27). Thus, it has been proposed that the vlPAG triggers the onset of hypotension (18) and sympathoinhibition (27) characteristic of phase 2.

The goal of this study was to evaluate the response of vlPAG neurons to simulated hypotensive hemorrhage in conscious, chronically prepared rabbits. If the vlPAG is important for producing hypotension and bradycardia during phase 2, then vlPAG neurons should modify their discharge rate immediately prior to the onset of hemorrhagic hypotension. Thus, our first objective was to test the hypothesis that vlPAG neurons change their firing frequency prior to the onset of hypotension during simulated hemorrhage. We recorded responses of dorsolateral PAG (dlPAG) neurons to simulated hemorrhage as an anatomic control. Our second objective was to test the hypothesis that electrical activation of the vlPAG produces hypotension in the conscious rabbit.

METHODS

Animals. All procedures were approved by the University of Missouri Animal Care and Use Committee, and conducted in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* [DHEW

(DHHS) Publication No. (NIH) 85-23, revised 1985, Office of Science Health Reports, DRR/NIH, Bethesda, MD 20205]. Ten New Zealand White rabbits (3 female) weighing 2.75 ± 0.09 kg (mean ± SE) were chronically prepared with arterial and venous catheters, an intrathoracic vena caval occluder, and midbrain recording electrodes midbrain.

Surgical preparation and anesthesia. Each rabbit underwent three surgical procedures separated by at least 2 weeks: 1) laparotomy for catheter placement; 2) thoracotomy for placement of a vena caval occluder; and 3) craniotomy for implantation of midbrain recording electrodes. Antibiotics (22.7 mg enrofloxacin, sc; Baytril, Bayer) were administered the day prior to surgery. Food but not water was withheld 15 – 20 hours prior to surgery. For catheter implantation, anesthesia was induced and maintained with halothane in oxygen via face mask. Anesthesia for the thoracotomy or craniotomy was induced and maintained with intravenous sodium pentobarbital (Nembutal, Abbott). Following induction of anesthesia with pentobarbital, the rabbit was orally intubated and mechanically ventilated with room air. Adequacy of anesthesia was assessed by absence of withdrawal or blood pressure changes in response to noxious stimuli. The analgesic buprenorphine hydrochloride (Buprenex, Reckitt Benckiser) was administered to all rabbits after each surgery. Rabbits were allowed to recover a minimum of 2 weeks after the last surgery before the start of experiments.

Catheters. A midline laparotomy was performed for implantation of intra-abdominal venous (vena cava) and arterial (aorta) catheters (44). Catheters were tunneled under the skin and exteriorized at the base of the neck. We used

the abdominal venous and arterial catheters for injection of drugs and recording of arterial pressure, respectively.

Vena caval occluder. A right lateral thoracotomy was performed at the third intercostal space for placement of an intrathoracic vena caval occluder. The cuff of the pneumatic occluder was placed around the caudal vena cava. Tubing enabling inflation of the occlusive balloon was tunneled under the skin and exteriorized at the base of the neck. Inflation of the occluder simulated hemorrhage by reducing venous return (76).

Midbrain electrodes. Rabbits were mounted in a rabbit stereotaxic frame (David Kopf). The head was leveled and fixed with lambda 1.5 mm below bregma. Four stainless steel machine screws (1-72X1/8”; Small Parts) were implanted in the skull and covered with dental acrylic (Stoelting). A midline craniotomy was performed and the dura opened to expose the cortex. A bundle of electrodes attached to a microdrive was implanted in the midbrain. The electrode bundle consisted of 10 nichrome microwires (California Fine Wire; 5 each of 32 and 64 μm diameter) (42). A single turn of the microdrive advanced the electrode bundle 0.3 mm. Each microwire, in addition to the ground electrode, was connected to a multipin plug (Viking TKR24-101-V).

The stereotaxic location of the electrode bundle at the time of implantation was 1 mm lateral to midline (right = 5 animals; left = 5 animals), 10.5 to 11 mm caudal to bregma, and 10.5 to 12 mm below bregma (skull zero). A single ground electrode was implanted in the subcutaneous layers of the neck. The hole in the skull was filled with rehydrated absorbable gelatin powder (Gelfoam,

Pharmacia & Upjohn) then covered with dental acrylic. The dental acrylic head plate was shaped to secure the microdrive and the electrode plug and to incorporate the 4 screws.

Experimental procedures: *General.* All experiments were performed on conscious rabbits accustomed to sitting quietly in a box that restricted their movement (33 X 15 X 18 cm). Animals were fasted for 15 – 20 hours prior to each experiment. We performed experiments as often as every day in individual rabbits. On the day of the experiment, rabbits were heparinized (sodium heparin, 2000 units, iv, Elkins-Sinn), the arterial catheter was connected to a pressure transducer, and the electrode plug was connected to a flexible cable. Rapid, manual inflation of the intrathoracic vena caval occluder was performed three times a week during recovery, and subsequently on each experimental day to free the balloon of connective tissue, and "warm up" the silastic balloon prior to slow inflation using a syringe pump (Sage Instruments Model 351). Arterial and venous blood pressure and extracellular neuronal activity were monitored on an oscilloscope and recorded using commercially available software (Spike2; CED). An audio speaker was used to monitor neuron activity. Heart rate was triggered on-line by pulsatile arterial pressure. Heart rate and blood pressure were allowed to stabilize before each experiment. The experimental protocol we used involved recording neuronal responses to simulated hemorrhage, then to iv pressor and depressor agents, and, finally, recording cardiovascular responses to electrical stimulation of recording sites.

Multiple neuron extracellular recording. We recorded the voltage difference across a pair of microwire electrodes. A custom-built amplifier (10X; incorporated into head plug) and switch box allowed us to select electrode combinations displaying neuron activity. The presence of one or more spikes with greater than 2:1 signal-to-noise ratio was required for recording from a particular electrode combination. Neuron activity was amplified (gain 1000 – 10,000), filtered (0.5 – 10 KHz bandpass) and digitized (1401 system, CED). We acquired one or two channels of neuron activity at 10 – 20 KHz.

Simulated hemorrhage. A typical simulated hemorrhage experiment is illustrated in Figure 1. The inflation rate of the vena caval occluder was adjusted to mimic changes in arterial pressure and heart rate observed during blood loss in conscious rabbits (115;116;119). When mean arterial pressure fell to 40 mmHg, inflation of the vena caval occluder was reduced to maintain mean arterial pressure $\leq$ 40 mmHg for 1 minute. Pressure in the occluder was then released allowing arterial pressure to return rapidly to normal. We divided the experiment into 4 periods: 1) Baseline (not shown in Figure 1); 2) Control; 3) Occlusion; and 4) Hypotension. Baseline was a 1 minute period during which we recorded neuronal activity prior to initiating inflation of the vena caval occluder. This 1 minute period was used to characterize Baseline firing frequency of the neuron(s) and assess the presence of arterial pulse modulated activity. The syringe pump was on during the Control period but there was no apparent change in heart rate or blood pressure. The period defined as Occlusion began at the point when heart rate first started to rise but arterial pressure was

maintained. In some animals, in which we monitored central venous pressure, the change in central venous pressure immediately preceding the rise in heart rate was used as the start of Occlusion. The end of Occlusion was defined as the time when mean arterial pressure fell to 40 mmHg. Hypotension was the 1 minute period when mean arterial pressure remained $\leq$ 40 mmHg.

Neuron barosensitivity. Some neurons were tested for sensitivity to pharmacologic changes in blood pressure. Cardiovascular variables and neuronal activity were recorded for 1 minute prior to and 2 minutes following iv injection of a pressor (phenylephrine 10 µg/kg, Sigma) or depressor (sodium nitroprusside, 0.1 mg/kg, Fisher Scientific) or nitroglycerin, 80 µg/kg, Nitrostat, Parke-Davis) agent. All injection volumes equaled 0.2 ml/kg and were followed by 2 ml of 0.9% sodium chloride flush.

Electrical stimulation. Electrical stimulation consisted of a 10 second train of 500 µsec biphasic pulses (100 Hz; 25, 50, 75 and 100 µA) through a single microelectrode (32). Cardiovascular variables, but not neuronal activity, were recorded for 1 minute prior to electrical stimulation. Adequate time for blood pressure and heart rate to stabilize was allowed between each stimulus train (at least 30 seconds).

Histology. The end of the electrode track was marked using the Prussian blue reaction. Iron ions were deposited at the site of the electrode tip (50 µA DC for 30 seconds; brain electrode positive). Rabbits were euthanized with an overdose of sodium pentobarbital (3 ml, iv, Sleepaway, Fort Dodge Animal Health) and perfused transcardially with 250 ml of 0.9% sodium chloride followed

by 1 liter of 15% potassium ferrocyanide in 10% formaldehyde. The brain was removed and stored in 10% formaldehyde followed by 15% sucrose in 10% formaldehyde. Frozen sections (40 to 60 μm) were stained with neutral red and evaluated every 100 μm. The blue dot, or bottom of the electrode track, was used as the reference to back-calculate the location of recording and stimulation sites. A horizontal line bisecting the cerebral aqueduct was used to define the border between the dlPAG and vlPAG. The shape of the PAG, and anatomical landmarks (e.g. red nucleus, and rostral and caudal colliculi) were used to determine the relative rostrocaudal location of each electrode track (Figures 4 and 7) (129).

Data analysis: *Spike sorting and neuron discrimination*. We used commercially available software (Spike2, CED) to generate templates of spike waveforms off-line. The threshold for spike detection was set at twice the noise level. Principal component analysis was applied to the templates and used to aid in spike discrimination. Templates were considered to represent single unit activity if principal component analysis yielded a discrete cluster and the interspike interval was greater than 1 millisecond (84). In cases where the interspike interval was less than 1 millisecond and spikes could not reliably describe the activity of a single unit, the templates were considered to represent a population of neurons with similar waveforms. In this study we describe the activity of single units and multiple-unit populations and refer to both as “unit” activity.

Response of neurons to simulated hemorrhage. The average firing frequency for each of the three periods Control, Occlusion and Hypotension were compared (Table 1). Additionally, the firing frequency of all units was closely evaluated for the presence of patterns consistent with a role in triggering the onset of hypotension (i.e. a change in firing frequency immediately prior to the drop in blood pressure during the period of Occlusion; gray arrow in Figure 1).

Pulse modulation of neuronal activity. We used cross-correlation histograms of unit activity triggered by heart rate to visually assess the existence of arterial pulse modulation during the Baseline period. Pulse modulation was defined as the presence of visible peaks in the cross-correlation histogram at the same frequency as heart rate (Figure 2) (92).

Neuron barosensitivity. Only units that changed firing frequency during simulated hemorrhage were evaluated for sensitivity to pharmacologic changes in blood pressure. We used waveform and interspike interval histogram characteristics to verify the presence of the same unit in the record, and compared unit activity before and after injection of the pressor and depressor agents.

Response to electrical stimulation. The peak change in diastolic arterial blood pressure associated with electrical stimulation ($\leq$ 100 μA) was used to determine the cardiovascular response to stimulation. A change in diastolic arterial pressure was defined as a change greater than 10 mmHg.

Statistical analysis. We used *t*-tests and two way repeated-measures ANOVA with Fisher's least significant difference test to compare unit

characteristics between the dlPAG and vlPAG, and the effects of simulated hemorrhage on unit activity within the dlPAG and vlPAG. Significance was set at $P < 0.05$. Data are presented as mean ± SE unless otherwise noted.

RESULTS

General. In this study, intrathoracic vena caval occlusion reliably produced changes in blood pressure and heart rate characteristic of phase 1 and phase 2 of hypotensive hemorrhage (e.g. Figure 1) (118). As stated previously (see Methods), the simulated hemorrhage experiments were divided into 4 periods: Baseline, Control, Occlusion, and Hypotension. Baseline mean arterial blood pressure (66 ± 1 mmHg) was not significantly different from Control (66 ± 1 mmHg). Baseline heart rate (138 ± 3 bpm) was slightly, but significantly different from Control (141 ± 3 bpm). During Hypotension period, mean arterial blood pressure was 30 ± 1 mmHg and heart rate was 235 ± 3 bpm. The duration of Occlusion, defined as the time from when heart rate began to rise to the point when mean arterial pressure fell to 40 mmHg, was 95 ± 7 seconds (median=84 seconds; range=12 to 215 seconds).

Response of neurons to simulated hemorrhage. We successfully recorded extracellular activity in the midbrain of conscious rabbits for up to 18 months following instrumentation. Vena caval occluders failed in 2 of the 10 rabbits. Thus, we were able to evaluate the response of PAG neurons to simulated hemorrhage in 8 rabbits. A total of 84 PAG units (3 – 23 units per

rabbit) were evaluated: 62 vlPAG units and 22 dlPAG units from 58 recording sessions.

Figure 3 illustrates the variety and range of changes in firing frequency of vlPAG and dlPAG units recorded during simulated hemorrhage. Some units displayed bursting activity (*A-1, B-1*), others fired in rhythmic bursts (*B-3;* 1 Hz), and some fired at a relatively constant rate (not shown in Figure 3). The firing frequency and/or rhythmicity of some units changed dramatically during the protocol (*A-2, B-2,3*), while others changed gradually over the entire protocol (*B-4*). In an effort to identify patterns of neuronal response to simulated hemorrhage, we categorized units as those that: 1) increased (Figure 3A); 2) decreased (Figure 3B); or 3) did not change firing frequency (not shown) over the periods of Control, Occlusion and Hypotension.

Firing frequency changed during simulated hemorrhage in 41 of 62 vlPAG (66%) and 17 of 22 dlPAG (77%) units. There was no statistical difference in the proportion of dlPAG and vlPAG units that changed activity. There was also apparent difference in rostrocaudal distribution of recording sites where units changed firing frequency in response to simulated hemorrhage (Figure 4). A change in firing frequency prior to the fall in arterial pressure would be consistent with a role of a vlPAG unit in triggering this event (see gray arrow Figure 1). Only 2 vlPAG units showed this pattern. Two dlPAG units also exhibited this pattern. In contrast to our hypothesis, the majority of vlPAG units that changed activity during simulated hemorrhage did so after the onset of hypotension (23 of 41; 56%).

Within the vlPAG, 22 units increased, 19 decreased, and 21 units did not change activity during simulated hemorrhage. The proportional distribution was similar in the dlPAG with 7 units increasing, 10 decreasing, and 5 units not changing activity during simulated hemorrhage. Table 1 describes the mean firing frequency of vlPAG and dlPAG neurons that increased, decreased, or did not change firing frequency in response to simulated hemorrhage. Baseline firing frequency was not different from Control for any category or location. The firing frequency of units that increased activity during simulated hemorrhage was significantly higher than Control during Occlusion (dlPAG) or Hypotension (vlPAG). Likewise, the firing frequency of units that decreased activity was significantly lower than Control during Hypotension (vlPAG and dlPAG). The firing frequency of vlPAG units that did not change activity was significantly higher than the other categories during each time period.

Pulse modulation of neuronal activity (see Figure 2). A smaller proportion of vlPAG units (30 of 62; 48%) than dlPAG units (17 of 22; 77%) demonstrated arterial pressure pulse-modulated activity. The percentage of units that changed activity during simulated hemorrhage and demonstrated pulse modulated behavior was smaller in the vlPAG (16 of 41; 39%) compared with the dlPAG (14 of 17; 82%).

Neuron barosensitivity. Injection of phenylephrine was associated with an increase in mean arterial blood pressure of 41 $\pm$ 3 mmHg and a decrease in heart rate of 59 $\pm$ 7 bpm. Administration of nitroprusside or nitroglycerin produced a decrease in mean arterial blood pressure of 20 $\pm$ 3 mmHg and an

increase in heart rate of 166 ± 9 bpm. We tested the 41 vlPAG and 17 dlPAG units that changed firing frequency during simulated hemorrhage for barosensitivity. Only 2 vlPAG units appeared to be sensitive to pharmacologic changes in arterial pressure (decreases only). No further quantitative analyses were performed.

Response to electrical stimulation. Electrical stimulation of the vlPAG and dlPAG was performed in 8 rabbits (not identical to the 8 used for simulated hemorrhage). Diastolic arterial blood pressure was increased ≥ 10 mmHg in response to electrical stimulation (≤ 100 μA) at 48 of 51 vlPAG sites (Figure 5) and 23 of 25 dlPAG sites. There was no change in blood pressure with electrical stimulation at the remaining 5 sites. The rostrocaudal distribution of blood pressure responses to electrical stimulation is illustrated in Figure 6. In addition to changes in blood pressure, we were able to evaluate the heart rate response to electrical stimulation at 48 vlPAG and 25 dlPAG sites. Electrical stimulation was associated with arterial pressure wave irregularities in some rabbits that, in the absence of electrocardiographic evidence, were suggestive of arrhythmias. Heart rate decreased by more than 30 bpm at 23 vlPAG and 16 dlPAG sites where electrical stimulation elicited an increase in blood pressure. Stimulation-induced increases in blood pressure were associated with an increase in heart rate at 16 vlPAG and 4 dlPAG sites. At 4 vlPAG and 3 dlPAG sites electrical stimulation through different electrodes produced opposite changes in heart rate (an increase and a decrease) even as blood pressure increased. No change in heart rate was noted at 2 vlPAG sites where blood pressure increased. A

decrease in heart rate of greater than 30 bpm, in the absence of blood pressure changes, was noted in response to electrical stimulation of 3 vlPAG and 2 dlPAG sites. In summary, electrical activation of both the vlPAG and dlPAG was predominantly associated with hypertension and bradycardia. Most notably, electrical stimulation of the vlPAG did not result in hypotension in our conscious rabbits.

Other observations. While no decrease in blood pressure was noted in response to electrical activation of the vlPAG or dlPAG, a striking behavioral change was occasionally observed. At 14 vlPAG and 14 dlPAG sites where we recorded the cardiovascular responses to electrical stimulation, we observed vigorous foot stomping well after the conclusion of the electrical stimulation. The latency for this behavior (time from end of stimulus to start of foot stomping) was longer in the vlPAG (43 $\pm$ 8 sec) than in the dlPAG (19 $\pm$ 2 sec). In several cases this behavioral change lasted longer than 2 minutes, and in one rabbit foot stomping persisted for 6 minutes. There was no apparent pattern of distribution of sites where this behavior was elicited (data not shown).

DISCUSSION

During blood loss in a variety of conscious animals, arterial pressure is initially maintained (phase 1) by sympathetic vasoconstriction (118). However, after a critical blood loss, there is a rapid decline in arterial blood pressure accompanied by a simultaneous decrease in sympathetic nerve activity (phase 2). The increase in sympathetic activity during phase 1 is clearly due to

unloading of arterial baroreceptors (118). While it is generally assumed that the sympathoinhibition of phase 2 is responsible for the decrease in arterial pressure, the central nervous system origin of this sympathetic modulation is not clear.

Recent studies in anesthetized (27) and conscious rats (18;19) have suggested that the site of the trigger for sympathoinhibition and the resultant decrease in arterial pressure during phase 2 may be the vlPAG. In anesthetized rats, inactivation of the vlPAG with muscimol, a gamma-aminobutyric $acid_A$ agonist, abolished the sympathoinhibition associated with hypotensive hemorrhage (27). In conscious rats, inactivation of the vlPAG with lidocaine or cobalt chloride (18) or blockade of delta opioid receptors with naltrindole (19) attenuated and/or delayed the phase 2 decrease in arterial pressure during blood loss. These results are consistent with a role for the vlPAG in triggering the onset of phase 2 during hemorrhage.

The purpose of this study was to evaluate changes in vlPAG neuronal activity during simulated hemorrhage in conscious, chronically prepared rabbits. If the vlPAG triggers phase 2, it seems reasonable to propose that some neurons in this structure should alter their activity prior to the fall in arterial pressure and the decrease in sympathetic nerve activity. In the present study, only 2 of 62 vlPAG units demonstrated a change in firing frequency consistent with a role in triggering the hypotension and sympathoinhibition. In addition, electrical activation of the vlPAG consistently produced increases rather than decreases in arterial pressure. Based on the vlPAG neuronal response to simulated hemorrhage, as well as the effects of electrical stimulation, it seems unlikely that

the vlPAG triggers the fall in blood pressure during hypotensive hemorrhage in conscious rabbits. In contrast, most of the recorded vlPAG neurons altered their activity after the onset of hypotension. This latter pattern is consistent with neurons in the vlPAG responding to, rather than causing, the rapid and profound fall in arterial pressure.

The apparent lack of agreement between earlier studies and the present results could be due to differences in the animal preparations (e.g. species, anesthesia, etc.). However, it also appears that a simple reexamination of the timing of sympathoinhibition and hypotension during hemorrhage in the various studies may directly address the differences. In conscious rats (122) and rabbits (14;45;87), the fall in blood pressure parallels the decrease in sympathetic nerve activity. In contrast, in anesthetized rats, the decrease in sympathetic activity appears to lag the fall in blood pressure during hemorrhage (5;27;131;145). The length of this delay is significant, and in several cases appears to be greater than 60 seconds (5;27;131). In addition, some investigators have observed an increase in renal sympathetic nerve activity coincident with the onset of hypotension (131;145). Thus, while this earlier data in anesthetized rats is consistent with a role for the vlPAG in producing sympathoinhibition, it is apparently not consistent with the resulting sympathoinhibition causing the hypotension. Rather, it appears to suggest that under anesthesia the fall in blood pressure triggers the sympathoinhibition.

Studies assessing the role of serotonin in cardiovascular control during hemorrhage may also shed light on the timing of hypotension and

sympathoinhibitory events. Intracerebroventricular administration of methysergide (a mixed serotonin agonist/antagonist) (122) or a specific 5-HT_{1A} receptor agonist (121) appears to uncouple the relatively tight temporal association of sympathoinhibition and hypotension during blood loss. Central administration of either of these compounds in conscious rats leads to increased sympathetic nerve activity while blood pressure is falling, and a delayed decrease in sympathetic activity (122). Thus, the results from the present study, as well as previously published results, open the possibility that sympathetic withdrawal during hemorrhage may involve more than one active neural pathway. For example, our results are entirely consistent with a potential sympathoinhibitory role for the vlPAG once blood pressure has fallen, since the majority of vlPAG neurons altered their firing rate after the development of hypotension.

Classically, the vlPAG and dlPAG have been described as functionally discrete longitudinal columns (6;7). Activation of the vlPAG is characterized by hypotension and bradycardia, compared with hypertension and tachycardia resulting from activation of the dlPAG (72). Surprisingly, we were unable to distinguish between the vlPAG and dlPAG based upon cardiovascular responses to electrical stimulation. In addition, foot stomping, a defensive behavior in rabbits, could be elicited by electrical activation of both areas. However, the latency of foot stomping was significantly longer in the vlPAG compared to the dlPAG. Others have described behavioral quiescence evoked by activation of

the vlPAG in sharp contrast to active defense behaviors resulting from chemical activation of the dlPAG (6).

Activation of the vlPAG has yielded inconsistent results relative to a clear role in triggering sympathoinhibition during hemorrhage. Many investigators have observed that chemical activation of the vlPAG in anesthetized rats produces hypotension, bradycardia (17;48;59;73) and sympathoinhibition (5). In conscious rats, however, chemical activation of the vlPAG has produced bradycardia with (19) and without hypotension (86). Electrical activation of the vlPAG in anesthetized rats has been reported to produce: hypotension and bradycardia (51); no consistent change in blood pressure or heart rate (72); or increases in arterial pressure and sympathetic activity (143). Species differences may exist, since in anesthetized rabbits, electrical activation of the vlPAG produced elevations in arterial pressure and bradycardia (78), and we regularly observed a marked pressor response to electrical stimulation of the vlPAG in our conscious rabbits.

While there is some inconsistency in the reported cardiovascular effects of vlPAG stimulation, results from stimulation of the dlPAG are more consistent. Chemical or electrical activation of the dlPAG consistently produces cardiovascular changes suggestive of a defense response (i.e. hypertension and tachycardia) (72). In addition, previous work has suggested that the vlPAG has an inhibitory effect on the dlPAG (73). What then might be the result of the interaction of these two areas during blood loss? We have recently reported (112) that stressful stimuli, such as air jet, that produce a cardiovascular defense

response (i.e. increased arterial pressure and tachycardia) extend the blood loss necessary to produce hypotension. Thus, if the vlPAG normally inhibits the dlPAG, inactivation of the vlPAG (18;27) during hemorrhage might have the same effect as sensory stressors. That is, by disinhibiting the dlPAG, blood pressure would be maintained longer in the face of continuing blood withdrawal. Thus, the earlier results reported by Cavun and Millington (18) and Dean (27) might be consistent with vlPAG inactivation affecting the response to hemorrhage in a similar way to sensory stressors (115). However, because these investigators (18;27) both used time (as opposed to arterial pressure) as an end-point during their hemorrhage experiments, it is difficult to assess whether inactivation of the vlPAG in their studies delayed or prevented the transition to phase 2.

Based upon the positive response of individual units to simulated hemorrhage, we predicted baroreflex modulation of their activity. On the contrary, we were unable to demonstrate clear barosensitivity of most PAG units. Other characteristics of neuronal activity within the vlPAG differed from dlPAG in two ways. First, a smaller percentage of neurons in the vlPAG (48%) compared to the dlPAG (77%) demonstrated pulse-modulated activity. This suggests a greater degree of cardiovascular modulation of neuronal activity in the dlPAG. Our results are similar to a previous report that 76% of primarily dlPAG neurons demonstrated cardiovascular modulation in conscious cats (92). Secondly, the mean firing frequency of vlPAG neurons that did not change their activity during

simulated hemorrhage was significantly higher than the same category of neurons within the dlPAG. The significance of this finding is unknown.

We were unable to identify firing characteristics of neurons within the vlPAG or dlPAG that would enable us to identify antemortem the site of recording. We can predict that units with very high firing frequencies will be less likely to respond to simulated hemorrhage than those units with slower rates of discharge (see Table 1). In the future, a longer time period may be allowed to assess resting neuronal activity (5 – 15 min). This longer time period may make it possible to identify firing characteristics that are unique to different regions within the PAG similar to what has been done with serotonergic cells in the pontomedullary region of the rat (79).

A necessary limitation of the present study was the use of simulated hemorrhage rather than actual blood removal. These two hypotensive stressors are not identical. In contrast to progressive blood loss, intra-abdominal venous pressure increases as the intrathoracic balloon is inflated. In addition, in our hands the time course for the development of hypotension differs between vena caval occlusion and progressive blood loss. Experimental blood withdrawal typically takes longer. Both these factors may contribute to differences in central neuronal processing of the respective hypotensive stimuli. However, Ludbrook and coworkers (76) compared intrathoracic vena caval occlusion with blood withdrawal for producing hypotensive hemorrhage and reported similar changes in blood pressure, heart rate, vascular conductance, and plasma levels of neurohumoral agents (76). In our own laboratory, we have compared vena caval

occlusion and blood loss in conscious rabbits. We recorded comparable changes in heart rate, arterial blood pressure and vascular conductance (unpublished observations). It seems likely that the rapid fall in blood pressure seen during vena caval occlusion is driven by the same central process that triggers hypotension during blood withdrawal.

In summary, while the activity of vlPAG neurons changes during simulated hemorrhage, the change is not consistent with a role for these neurons in triggering phase 2 sympathoinhibition. The midbrain may well play an important role in modulating the sympathetic and cardiovascular responses to blood loss (140). However, it seems more likely that the role of the vlPAG may be in maintaining hypotension and sympathoinhibition, rather than triggering the transition from phase 1 to phase 2.

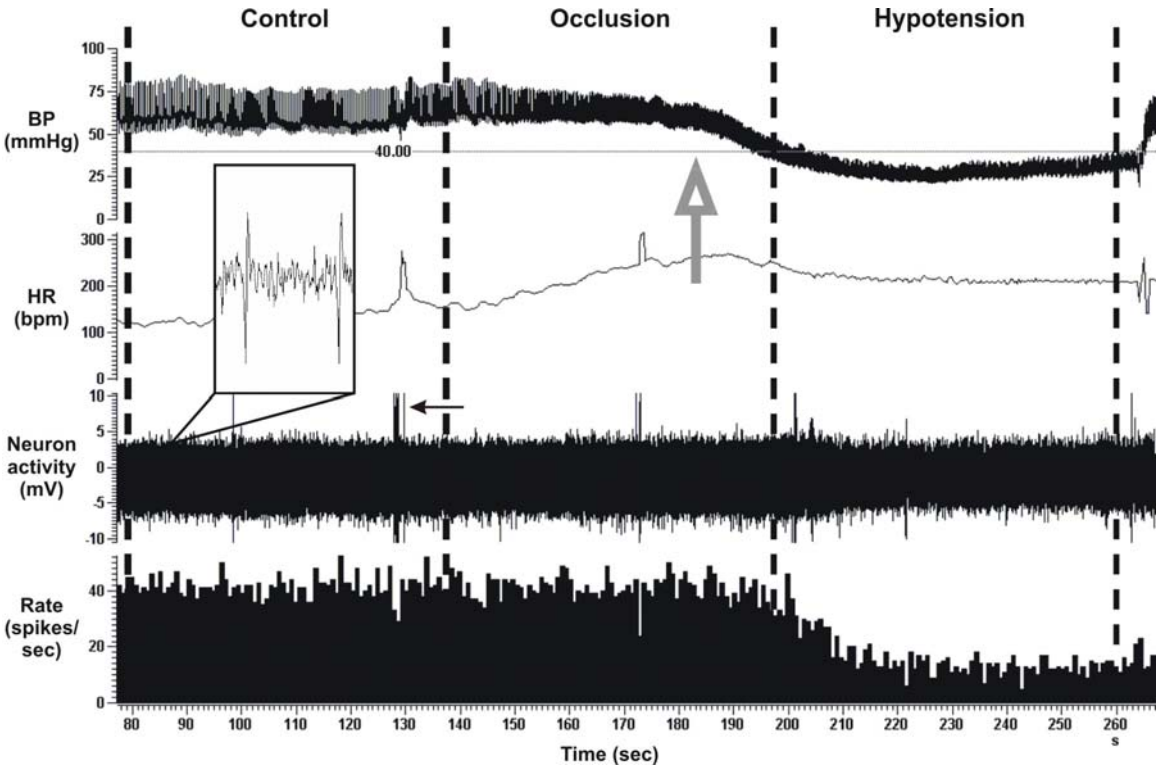

Figure 1. Experimental record illustrating changes over time in pulsatile arterial blood pressure (BP), heart rate (HR), raw neuronal activity (Neuron activity), and the rate histogram (Rate) of the single unit present within the neuronal record (inset) during a simulated hemorrhage experiment. The black arrow indicates one of several movement artifacts. The neuronal response to simulated hemorrhage was determined by comparing the average firing frequency of the three periods shown: Control, Occlusion and Hypotension. If neurons in the vlPAG trigger sympathoinhibition and hypotension during hemorrhage, we would expect their activity to change prior to the fall in blood pressure (gray arrow). The unit represented in this record changes firing frequency after the onset of hypotension. Note the rapid rise in BP following release of the balloon occluder after the period of hypotension.

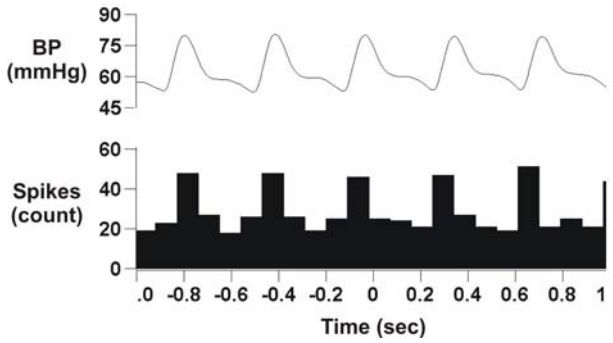

Figure 2. Illustration of arterial pulse modulation of PAG unit activity. The arterial blood pressure (BP) trace overlays the cross-correlation histogram of PAG unit activity triggered by heart rate (Spikes). The peaks in the histogram occur at the same frequency as heart rate demonstrating pulse-modulated PAG unit activity.

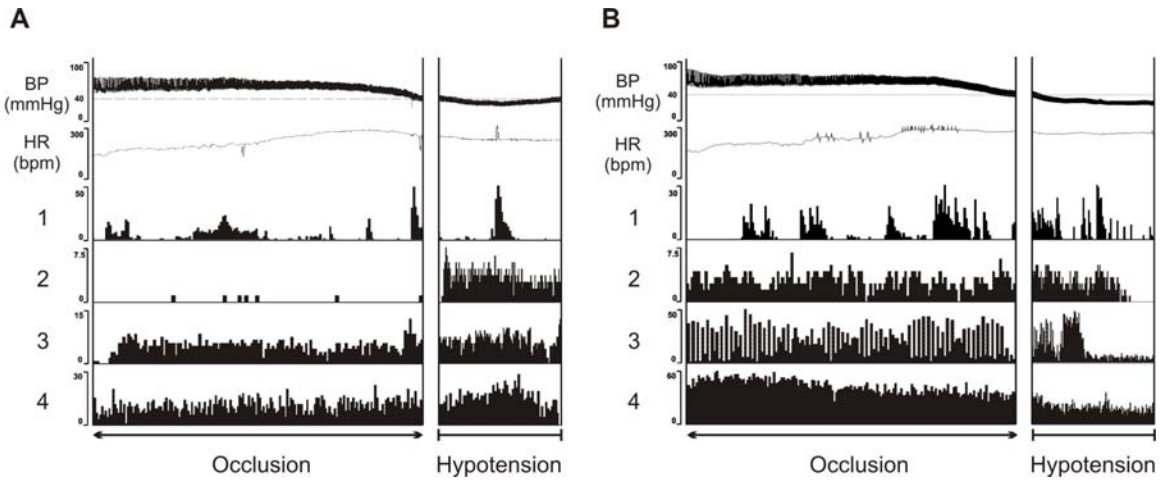

Figure 3. This figure illustrates the observed diversity of vlPAG and dlPAG unit responses to simulated hemorrhage. The top two traces represent arterial blood pressure (BP) and heart rate (HR), and the bottom four panels represent rate histograms for 4 units (1 – 4). Eight separate experiments in 6 different rabbits are represented. Units that quantitatively increased (*A*) or decreased (*B*) activity by more than 20% of Control over the course of the simulated hemorrhage experiment are illustrated. The Control period is not shown. The BP and HR traces were obtained during the individual experiments that yielded the units represented in *A-1* and *B-1*. The time scale for the period of Occlusion in *A-2,3,4* and *B-2,3,4* is normalized to the first example (*A-1, B-1*), while the time period for Hypotension is 1 minute in all examples. Examples of both vlPAG (*A-1,2,4; B-2,3*) and dlPAG units (*A-3; B-1,4*) are displayed.

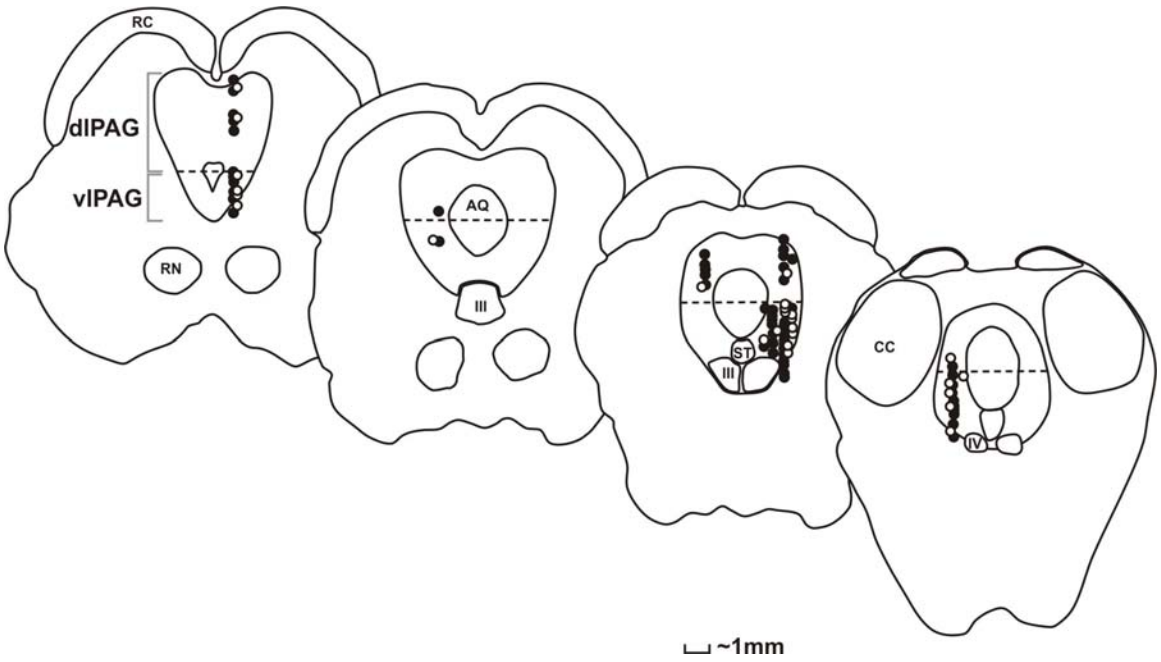

Figure 4. Rostrocaudal distribution of the 62 vlPAG and 22 dlPAG units evaluated in this study drawn on representative transverse sections of the rabbit midbrain (129). There was no apparent difference in distribution of units that changed (filled circles) or did not change (open circles) activity during the simulated hemorrhage experiment. The PAG was subdivided into the dlPAG and vlPAG using a horizontal line that bisected the cerebral aqueduct (AQ). CC, caudal colliculus; RC, rostral colliculus; RN, red nucleus; ST, supratrochlear nucleus; III, nucleus of the cranial nerve III; IV, nucleus of cranial nerve IV.

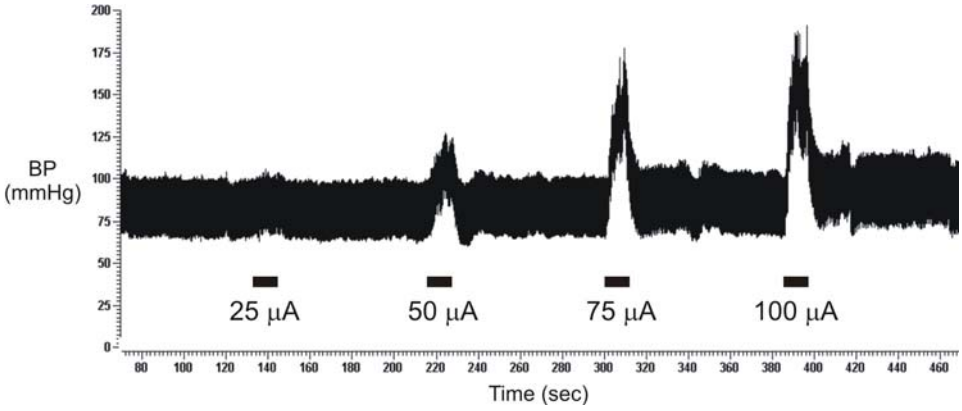

Figure 5. Pulsatile arterial blood pressure (BP) during electrical activation of the vlPAG. Bars represent the 10 second duration of stimulus; numbers indicate the current intensity. This record illustrates a typical intensity-dependent increase in BP in response to electrical stimulation within the vlPAG.

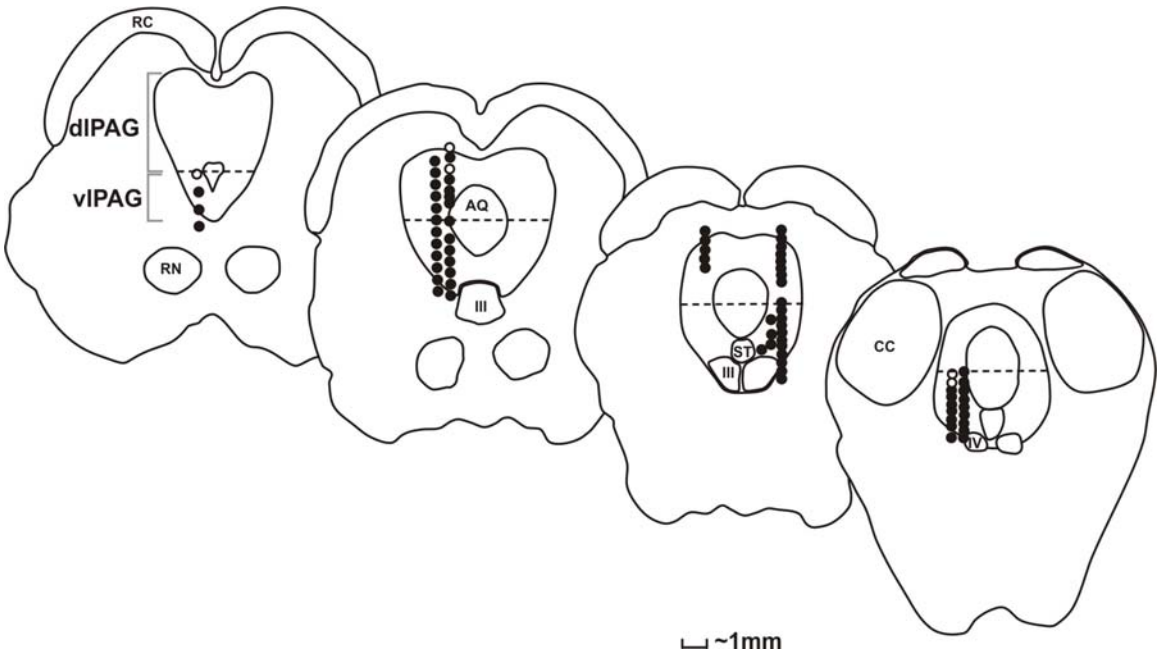

Figure 6. Rostrocaudal distribution of the 51 vlPAG and 25 dlPAG sites of electrical stimulation drawn on representative transverse sections of the rabbit midbrain (129). Filled circles represent 48 vlPAG and 23 dlPAG sites where electrical stimulation increased diastolic arterial blood pressure greater than 10 mmHg. Open circles represent the 5 sites where blood pressure did not change in response to electrical stimulation. No hypotension was seen in response to electrical activation of the PAG in this study. See Figure 4 for abbreviations.

Table 1. Firing frequency (spikes/sec; mean ± SE) of 3 categories of units within the vlPAG and dlPAG during simulated hemorrhage

Location *Category*	*Baseline*	*Control*	*Occlusion*	*Hypotension*
vlPAG				
Increase (22)	9 ± 1	9 ± 1	10 ± 1	13 ± 1‡
Decrease (19)	13 ± 1	12 ± 1	10 ± 1	6 ± 1‡
No change (21)	33 ± 1¥	34 ± 1¥	35 ± 1¥	34 ± 1¥
dlPAG				
Increase (7)	10 ± 3	10 ± 3	20 ± 3*	15 ± 3
Decrease (10)	22 ± 3	21 ± 3	19 ± 3	9 ± 3‡
No change (5)	10 ± 4	9 ± 4	10 ± 4	10 ± 4

Number of units in parentheses. * Different from Baseline and Control; ‡ Different from Baseline, Control and Occlusion; ¥ Different from Increase and Decrease; $P < 0.05$

CHAPTER 3

Periaqueductal gray neurons respond to intero- and exteroceptive sensory stimuli in conscious rabbits

ABSTRACT

As an integrative site for autonomic control, the midbrain periaqueductal gray (PAG) receives both internal and external sensory inputs. The purpose of this study was to evaluate multi-sensory modulated neuronal activity in the PAG. We hypothesized that the activity of individual PAG neurons is modified by both intero- and exteroceptive sensory stimuli. Six rabbits were chronically instrumented with arterial and venous catheters and midbrain microwire electrodes on a microdrive. The responses of PAG neurons to changes in arterial blood pressure, cardiopulmonary chemoreceptor stimulation, and external alerting visual, auditory and tactile stimuli were recorded in conscious rabbits. Interoceptive stimuli were intravenous boluses of phenylephrine, sodium nitroprusside and phenylbiguanide. Exteroceptive stimuli (light, sound, air puff) were presented in a random order and separated by > 60 s. Neuronal firing frequency over a 10 s period immediately preceding and following each stimulus was compared. A 40 percent change in peak firing frequency was considered a response. Arterial pulse modulated activity was evaluated during a 60 s control

period. Pulse modulated activity was apparent in 18 of 25 dorsolateral PAG and 16 of 29 ventrolateral PAG neurons. The majority of PAG neurons responded to one or more intero- and exteroceptive sensory stimuli, and of these, the majority also displayed pulse modulated activity. This study demonstrates that PAG neurons in conscious rabbits respond to both internal and external stimuli. Our results are consistent with an integrative role for the PAG in cardiovascular control.

INTRODUCTION

Homeostasis is the active and ongoing process of maintaining optimal physiologic function in dynamic internal and external environments. Interoceptors and exteroceptors are sensory receptors that detect changes in the internal and external environments, respectively. Afferent sensory input is relayed from these receptors to the central nervous system where integration of sensory inputs leads to a coordinated physiologic and behavioral response with the goal of maintaining homeostasis. Alerting and noxious stimuli, as well as internal stressors such as blood loss can all signal a potential threat to survival and, thus, trigger autonomic and behavioral changes. Therefore, it is not surprising that anatomical and functional overlap exists between central nervous system circuits that process internal and external stressful sensory stimuli.

Multiple lines of evidence suggest that the midbrain PAG is one structure capable of integrating multiple sensory inputs. The PAG receives diverse afferent input from sensory (e.g. dorsal horn; sensory cortex), motor (e.g. motor

cortex), limbic (e.g. amygdala), autonomic (e.g. nucleus of the solitary tract) and endocrine centers (e.g. hypothalamus) (82). Outputs from the PAG are no less diverse, suggesting that the PAG may be involved in modulating and expressing complex autonomic and behavioral functions (83). The functional roles of the PAG have been characterized in multiple species and grouped into five major areas including 1) pain processing and modulation, 2) autonomic regulation, 3) defense behavior, 4) vocalization and 5) sexual behavior (8;82;83). Thus, PAG structure and function suggests that it may be important in maintenance of homeostasis through coordination of autonomic and behavioral responses to sensory stimuli. This study focuses on the response of PAG neurons to a variety of sensory stimuli. We hypothesized that individual neurons within the PAG would alter their firing rate in response to multiple intero- and exteroceptive sensory stimuli.

METHODS

Animals and instrumentation. All procedures were approved by the University of Missouri Animal Care and Use Committee, and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (50). Six New Zealand white rabbits (2 female) weighing 3.0 $\pm$ 0.1 kg (mean $\pm$ SE) were chronically instrumented with catheters, diaphragmatic electromyographic (dEMG) electrodes, and midbrain recording electrodes.

Catheters, dEMG and midbrain electrodes. Each rabbit underwent two surgical procedures: 1) laparotomy for catheter and dEMG electrode placement;

and 2) craniotomy for implantation of midbrain recording electrodes. Two rabbits underwent a third surgery (thoractomy) for placement of dEMG electrodes. All surgeries were separated by at least 2 weeks. Antibiotics (22.7 mg enrofloxacin, sc; Baytril, Bayer) were administered the day prior to surgery. Food but not water was withheld 15 – 20 hr prior to surgery. The analgesic buprenorphine hydrochloride (Buprenex, Reckitt Benckiser, 0.06 mg, sc) was administered to all rabbits for perioperative analgesia.

For catheter implantation, anesthesia was induced and maintained with halothane in oxygen via face mask (5% for induction, 1 – 3% for maintenance). A midline laparotomy was performed for implantation of intra-abdominal venous (vena cava) and arterial (aorta) catheters (44). We used the abdominal venous and arterial catheters for injection of drugs and recording of arterial pressure, respectively. EMG electrodes were implanted in the diaphragm (126). Catheters and EMG wires were tunneled subcutaneously and exteriorized at the base of the neck.

Intravenous sodium pentobarbital was used for induction and maintenance of anesthesia for thoracotomy and craniotomy. Following induction with pentobarbital, the rabbit was intubated and mechanically ventilated with room air.

Extracellular neuronal recording electrodes were placed as described previously (120). Briefly, the rabbit's head was mounted in a stereotaxic frame (David Kopf) and leveled with lambda fixed at 1.5 mm below bregma. A bundle of 10 electrodes attached to a microdrive (81) was implanted in the midbrain through a midline craniotomy. The stereotaxic location of the recording

electrodes at the time of implantation was 1.0 – 1.2 mm lateral to midline (right = 3 animals; left = 3 animals), 10 to 11 mm caudal to bregma, and 10 to 12 mm below bregma (skull zero). One turn of the microdrive advanced the electrode bundle 0.3 mm. Rabbits were allowed to recover a minimum of 2 weeks after the last surgery before the start of experiments.

Experimental procedures. All experiments were performed on conscious rabbits accustomed to sitting quietly in a box that restricted their movement (33 X 15 X 18 cm). Animals were fasted for 15 – 20 hr prior to each experiment. We performed experiments as often as every day in individual rabbits. On the day of the experiment, rabbits were heparinized (sodium heparin, 2000 units, iv, Elkins-Sinn), the arterial catheter was connected to a pressure transducer, and the electrode plug was connected to a flexible cable. Arterial blood pressure and extracellular neuronal activity were monitored on an oscilloscope and recorded using commercially available software (Spike2, CED). An audio speaker was used to monitor neuronal activity. Heart rate was triggered on-line by pulsatile arterial pressure. Heart rate and blood pressure were allowed to stabilize before each experiment. Baseline neuronal activity was recorded during a 1 – 5 min control period prior to initiating sensory stimulation. After the control period, we recorded the neuronal responses to interoceptive sensory stimuli and then responses to exteroceptive sensory stimuli.

Sensory stimulation. Three interoceptive stimuli were performed in the following order: phenylephrine (10 μg/kg, Sigma), sodium nitroprusside (0.05 mg/kg, Fisher Scientific) and phenylbiguanide (100 μg/kg, Aldrich Chemical Co.).

All intravenous injection volumes equaled 0.2 ml/kg and were followed by 2 ml of heparinized (10 U/ml) 0.9% sodium chloride flush. Cardiovascular and respiratory parameters were recorded for 1 min prior to and 2 min following all injections.

Four exteroceptive sensory stimuli were delivered in a random order separated by at least 30 sec: Light, Sound, Air and Sham. The visual stimulus was a single flash of a xenon strobe light. The acoustic stimulus was a short burst of sound (1 sec, 10 Hz). The tactile stimulus was a puff of air (1 sec, 25 psi) directed towards the rabbit's nose through a hole in the rabbit box.

Data acquisition and analysis. The voltage difference across a pair of microwire electrodes was recorded. A custom-built amplifier (10X; incorporated into head plug) and switch box allowed us to select electrode combinations displaying neuron activity. The presence of one or more spikes with greater than 2:1 signal-to-noise ratio was required for recording from a particular electrode combination. Neuron activity was amplified (gain 1000 – 10,000), filtered (0.5 – 10 KHz bandpass) and digitized (1401 system, CED). We acquired one or two channels of neuron activity at 20 KHz.

We used commercially available software (Spike2, CED) to generate templates of spike waveforms off-line. The threshold for spike detection was set at twice the noise level. Principal component analysis was applied to the templates and used to aid in spike discrimination. Templates were considered to represent single neuron activity if principal component analysis yielded a discrete cluster and the minimum interspike interval was greater than 1 msec (84).

Pulse modulation of neuronal activity. We constructed cross-correlation histograms of neuronal activity triggered by heart rate and peristimulus time histograms of heart rate triggered by heart rate during the control period. These histograms were overlayed and assessed visually for the existence of arterial pulse modulation. Pulse modulation was defined as the presence of visible peaks in the cross-correlation histogram coincident with or at a constant distance from peaks in the heart rate histogram (Figure 3) (92).

Histology. The end of the electrode track was marked by depositing iron ions at the tip of one electrode (50 μA DC for 30 seconds; brain electrode positive)(120). Following euthanasia, rabbits were perfused transcardially with 0.9% sodium chloride followed by 15% potassium ferrocyanide in 10% formaldehyde to produce a blue reaction product at the end of the recording electrodes. This blue dot was used as a reference to back-calculate the location of recording sites. Brains were removed and stored in 10% formaldehyde followed by 15% sucrose in 10% formaldehyde. Frozen sections (40 to 60 μm) were stained with neutral red and evaluated every 100 μm. The border between the dorsolateral (dl) and ventrolateral (vl) PAG was defined by a horizontal line bisecting the cerebral aqueduct. The shape of the PAG, and anatomical landmarks were used to determine the relative rostrocaudal location of each electrode track (Figure 1) (129).

RESULTS

We recorded the response of 25 dlPAG and 29 vlPAG neurons to multiple intero- and exteroceptive sensory stimuli in conscious, chronically instrumented rabbits. These neurons were located within the midsection of the rostrocaudal extent of the PAG (Figure 1). During the control period, the mean firing frequency of neurons within the dlPAG (8.4 $\pm$ 1.5 Hz) was less than that of neurons within the vlPAG (21.0 $\pm$ 5 Hz) (P=0.03). The median firing frequency was 5.4 Hz and 10 Hz for dl- and vlPAG neurons, respectively. There was a wider range of firing frequency among vlPAG neurons (Figure 2).

During the control period preceding intero- and exteroceptive sensory stimulation, neuronal activity was evaluated for pulse modulated activity. Figure 3 is an example of a pulse-triggered histogram illustrating changes in firing frequency co-incident with pulsatile arterial pressure. The majority of neurons within the dlPAG (18 of 25, or 72%) and vlPAG (16 of 29, or 55%) demonstrated pulse modulated activity.

An example of a single vlPAG neuron that responded to all three interoceptive stimuli is shown in Figure 4. In the example shown, the neuron increased its firing frequency in response to each of the three stimuli. Figure 5 is a raw experimental record from a different rabbit illustrating a dlPAG neuron that fired intensely immediately after the burst of sound and air puff but not after the flash of light. The number of neurons within the dl- and vlPAG responding to specific intero- and exteroceptive sensory stimuli is illustrated in Figure 6. A few neurons responded to single stimuli, while most responded to more than one

stimulus within a modality (intero- or exteroceptive). The nature of the response of neurons to multiple stimuli was varied. For example, some neurons increased firing frequency in response to one stimulus and decreased firing frequency in response to a second, and vice versa. The majority of neurons in the dl- and vlPAG responded in a similar fashion to multiple stimuli (e.g. increased or decreased in response to multiple stimuli). Six dlPAG and 6 vlPAG neurons showed mixed responses (e.g. increases and decreases in firing frequency) to interoceptive stimuli. Three dlPAG and 1 vlPAG neuron showed mixed responses to exteroceptive sensory stimuli.

Figure 6 does not show the overlap between neurons that responded to both intero- and exteroceptive stimuli. Instead, Figure 7 illustrates the percentage of neurons responding to intero- and/or exteroceptive stimuli. The majority of dl- and vlPAG neurons responded to at least one intero- and exteroceptive sensory stimulus. Less than 20 percent of neurons within the PAG were not responsive to either intero- or exteroceptive stimuli.

DISCUSSION

Integration of sensory inputs is critical to coordinated autonomic and behavioral responses to internal and external sensory stimuli. Functional and anatomical evidence demonstrates that the PAG receives and integrates sensory information and coordinates behaviors. Thus, we predicted that neurons within the PAG would respond to a variety of sensory stimuli. We are not aware of other studies evaluating the response of individual neurons within the PAG to

intero- and exteroceptive sensory stimuli. We recorded from neurons in the PAG of conscious, chronically instrumented rabbits that were exposed to changes in arterial pressure, cardiopulmonary chemoreceptor stimulation, and alerting visual, auditory, and tactile stimuli. The important new finding of this study is that neurons within the PAG respond to both intero- and exteroceptive sensory stimuli.

Chemical or electrical stimulation has provided functional evidence for organization of the PAG into longitudinal columns. Specifically, excitatory amino acid microinjections into the dlPAG have elicited tachycardia, hypertension, non-opioid analgesia, and active defense behaviors. In contrast, excitatory amino acid activation of the vlPAG has evoked bradycardia, hypotension, opioid-mediated analgesia, and decreased behavioral activity (6;53;141). Labeling of neuronal projections with antero- and retrograde tracers has demonstrated further differences in regional distribution of afferent inputs into columns within the PAG. Both the dlPAG and vlPAG receive a variety of somatosensory and visceral afferents (e.g. somatosensory cortex, nucleus of the solitary tract, spinal cord). The vlPAG appears to receive the largest afferent input from autonomic sites, such as the hypothalamus (82). In contrast, the largest amount of afferent input to the dlPAG comes from the vlPAG, and appears to be inhibitory in nature (60;82). Efferent information from the dlPAG and vlPAG project to major cardiovascular, respiratory and anti-nociceptive regions in the midbrain and medulla (e.g. rostral and caudal ventrolateral medulla, ventral respiratory group, raphe nuclei) (6;83). Studies quantifying c-Fos, a gene product expressed

following synaptic activation, have reported differential activation of neurons within the PAG following interoceptive sensory stimulation. For example, sustained hypotension appears to preferentially activate cells in the vlPAG of rats (88). In contrast, sustained hypertension activated the dlPAG in rats, and failed to activate cells in the vlPAG in rats (88) and rabbits (65).

Based upon functional and anatomical evidence for integration of cardiovascular inputs in the PAG, we were not surprised to find that the majority of neurons within the dl- and vlPAG demonstrated pulse modulated activity. The percentage of neurons with pulse modulated activity is consistent with those reported elsewhere (92;120). What was surprising was the apparent lack of baroreflex modulated neuronal activity. Nine dlPAG and 12 vlPAG neurons responded in some way to phenylephrine and sodium nitroprusside (see Figure 6). These two interoceptive stimuli increase and decrease arterial blood pressure, respectively, and engage the baroreflex. One would expect baroreflex modulated neurons to respond to both increases and decreases in arterial pressure with increases and decreases in firing frequency, or vice versa. However, only 2 dl- and 3 vlPAG neurons displayed changes in firing frequency consistent with being baroreflex modulated. Of these, four increased firing frequency following an increase in arterial pressure and decreased firing frequency following a decrease in arterial pressure. The remaining potentially baroreflex modulated neuron decreased firing frequency following an increase in arterial pressure and increased firing frequency in response to decreased arterial pressure. Despite the fact that few neurons in the PAG displayed baroreflex

activity, the prevalence of efferent projections from the PAG to cardiovascular nuclei (83) suggests that neurons in this midbrain region may be involved in integration of cardiovascular inputs. That is to say that PAG neurons may integrate sensory inputs from multiple interoceptors rather than specifically responding to changes in arterial pressure in a pattern associated with baroreflex activation.

Determining specific sensory stimuli that neurons in a particular brain region respond to is one step towards antemortem identification of neuronal location. The functional columnar organization of the PAG is supported by studies that have demonstrated differential activation of dl- and vlPAG neurons in response to changes in arterial pressure (65;88). We identified two consistent differences between dl- and vlPAG neurons. First, the spontaneous firing frequency of vlPAG neurons is higher than that of dlPAG neurons. Second, more dlPAG neurons demonstrate pulse modulated activity. Unfortunately, these characteristics are not specific for neurons within the vlPAG or dlPAG, respectively. For example, there is considerable overlap of the distribution of neuronal firing frequency within the dl- and vlPAG (see Figure 2). Also, a substantial percentage of vlPAG neurons are also pulse modulated. It is possible that by limiting the present study to neurons with spontaneous activity we may have missed important differences in PAG neuronal responses to intero- and exteroceptive stimuli.

To our knowledge, this is the first study to demonstrate that neurons in the PAG respond to both internal and external sensory stimuli in conscious

chronically instrumented animals. Others have successfully classified hypothalamic paraventricular neurons in conscious rats as vasopressin-secreting based upon single neuron responses to intero- and exteroceptive stressors (146). We hope that further evaluation of neuronal responses to multiple sensory stimuli will aid in functional, antemortem identification of neurons within the anatomically described columns of the midbrain periaqueductal gray.

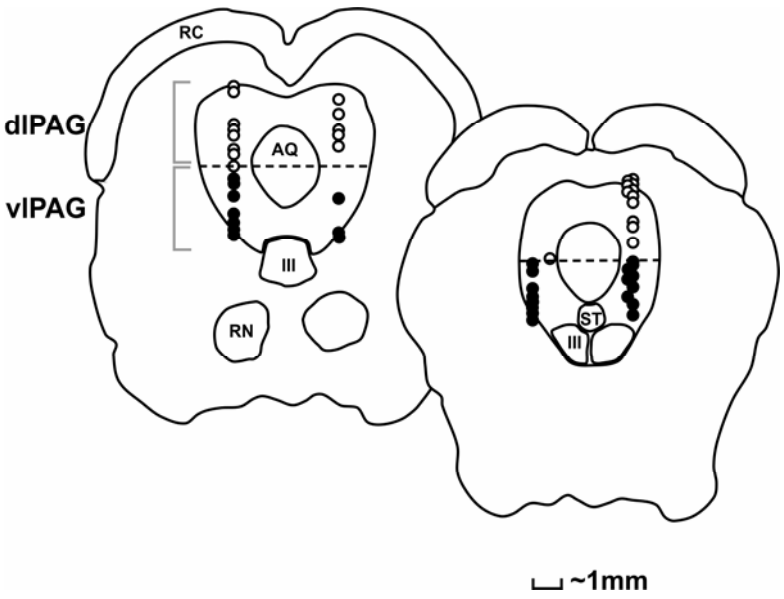

Figure 1. Rostrocaudal distribution of the 25 dlPAG (open circles) and 29 vlPAG (filled circles) neurons evaluated in this study drawn on representative transverse sections of the rabbit midbrain (129). The dashed line indicates the boundary between the dl- and vlPAG. AQ, cerebral aqueduct; RC, rostral colliculus; RN, red nucleus; ST, supratrochlear nucleus; III, nucleus of cranial nerve III.

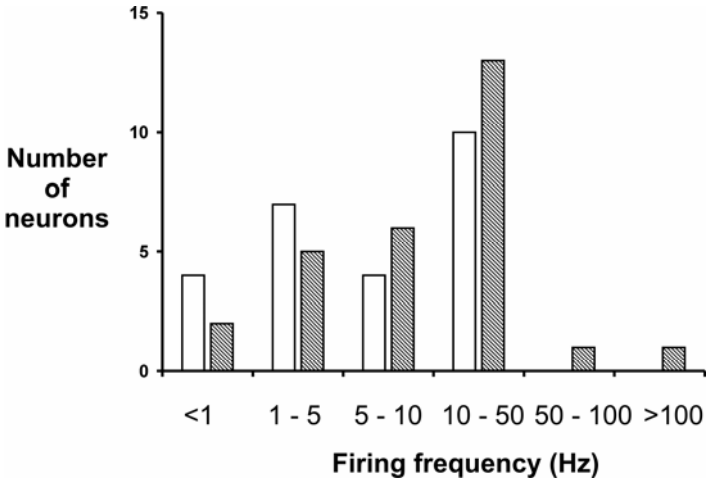

Figure 2. Firing frequency of neurons within the dlPAG (open bars) and vlPAG (cross hatched bars). The distribution of control firing frequency within a range of firing frequencies is shown on the x-axis. The number of neurons within each category is shown on the y-axis.

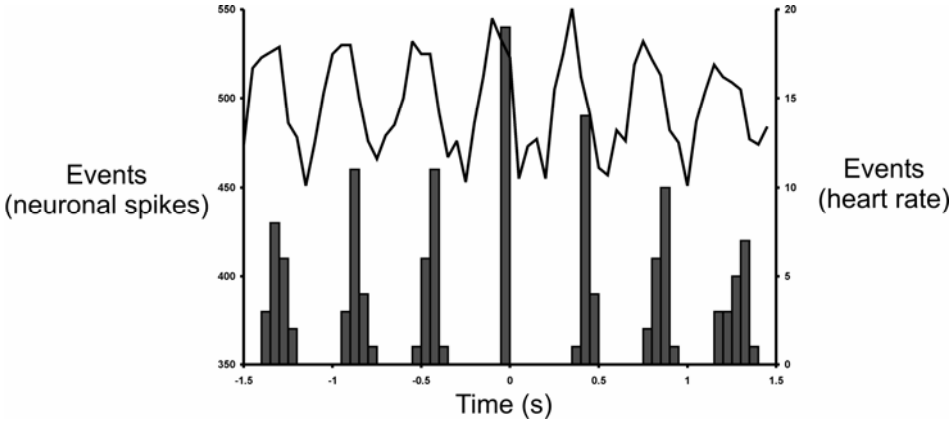

Figure 3. Example of pulse modulated activity in a single dlPAG neurons. Top trace is the arterial pulse-triggered average of neuronal activity during the control

period. Bars are the heart rate histogram. The change in neuronal firing frequency is coincident with heart rate, thus demonstrating pulse modulated activity.

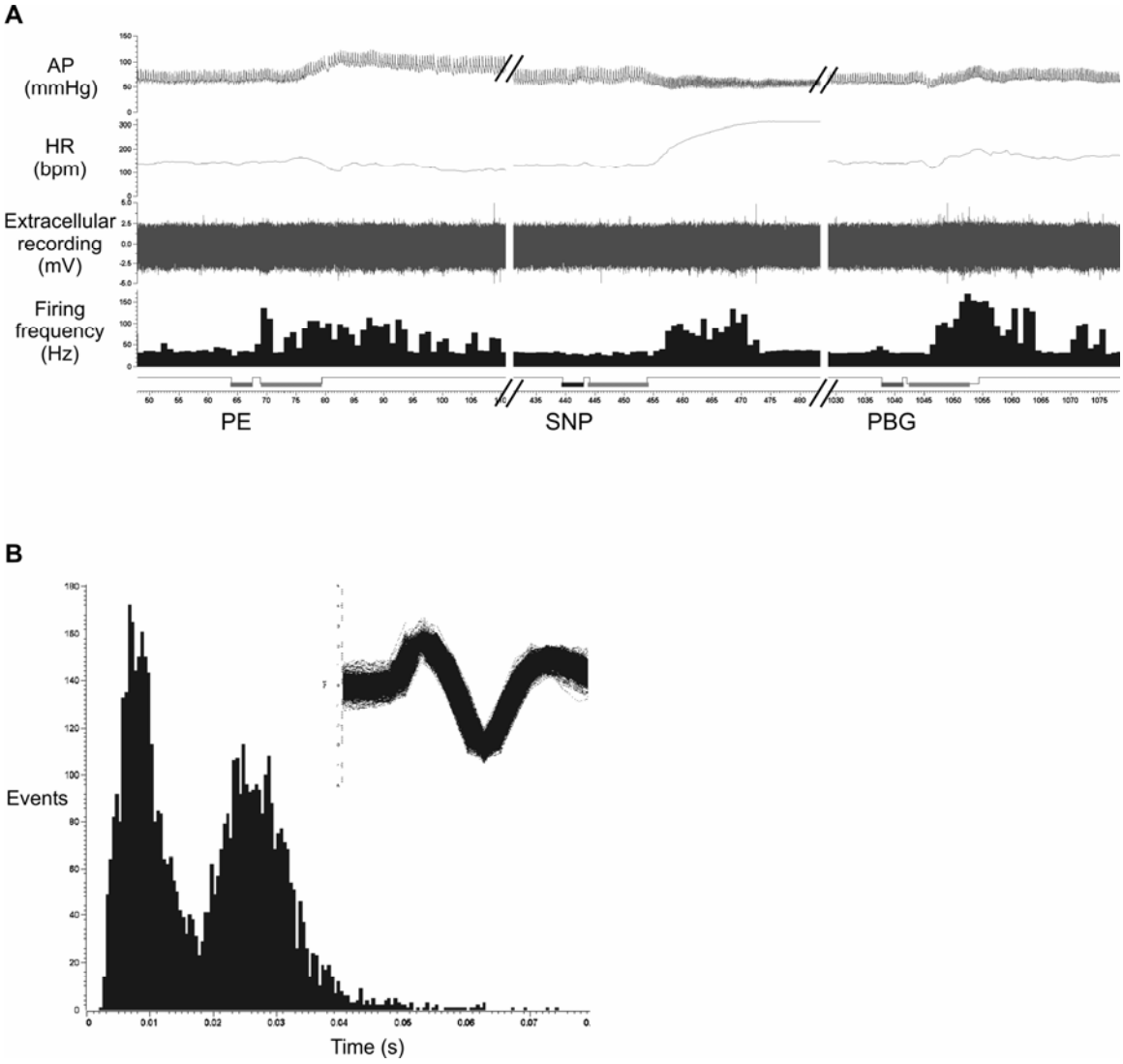

Figure 4. Response of a vlPAG neuron to interoceptive sensory stimuli. A) Changes in arterial pressure (AP), heart rate (HR), extracellular neuronal activity

(Extracellular activity) and neuronal firing frequency (Firing frequency) are shown prior to, and following intravenous injections of phenylephrine (PE), sodium nitroprusside (SNP) and phenylbiguanide (PBG). Neuronal firing frequency increased after all three interoceptive stimuli. B) Interspike interval histogram of the single neuron present in (A). Inset is the spike waveform overlay.

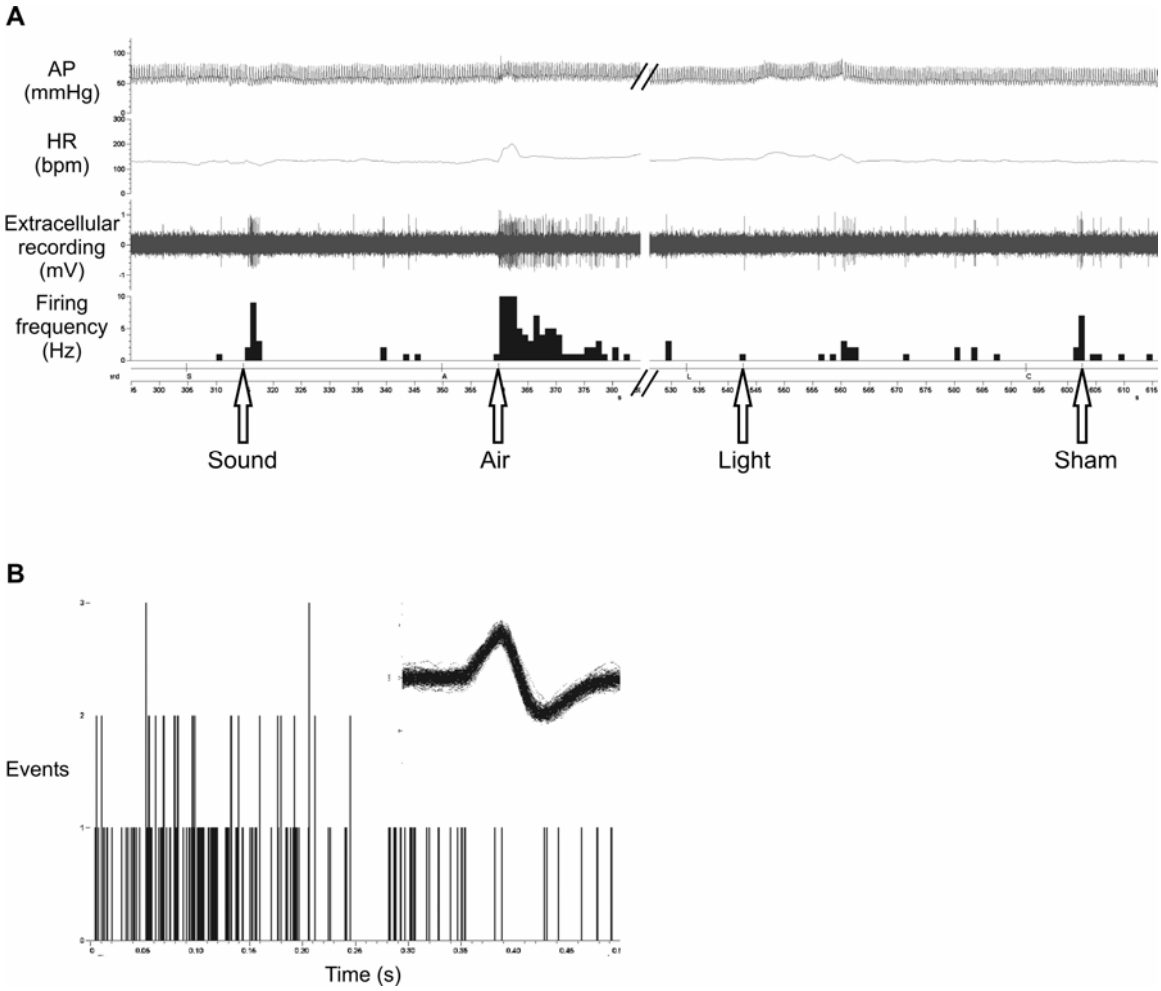

Figure 5. Response of a dlPAG neuron to exteroceptive sensory stimuli. A) Changes in arterial pressure (AP), heart rate (HR), extracellular neuronal activity

(Extracellular activity) and neuronal firing frequency (Firing frequency) are shown prior to, and following exposure to Sound, Air, Light and Sham stimuli. The arrows indicate the timing of each stimulus. Because the basal firing frequency of this neuron is slow, the increases in firing frequency after Sound and Air stimuli are visible in the raw extracellular recording. B) Interspike interval histogram of the single neuron present in (A). Inset is the spike waveform overlay.

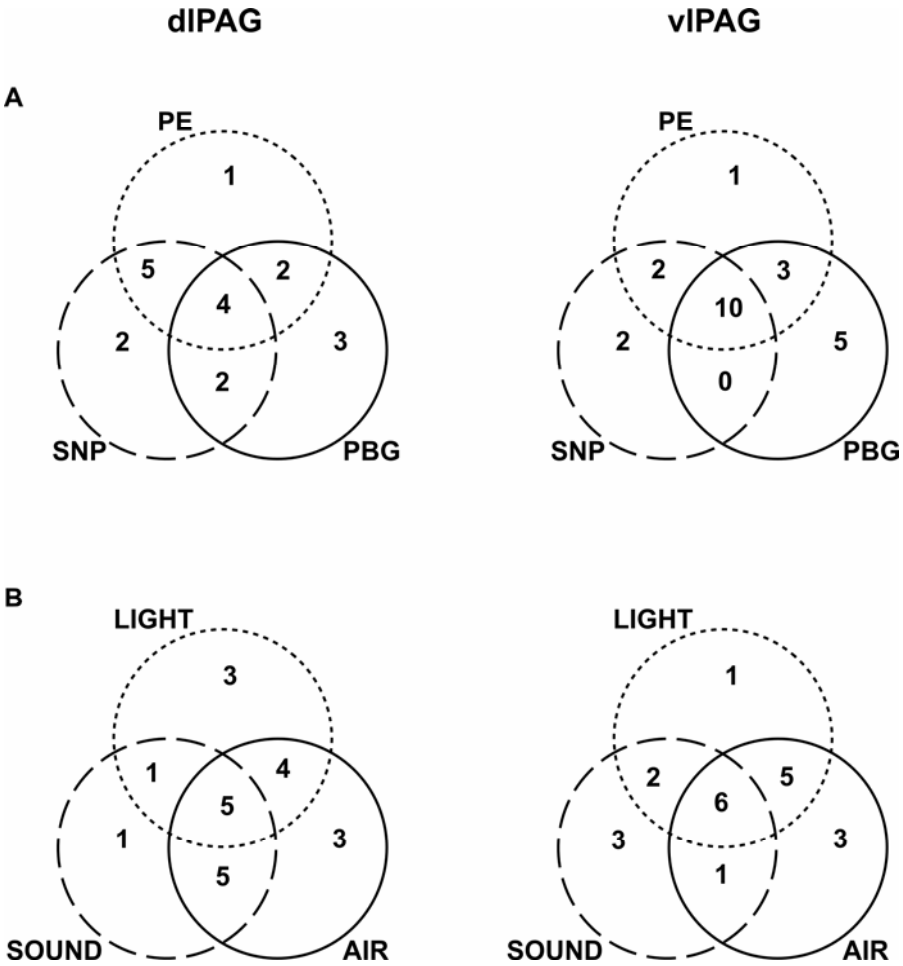

Figure 6. Number of dl- and vlPAG neurons responding to intero- and exteroceptive sensory stimuli. A) Number of neurons that responded by increasing or decreasing firing frequency following phenylephrine (PE), sodium nitroprusside (SNP) or phenylbiguanide (PBG) are represented within their respective circles. Numbers within overlapping circles indicate neurons that responded to more than one interoceptive stimulus. B) Number of neurons that responded to Light, Sound or Air stimuli are represented within their respective

circles. As in (A), overlapping circles indicated neurons that responded to more than one exteroceptive stimulus. For both intero- and exteroceptive stimuli, most PAG neurons responded to multiple sensory stimuli.

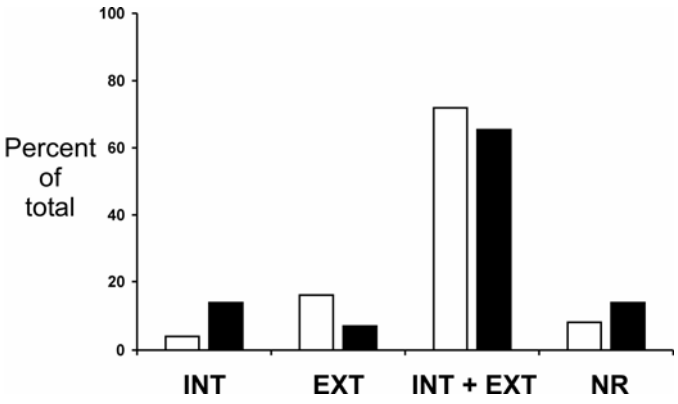

Figure 7. Percentage of PAG neurons responding to intero- and exteroceptive sensory stimuli. Percentage of dlPAG (open bars) and vlPAG (black bars) that responded to interoceptive (INT), exteroceptive (EXT), intero- and exteroceptive (INT & EXT) stimuli, and those that did not respond (NR) to any sensory stimuli are indicated.

CHAPTER 4

A novel electrode design for chronic recording of electromyographic activity

ABSTRACT

We describe a simple, self-retaining electromyography (EMG) electrode for use in chronic recording of EMG activity. The EMG electrode is helical in shape, resembling the screw-in fixation device on many cardiac pacing electrodes. Screw-like placement of the electrode secures it in the musculature without sutures. We have been using this electrode design to obtain months of quality diaphragmatic EMG recording in conscious rabbits. By changing the electrode wire size and coil dimensions, this design could be applied to chronic EMG recording in a variety of muscles and species.

INTRODUCTION

Electromyographic (EMG) activity in chronically instrumented animals is commonly used to describe changes in respiratory rate, sleep states, and abdominal motor responses to a variety of interventions. Chronic recording of EMG in conscious animals presents unique instrumentation challenges. For example, electrodes must be securely implanted and provide reliable, lasting

signal quality. We are interested in recording diaphragmatic EMG activity in conscious, chronically instrumented rabbits. To facilitate implantation in a thin, rhythmically contracting muscle, we sought to create an easy-to-construct electrode that would secure in the muscle without sutures and require minimal muscle exposure.

The recording electrode we describe has a helical shape similar to screw-in leads used for fixation of many cardiac pacemaker electrodes (85). The screw-in technique was developed to improve anchoring of pacing leads without the need for suturing. We have applied the screw-in fixation design to EMG recording electrodes and have successfully recorded diaphragmatic EMG in conscious rabbits for more than nine months. While this electrode was originally developed for use in the diaphragm, we have recently extended its application to chronic recording of EMG activity in abdominal muscles.

MATERIALS AND METHODS

EMG electrode fabrication

Approximately 7 mm of insulation is removed from a piece of multi-stranded silver-plated copper wire (29 gauge, Calmont Engineering & Electronics Corp., Santa Ana, CA). A short length (3 cm) of uninsulated stainless steel wire (0.01” diameter, California Fine Wire Co., Grover Beach, CA) that will ultimately become the recording electrode is inserted 1 – 2 mm under the insulation of the multi-stranded wire. The joint between the copper wire and the electrode wire is soldered (Figure 1 A). The solder joint is checked for security and covered with

heat-shrink tubing (Alpha Wire Corp., Elizabeth, NJ). The stainless steel electrode wire is wrapped counter-clockwise around a wire stylet (0.03” diameter, Small Parts Inc., Miami, FL) creating a right-handed thread of internal diameter 0.03” (Figure 1 B). In our hands, two turns of the electrode wire and a final electrode coil length of 2 mm are preferred for implantation in the diaphragm of 2.5 – 3.0 kg rabbits. The excess wire is removed by cutting the electrode at an angle where the wire is still coiled (Figure 1 C). This creates a sharp tip to aid in initial penetration of the muscle.

Ground electrode fabrication

Approximately 2 cm of insulation is removed from multi-stranded silver-plated copper wire (29 gauge, Calmont Engineering & Electronics Corp., Santa Ana, CA). The strands are wrapped around a cylindrical object (4 mm diameter), and twisted back around themselves to create a loop. The loop is filled with solder. A short length of heat-shrink tubing is placed over the junction between the wire insulation and the solder. The completed EMG and ground electrodes (Figure 1 C & D) are sterilized using a non-thermal method.

Surgical implantation

We use a midline laparotomy (halothane anesthesia) to implant arterial and venous catheters in the abdominal aorta and vena cava, respectively, of young adult New Zealand white rabbits (44). We use this abdominal approach for placement of chronic diaphragmatic EMG leads. Three EMG leads are implanted into the left ventrolateral diaphragm in a triangular configuration with leads approximately 1 cm apart. The third electrode serves as a reserve in the

event that one electrode becomes dislodged or nonfunctional. The heat-shrink tubing of the EMG electrode is grasped with a pair of hemostats and the electrode coil is screwed into the muscle. There is no need to suture the electrodes in place. Stress-relieving loops in each lead are left in the abdomen before passing the wires through the abdominal wall approximately 2 cm lateral to the midline incision. Knots are tied in the lead wires on either side of the abdominal wall to prevent excess motion of the wires. The ground electrode is secured to the external abdominal wall with sutures during closure of the abdominal incision. All leads are tunneled subcutaneously and exteriorized at base of neck.

RESULTS

During experiments, electrodes are connected to a custom-built amplifier(10X) placed close to the animal. Data is acquired at 4 – 10 kHz and high-pass filtered (200 Hz) to minimize ECG artifact. The signal baseline is set to zero and then rectified and integrated (time constant = 50 – 100 msec). Representative diaphragmatic EMG recordings obtained 4 and 9 months after electrode implantation from the same conscious rabbit are shown in Figure 2. The burst frequency is used to calculate respiratory rate. The peak amplitude and area under the curve of each inspiratory burst may be used as an index of tidal volume (34;47).

To date, our lab has successfully instrumented 32 rabbits with chronic diaphragmatic EMG electrodes. Quality recordings from electrodes have been

made for as long as 9 months after implantation (see Figure 2 B). Signal quality does not appear to deteriorate with time (Figure 2). At the time of euthanasia (2 – 9 months post-implantation), all rabbits have had functioning diaphragmatic EMG electrodes with a clear signal. Post-mortem examination of the diaphragm and electrodes has revealed neither muscle damage nor corrosion of the electrode tips. In two rabbits, one of the three diaphragmatic EMG electrodes was found dislodged from the diaphragm at necropsy. The steel coil of all other remaining electrodes has been found securely adhered to the diaphragm with connective tissue.

DISCUSSION

Simplicity has been advocated as a guiding principle in electrode design (70). We describe a simple, yet effective electrode design for chronic recording of EMG activity in conscious animals. The electrode is easy to construct and can be placed into muscles with limited accessibility. The unique coiled tip allows for simple, secure fixation without the need for sutures. This electrode provides high quality signal recordings with remarkable longevity.

Placement of our diaphragmatic electrode does not require entering the chest cavity as is the case with some other techniques (20). Our lab uses the abdominal approach to the diaphragm as we combine this procedure with laparotomy for placement of indwelling abdominal vascular catheters. While the abdominal approach eliminates the need for a thoracotomy, the electrodes could also be placed from within the chest cavity. We routinely place three electrodes

to insure against potential electrode failure over months of recording. This can be done at little cost in terms of construction or surgery time and has a large potential gain. Placement of three electrodes into the diaphragm has also allowed us to select the differential diaphragmatic EMG signal with the least ECG artifact.

By changing wire size or coil dimensions this electrode design may be applied to recording EMG activity in muscles other than the diaphragm or in other species. We have recently implanted electrodes into the internal abdominal oblique muscles using a coil of slightly longer dimensions. In summary, we feel this screw-in type electrode is simple, versatile and effective for recording EMG signals in chronically instrumented animals.

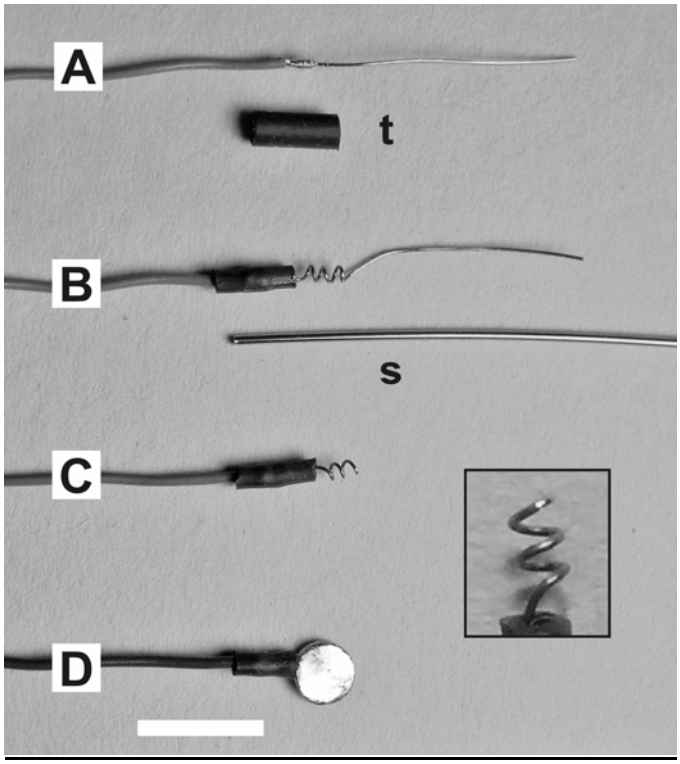

Figure 1. Photograph of EMG and ground electrodes in various stages of construction. (A) Multiple-strand insulated wire soldered to uninsulated stainless steel electrode wire with short piece of heat shrink tubing (t) to cover solder joint. (B) Electrode wire is wrapped counter-clockwise around a stylet (s) to create a right-handed thread. (C) Completed EMG electrode. (D) Ground electrode. Scale bar is 1 cm. Inset is enlarged view of coiled electrode tip.

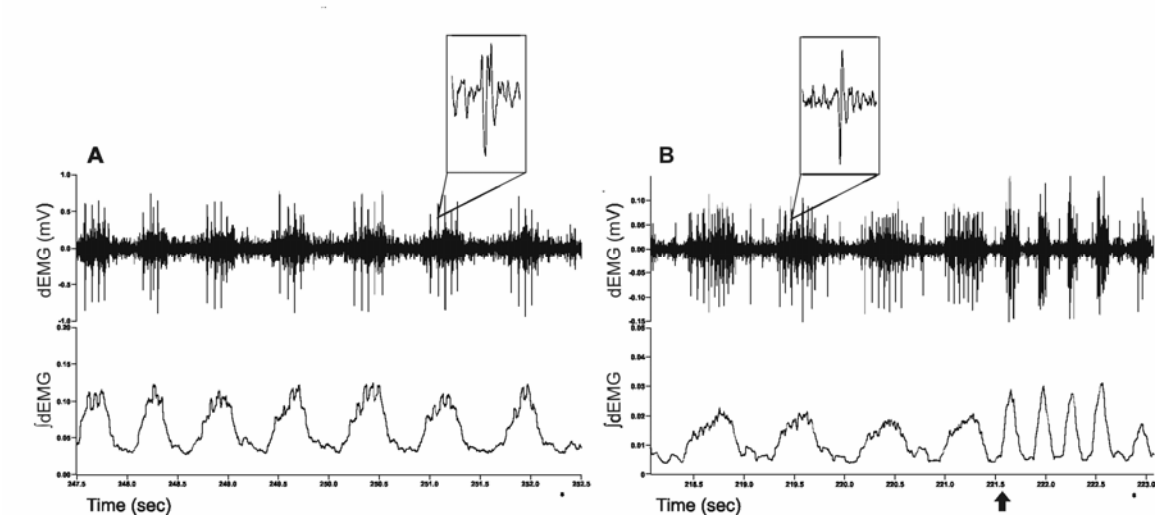

Figure 2. Five seconds of diaphragmatic EMG (dEMG) signal recorded in the same conscious rabbit 4 (A) and 9 months (B) after electrode implantation. Top panel shows high-pass filtered (200 Hz) dEMG signal. Bottom panel is the rectified and smoothed dEMG signal (time constant = 50 msec). Insets (200 msec) show a single motor unit present within the dEMG signal on each recording day. Signal is amplified 1000 times in A and 100 times in (B). Arrow in (B) indicates onset of sniffing.

CHAPTER 5

Evaluation and validation of a model of noxious stimulation in conscious rabbits

Our laboratory has been gathering data about cardiovascular, respiratory and central nervous system responses to hypotensive hemorrhage in conscious rabbits in an effort to improve understanding of, and therapies for, blood loss in all animals. Our research has explored the interaction of environmental stressors and the response to hemorrhage, and we have demonstrated that such stress alters cardiovascular control of arterial blood pressure during blood loss (114;117).

In addition to other environmental stressors, in the human and veterinary clinical setting, trauma leading to blood loss is associated with pain. While it is known that painful stimuli alter cardiovascular (1;11;12;97) and respiratory function (108), the effect of pain on the cardiovascular and respiratory response to hemorrhage in conscious animals is not well understood. To better understand the effect of pain on the integrated cardiorespiratory response to blood loss in conscious animals including humans, we sought to establish a model of noxious stimulation for use in conscious, chronically instrumented rabbits. This project, divided into two phases (Figure 1), was a first step toward

establishing a model of pain in rabbits associated with short-lived physiological responses that can be ameliorated by analgesic administration, and causes no lasting tissue damage. During Phase 1, noxious somatic and visceral stimuli were tested on anesthetized rabbits to evaluate physiological responses. If a painful stimulus caused temporary changes in cardiorespiratory parameters that could be attenuated by analgesics, and was not associated with tissue damage in Phase 1, the stimulus was tested in conscious rabbits during Phase 2. Phase 2 involved validation of the model through repeated testing of noxious stimuli in conscious rabbits. There were three objectives for each phase. The first objective was to evaluate the cardiovascular and respiratory responses to somatic and visceral noxious stimuli. The second objective was to evaluate the effect of the analgesic, buprenorphine, on the cardiovascular and respiratory response to noxious stimulation. Finally, the third objective was to evaluate tissue for gross evidence of damage resulting from noxious stimulation.

New Zealand White rabbits were used for these experiments because 1) their response to blood loss closely parallels that in humans; 2) their size allows the necessary instrumentation to be chronically implanted; and 3) they are docile animals that can be trained to sit quietly during experiments. Conscious, chronically instrumented rabbits have been used extensively in our laboratory to evaluate the integrated physiological response to blood loss, as well as potential interventions for the treatment of blood loss.

METHODS

Animals and instrumentation. All procedures were approved by the University of Missouri Animal Care and Use Committee. New Zealand White rabbits [3.5 ± 0.1 kg (mean ± SE)] were chronically instrumented with catheters and electromyographic (EMG) electrodes under halothane anesthesia. A midline laparotomy was performed for implantation of non-occlusive arterial and venous catheters which allowed monitoring of arterial pressure and injection of drugs, respectively. EMG electrodes (126) were implanted in the diaphragm (Phase 1 & 2) to record diaphragmatic muscle (dEMG) activity and allow monitoring of respiratory rate. All rabbits prepared for Phase 2 were instrumented with EMG electrodes in the ventral abdominal musculature, 2 cm lateral to midline, to record abdominal muscle (aEMG) activity. A single ground electrode was secured to the abdominal wall. Catheters and EMG leads were tunneled subcutaneously and exteriorized at the nape of the neck. Buprenorphine was administered for perioperative analgesia. For both Phase 1 and Phase 2, rabbits were allowed to recover for a minimum of two weeks before undergoing any experiments.

Noxious stimuli and selection criteria. *Visceral stimuli.* In this study, visceral stimulation refers to stimuli applied to the intestinal tract. We considered noxious visceral stimuli that have been used in conscious rabbits (25;54;55;95), horses (107;130), and human volunteers (43;75;90;144). Specifically, we evaluated the mechanical stimulus, colorectal distension, in the absence and presence of an inflammatory substance, acetic acid, instilled rectally. A double-

lumen catheter with a 5 cm balloon fashioned from the finger of a surgical glove (Figure 2) was lubricated and inserted 9 cm into the rectum. The balloon was progressively inflated using distilled water. Colorectal balloon pressure was measured continuously. A maximum balloon pressure of 90 mmHg was used to avoid tissue damage (55).

Somatic stimuli. In this study, somatic stimulation refers to stimuli applied to skin, subcutaneous tissues, and skeletal muscle. We considered noxious somatic stimuli that have been used in conscious rabbits (137), cats (103), and human volunteers (16;29;64;104;135;136). The following algesic substances were injected subcutaneously and intramuscularly: saline (0.9%, 5% and 9%), glutamate (0.1M, 0.5M and 1.0M), bradykinin (1 μg/ml, 10 μg/ml and 0.1 mg/ml), and capsaicin (5 μg/ml and 50 μg/ml). Subcutaneous injections of 1.0 ml were made over the craniolateral thorax. Intramuscular injections of 0.2 ml were made in the muscles overlying the thoracic vertebrae (e.g. epaxial muscles).

Selection criteria. We set the following criteria for selection of a noxious stimulus for use in our conscious rabbits. The noxious stimulus should be:

A. Titratable (i.e. variable intensity)

B. Short-lived (only present while the stimulus is “on”)

C. Associated with a change in blood pressure of 10 – 15 mmHg

D. Repeatable

E. Not associated with lasting tissue damage

F. Responsive to opioid analgesia, and

G. Feasible in a conscious, chronically instrumented rabbit.

Experimental procedures. *Phase 1*: Eight chronically instrumented rabbits (4 females) were anesthetized with halothane by mask. All animals were then acutely instrumented with abdominal EMG electrodes using barbed electrodes inserted through a needle. Diaphragmatic EMG electrodes were placed in animals with absent or poor dEMG signal quality. One animal required acute placement of a femoral arterial and venous catheters. Following acute instrumentation, the depth of anesthesia was adjusted to achieve a brisk withdrawal reflex in response to toe pinch. Heart rate, blood pressure, dEMG and aEMG activity were monitored throughout the experiment.

Heart rate and blood pressure were allowed to stabilize after checking the withdrawal response in both fore- and hindlimbs. Following a 1-min control period, brief colorectal distension was performed (30 sec – 3 min, up to 90 mmHg). Assessment of the withdrawal reflex was repeated and then somatic stimulation was performed. In one animal, 4 ml of 0.6% acetic acid were instilled in the distal colon, and colorectal distension was repeated one hour later. Intracolonic acetic acid did not appreciably alter the response to colorectal distension, so it was not performed in other rabbits. In 6 rabbits, buprenorphine was administered intravenously (iv) after initial testing of visceral and somatic stimuli. Ten or 20 min after buprenorphine administration, the visceral and somatic stimuli were repeated.

Rabbits were euthanized at the end of the experiment and necropsy was performed to evaluate tissues for gross signs of damage. The colorectal balloon

catheter was inflated with water and the volume required for colon rupture was noted. In addition, intramuscular placement of all EMG electrodes was verified after euthanasia.

Phase 2: Colorectal distension was the only noxious stimulus selected for further evaluation in conscious animals. Five male rabbits were chronically instrumented with arterial and venous catheters, and diaphragmatic and abdominal EMG electrodes. Acute placement of the colorectal balloon catheter in conscious rabbits was achieved by placing each rabbit on its back with its eyes covered. Rabbits become relaxed in 1 – 3 min and tolerate insertion of a well-lubricated balloon catheter into the rectum. The catheter was gently secured to the rabbit's tail. Following insertion of the colorectal balloon catheter, the rabbit was returned to the rabbit box (33 X 15 X 18 cm). All rabbits underwent training to accustom them to placement of the colorectal balloon catheter, and being in the rabbit box in the laboratory setting.

Based upon our results from 8 anesthetized rabbits (*Phase 1*, Figure 3), we expected colorectal distension to be associated with an increase in mean arterial pressure. The target end-point for adequate intensity of colorectal distension in the conscious rabbits was an increase in mean arterial blood pressure of 10 – 15 mmHg. Rabbits underwent progressively longer colorectal distensions over a 1 – 2 week period. Initially, the duration of the colorectal distension was 1 – 2 min, but this was increased progressively over 1 – 2 week period to 10 min total duration. The goal was to perform multiple 9.5 min colorectal distension experiments in each rabbit to assess reproducibility of the

cardiovascular and respiratory responses to colorectal distension. These experiments were performed no more than three days per week.

Rabbits underwent four 9.5 min colorectal distension experiments during a two week period. Food but not water was withheld for 15 – 20 hrs prior to each experiment. On the day of the experiment, the colorectal balloon catheter was placed and then rabbits were heparinized (sodium heparin, 2000 units, iv). The arterial and colorectal balloon catheters were connected to separate pressure transducers, and the EMG electrodes were connected to custom-built, differential amplifiers (10X). Blood pressure, heart rate, colorectal balloon pressure, dEMG and aEMG were monitored continuously during each experiment. A 30-sec test inflation (i.e. $\leq$ 20 mmHg) was performed at the beginning of each experiment to check for the ability to detect changes in pressure within the colorectal balloon.

We compared 30 sec averages for mean arterial pressure, heart rate, respiratory rate and balloon pressure at 4 time points: Baseline, Start and End of distension, and Release. Values were taken during a stable period prior to the test inflation (Baseline), during the first and last 2 min of the distension (Start and End of inflation), and within 2 min of release of pressure within the balloon (Release). Peak changes in arterial pressure and colorectal balloon pressure were also measured. To evaluate opioid-attenuation of the cardiovascular and respiratory response to colorectal distension, 4 rabbits underwent two separate experiments during which buprenorphine [60 μg (~20 μg/kg), iv] was administered 10 min prior to a 9.5 min colorectal distension. At the end of this

experimental series, rabbits were euthanized, and the distal colon was grossly evaluated for evidence of tissue damage.

Data acquisition and analysis. Data were acquired at 4 – 20 kHz using a PC-based data acquisition system (Power Lab, ADInstruments, Colorado Springs, CO, or 1401, CED, Cambridge, UK). Off-line analysis of all records was performed using commercially available software (Spike2, CED, Cambridge, UK). Heart rate was determined from the pulsatile arterial pressure signal. The dEMG signal was filtered (200 Hz) to minimize ECG artifact, the DC offset was removed, and then the signal was rectified and smoothed (time constant=50 msec). Respiratory rate was measured from the peaks in the dEMG activity. Data were evaluated subjectively in Phase 1. In Phase 2, a one-way repeated measures analysis of variance (ANOVA) was used to compare the effect of time (e.g. Baseline, Start, End, Release) or experiment number on measured variables (SigmaStat v3.1, Systat Software, Inc., Richmond, CA). The Bonferroni multiple comparison procedure was preformed for planned comparisons. Data are reported as mean $\pm$ SE. When possible, the SE was calculated from the pooled estimate of the population variance from the associated ANOVA.

RESULTS

Phase 1. Responses to noxious somatic stimuli in anesthetized rabbits were evaluated subjectively. Subcutaneous and intramuscular injections of hypertonic saline, glutamate, bradykinin and capsaicin produced variable changes in mean arterial pressure, heart rate, respiratory rate, and/or abdominal

EMG activity. Importantly, these substances did not produce short-lived responses (i.e. responses that were limited to the duration of the stimulus). The duration of the response to these substances was not quantified because it was deemed too long to be acceptable in a conscious rabbit. Finally, at necropsy, gross evidence of tissue damage associated with both subcutaneous and intramuscular injections was noted. Thus, no noxious chemical stimuli were evaluated further in conscious rabbits.

In halothane-anesthetized rabbits, colorectal distension produced changes in arterial pressure that appeared to be limited to the duration of the stimulus. Figure 3 is a representative experimental record illustrating the short-lived increase in arterial pressure during colorectal distension in an anesthetized rabbit. There were no noticeable changes in heart rate, respiratory rate or aEMG activity associated with colorectal distension in anesthetized rabbits.

Brief colorectal distension (30 sec to 3 min) with an average distension pressure of 68 $\pm$ 2 mmHg was associated with a 5 $\pm$ 1 mmHg increase in mean arterial pressure in anesthetized rabbits. Administration of the analgesic, buprenorphine, prior to colorectal distension blunted the increase in mean arterial pressure associated with colorectal distension (Figure 4).

While colorectal balloon pressure was not measured, inflation volumes of greater than 14 ml were required to rupture the colon at necropsy. Thus, in addition to the 90 mmHg colorectal balloon pressure limit, we set 12 ml as the maximum volume of distension in conscious animals.

Phase 2. As expected, the response to colorectal distension was amplified in conscious rabbits. Under anesthesia, colorectal distension of 68 ± 2 mmHg produced a 5 ± 1 mmHg increase in arterial pressure, whereas in conscious rabbits, colorectal distension of 54 ± 3 mmHg increased arterial pressure by 12 ± 1 mmHg.

Figure 5 is an example of the response to a 9.5 min colorectal distension in a conscious rabbit. It is possible to see that the increase in arterial pressure is maintained throughout the distension, and returns towards baseline following release of pressure within the balloon. The drop in respiratory rate occurs coincident with the increase in balloon pressure and arterial pressure, but remained below baseline levels after the end of the distension. Summary data from 9.5 min colorectal distension experiments in 5 rabbits is shown in Figure 6. Distension of the colon was associated with increases in mean arterial pressure, and decreases in respiratory rate. While mean arterial pressure returned to baseline levels following release, respiratory rate remained below baseline. While there was a significant effect of time on heart rate (P=0.03), the multiple comparison procedure failed to show statistical significance when comparing the Start (P=0.10) or End of distension (P=0.17) to Baseline. The time from peak colorectal balloon pressure to peak change in mean arterial pressure was 42 ± 8 sec.

Four colorectal distension experiments were performed in each rabbit during a two week time period. The results from these experiments are summarized in Table 1. Colorectal distension reproducibly increased arterial

pressure. It appears that baseline heart rate may have decreased over time (P=0.10). However, the results indicate that there were no statistically significant differences in cardiovascular or respiratory parameters among the four experiments.

Buprenorphine altered cardiovascular and respiratory parameters in conscious rabbits, as well as the cardiorespiratory response to colorectal distension (Figure 7). Arterial pressure was significantly higher 10 min after buprenorphine (75 $\pm$ 1 mmHg) compared with baseline (70 $\pm$ 1 mmHg). Heart rate was 150 $\pm$ 4 bpm at baseline, and remained unchanged at 10 min post-buprenorphine (150 $\pm$ 4 bpm). Buprenorphine administration reduced baseline respiratory rate from 214 $\pm$ 26 bpm to 42 $\pm$ 26 bpm.

Inflation of the colorectal balloon after administration of buprenorphine did not alter mean arterial pressure, heart rate or respiratory rate at the start of the distension (Figure 7). However, by the end of the 9.5 min distension experiments, arterial pressure was higher than post-buprenorphine values. After buprenorphine administration, heart rate and respiratory rate remained unchanged throughout the colorectal distension and following release.

In the absence of buprenorphine, the peak increase in arterial pressure associated with colorectal distension was 15 $\pm$ 2 mmHg. When colorectal distension was performed after administration of buprenorphine, the peak change in mean arterial pressure was 7 $\pm$ 2 mmHg. There was no difference in peak colorectal balloon pressure with (60 $\pm$ 3 mmHg) or without buprenorphine (63 $\pm$ 3 mmHg)(P=0.57). Thus, buprenorphine blunted the increase in arterial pressure

by approximately 50 percent. However, because buprenorphine was associated with an increase in arterial pressure, the absolute values of the peak mean arterial pressure were not different between experiments without (86 ± 1 mmHg) or with buprenorphine (82 ± 1 mmHg) (P=0.12).

Changes in aEMG activity did not appear to be predictably related to the onset or intensity of colorectal distension. Examples from 3 animals show that aEMG activity was variable during colorectal distension experiments (Figure 8). In many animals, aEMG activity was quiescent throughout the experiment. For this reason aEMG activity was not analyzed further.

In 5 rabbits, the average volume of distension for 9.5 min experiments was 5.2 ± 0.2 ml. This was substantially less than the 12 ml limit established during Phase 1. At necropsy, there was no evidence of tissue damage in the distal colon of any rabbits used in Phase 2 of this study.

DISCUSSION

The goal of this study was to evaluate noxious somatic and visceral stimuli for use in conscious, chronically instrumented rabbits. We sought a noxious stimulus that could be applied at variable intensities. A stimulus that reliably changed blood pressure was desirable since it is difficult to objectively assess pain in animals, and we felt a detectable change in blood pressure might be one indication of stimulus intensity. Because we intended to use the selected noxious stimulus in conscious animals, it was critical that the stimulus not cause long-lasting pain. The advantage of using chronically instrumented animals is

the ability to repeat experiments in individual animals, thus reducing variability. Therefore, it was important that the noxious stimulus be associated with reproducible changes in blood pressure and not cause tissue damage.

In Phase 1 of this study, we evaluated the cardiovascular and respiratory effects of noxious chemical and visceral stimuli in anesthetized rabbits. Algesic substances were injected subcutaneously and intramuscularly. These noxious chemical stimuli changed blood pressure and EMG activity, however, they produced unacceptable tissue damage. Under anesthesia, colorectal distension produced increases in blood pressure that were temporally limited to the duration of distension. At necropsy there was no evidence of tissue damage if inflation volumes were less than 14 ml. In anesthetized rabbits, colorectal distension met all but the last of the selection criteria (e.g. feasible in conscious rabbits). It seemed that colorectal distension would be feasible in conscious rabbits.

Further evaluation of colorectal distension in conscious animals was performed in Phase 2. All rabbits tolerated placement of the colorectal balloon catheter. During distension some rabbits moved forward or backward in the rabbit box. On occasion a rabbit stomped its feet, yet none vocalized or exhibited violent escape behavior during distension. Mean arterial pressure reproducibly increased during colorectal distension and returned to baseline following release of pressure within the balloon. Behavioral signs of discomfort, if displayed, ceased when pressure in the balloon was released. None of the rabbits exhibited behavioral signs of pain or distress at the end of a colorectal distension experiment, after the balloon had been removed, or when observed in

their home cages. Rabbits continued to gain weight normally during the experimental series. This preliminary study demonstrated that colorectal distension predictably and reproducibly increased arterial pressure, and decreased respiratory rate without causing any apparent tissue damage.

Others have evaluated the effect of opioid analgesics on the response to colorectal distension in rodents (26;62). One study administered opioid agonists intrathecally in conscious rats subjected to repeated colorectal distension. Intrathecal mu-opioid receptor agonists [morphine and D-Ala^{2},*N*-Me-Phe^{4},Gly^{5}-ol]enkephalin (DAMPGO)] attenuated the increase in arterial pressure associated with colorectal distension, and increased the threshold for visceromotor reflex (26). In conscious mice, subcutaneous administration of fentanyl, a mu-opioid agonist, significantly reduced the visceromotor response to colorectal distension. Thus, it appears that mu-opioids may provide analgesia in this model of visceral pain.

Buprenorphine is a partial mu-opioid agonist, commonly used as an analgesic in laboratory animals (105). We were interested in knowing if buprenorphine would provide visceral analgesia in conscious rabbits undergoing colorectal distension. Indeed, in anesthetized and conscious rabbits, buprenorphine administration blunted the rise in mean arterial pressure associated with colorectal distension by approximately 50 percent.

In conscious rabbits, buprenorphine caused an increase in arterial pressure, a dramatic decrease in respiratory rate, but no change in heart rate. While buprenorphine attenuated the change in arterial pressure associated with

colorectal distension, the absolute values for mean arterial pressure during colorectal distension were not different from those in the absence of buprenorphine. It is conceivable that colorectal distension produces a ceiling response with regard to increases in blood pressure, and, therefore, the increase in blood pressure was smaller following buprenorphine administration because starting pressure was higher. It is unclear whether the blunted increase in mean arterial pressure reflects analgesia produced by buprenorphine.

Pseudoaffective behaviors are indicative of an emotional or affective response to a noxious stimulus (89). Abdominal muscle contraction in response to noxious colorectal distension is considered a pseudoaffective reflex that is correlated with the intensity of the stimulus (91). Abdominal EMG activity is used to quantify the visceromotor response to colorectal distension in rodents (26;62;91). We recorded aEMG activity in anesthetized and conscious rabbits during colorectal distension. In anesthetized rabbits, there was little change in aEMG during distension. The changes in aEMG activity in conscious rabbits was variable and rarely associated with colorectal distension. Thus, in our preparation, aEMG activity did not appear to provide meaningful information about the pseudoaffective reflex in conscious rabbits. It is possible that we were recording from a small population of abdominal muscle fibers, and therefore our aEMG recordings did not reflect abdominal contractions. However, we would have expected to see aEMG activity consistent with previously reported visceromotor responses in at least one of our rabbits, or during one or more experiments. The lack of a visceromotor response was not due to insufficient

intensity of the noxious visceral stimulus. The colorectal balloon pressures reported here (50 – 60 mmHg) are within the range of pressures deemed noxious in rabbits (30 – 90 mmHg) (25;54;55;95).

In summary, we evaluated noxious stimuli for use in conscious, chronically instrumented rabbits. The purpose of Phase 1 experiments was to select a noxious stimulus for further evaluation in conscious rabbits. Thus, there was limited analysis of data gathered from evaluation of noxious stimuli in anesthetized rabbits. Colorectal distension, a model of noxious visceral stimulation, was the only stimulus evaluated in conscious rabbits. During Phase 2, we characterized the cardiovascular and respiratory response to colorectal distension in conscious, chronically instrumented rabbits. Rabbit sex was not a consideration in this feasibility study. Considering the cardiovascular and behavioral responses observed, we would characterize the discomfort experienced by rabbits during distension as mild to moderate. Based upon the data we have collected during colorectal distension in conscious rabbits, we are confident that this model of visceral pain is short-lived, reproducible, and causes no lasting tissue damage. We feel that colorectal distension is an appropriate, humane, and feasible noxious stimulus for use in future experiments evaluating the effect of pain on the response to blood loss in conscious rabbits.

Figure 1. Flow chart detailing evaluation and validation of noxious stimuli in rabbits.

Phase 1: TERMINAL experiments testing noxious stimuli in ANESTHETIZED rabbits

Experiments in Phase 1 were designed to evaluate noxious stimuli and assess whether they meet the following Selection Criteria. Specifically, is a particular noxious stimulus:

A. Titratable (of variable intensity)
B. Short-lived
C. Associated with a change in blood pressure of approx. 10 mmHg
D. Repeatable
E. Not associated with overt tissue damage
F. Responsive to opioid analgesia, and
G. Feasible in a conscious, chronically instrumented rabbit?

Yes → Test in conscious rabbits
No → No further testing

Phase 2: VALIDATION of noxious stimuli in CONSCIOUS rabbits

Experiments in Phase 2 were designed to:

1. Evaluate cardiovascular and respiratory responses to noxious stimulation repeated 4 times over 2 week period.
2. Evaluate the effect of buprenorphine on the cardiovascular and respiratory responses to noxious stimulation.
3. Evaluate tissues at necropsy for gross evidence of tissue damage

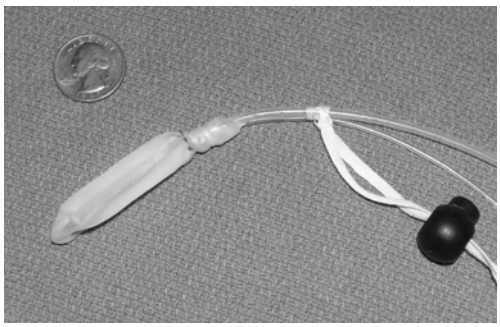

Figure 2. Colorectal balloon catheter. The double-lumen balloon catheter allows monitoring of balloon pressure while inflating. One lumen is made from PE 240 tubing (ID 0.066", OD 0.095") and the second is Tygon tubing (ID 0.04", OD 0.07”). The 5-cm balloon is made from the finger of a surgical glove. The balloon is attached to the tubing with silicone rubber and fastened down with dental floss. A strip of parafilm (American Can Co., Greenwich, CT) is wrapped around the dental floss knots to create a smooth surface. The balloon is inserted 9 cm and is secured to the rabbits tail with a loop of umbilical tape and a cord-lock.

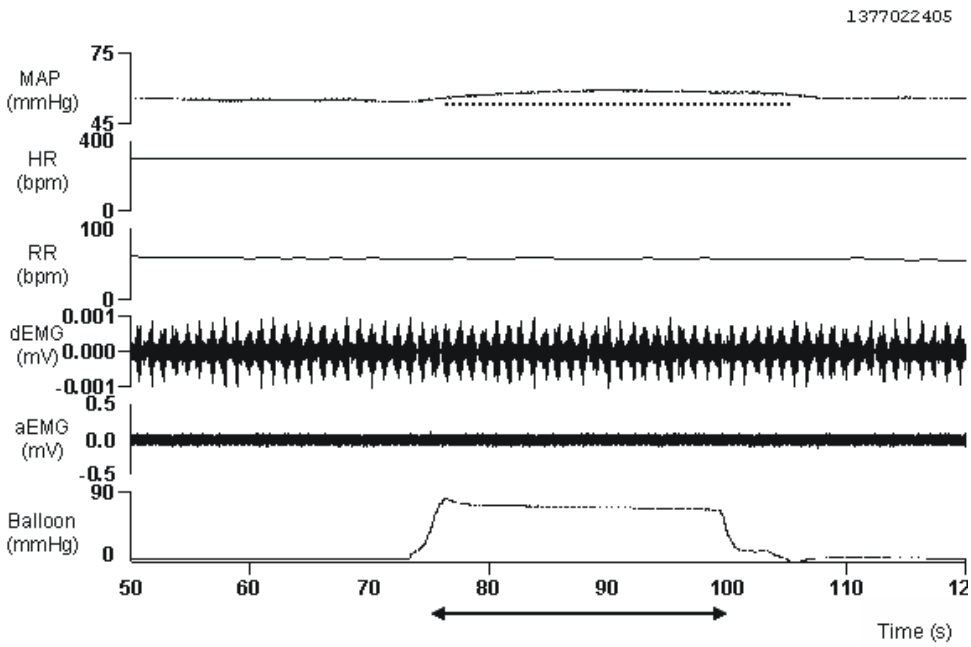

Figure 3. Brief colorectal distension in a halothane-anesthetized rabbit. Increasing colorectal balloon pressure (Balloon) to approximately 80 mmHg for 30 s was associated with an increase in mean arterial pressure (MAP). The dotted line accentuates the slight, short-lived increase in MAP. There were no changes in heart rate (HR), respiratory rate (RR), diaphragmatic EMG (dEMG) activity or abdominal EMG (aEMG) activity during colorectal distension.

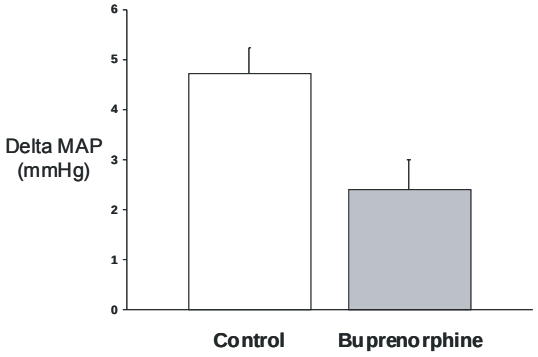

Figure 4. The change in mean arterial pressure (Delta MAP) associated with colorectal distension in halothane-anesthetized rabbits without (Control) and with Buprenorphine (0.06 mg, iv). There was no difference in the average colorectal balloon pressure during Control (68 ± 2 mmHg) and Buprenorphine (68 ± 2 mmHg) treatments (N=6).

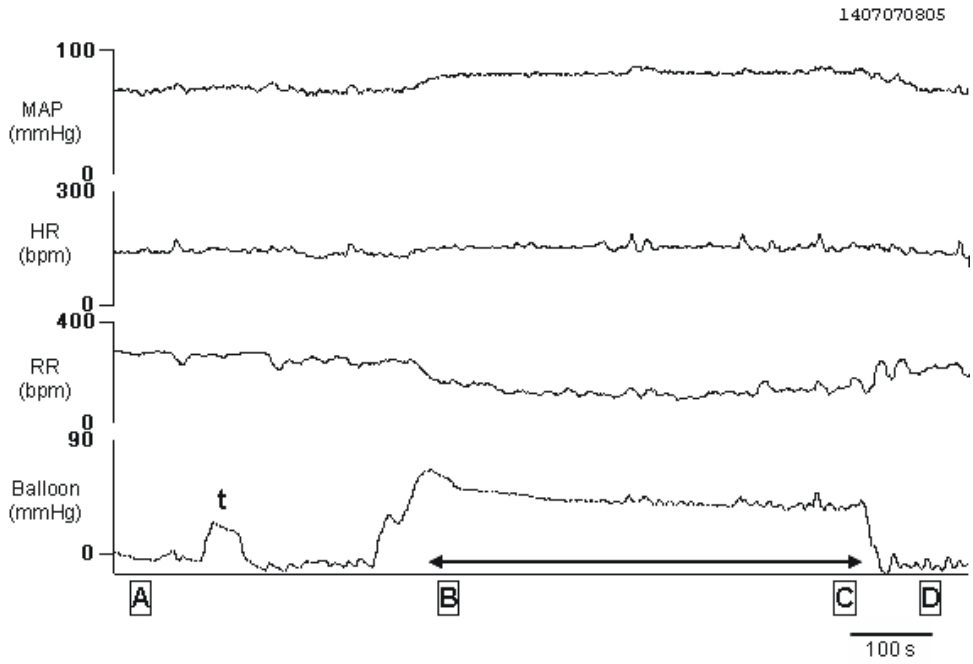

Figure 5. Experimental record of the cardiorespiratory response to colorectal distension (arrow) in a conscious rabbit. A brief test inflation (t) was performed and then the colorectal balloon was inflated until mean arterial pressure (MAP) increased by 10 – 15 mmHg. The peak colorectal balloon pressure (Balloon) was approximately 70 mmHg, and this decreased to approximately 50 mmHg for the duration of the distension (9.5 min). Values at 4 time points were statistically compared: Baseline (A), the Start (B) and End (C) of distension, and after Release (D). HR, heart rate; RR, respiratory rate.

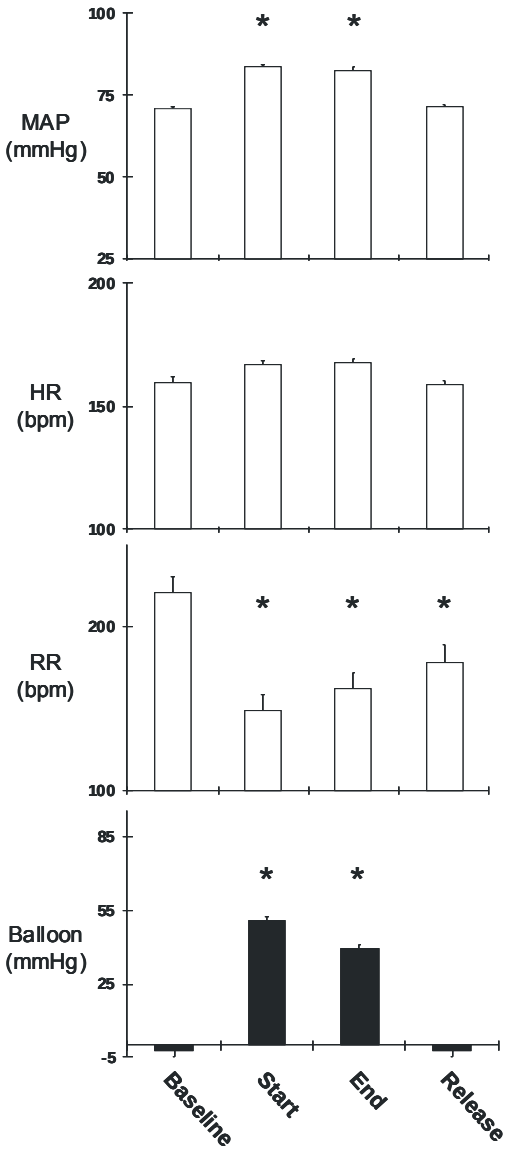

Figure 6. Cardiorespiratory response to 9.5 min colorectal distension in conscious rabbits. The four panels show changes in mean arterial pressure (MAP), heart rate (HR), respiratory rate (RR), and colorectal balloon pressure (Balloon) at Baseline, the Start and End of the distension, and after Release of the pressure within the balloon. Colorectal distension increased MAP, decreased RR and did not change HR. * Significantly different from Baseline ($P<0.05$).

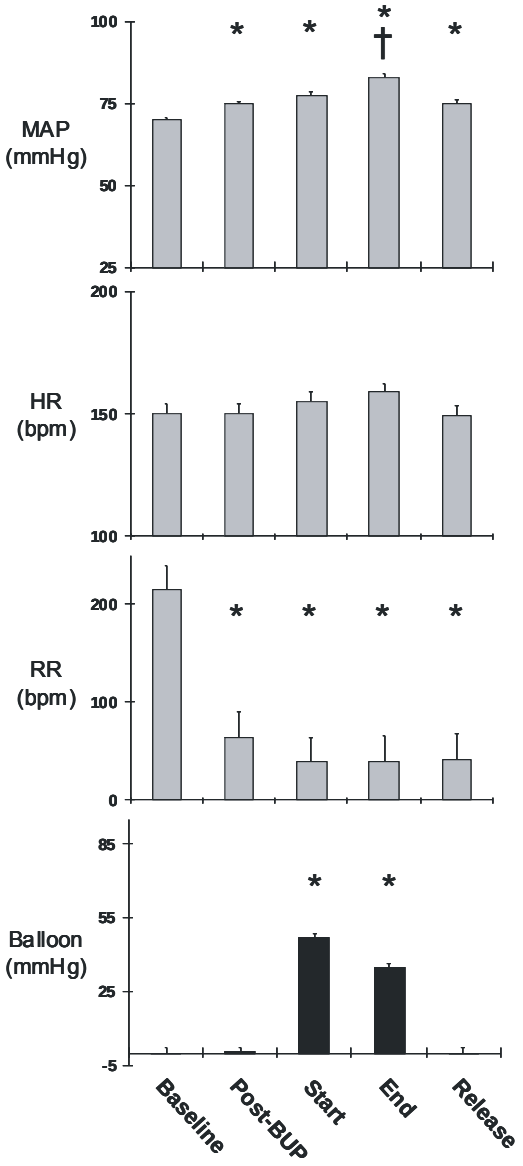

Figure 7. Effect of buprenorphine on cardiorespiratory response to 9.5 min colorectal distension in conscious rabbits. Abbreviations as in Figure 6. Post-BUP was 10 min after buprenorphine (0.06 mg, iv). Administration of buprenorphine increased MAP, decreased RR, but did not change HR.

* Significantly different from Baseline (*P*<0.05). † Significantly different from Post-BUP (*P*<0.05)

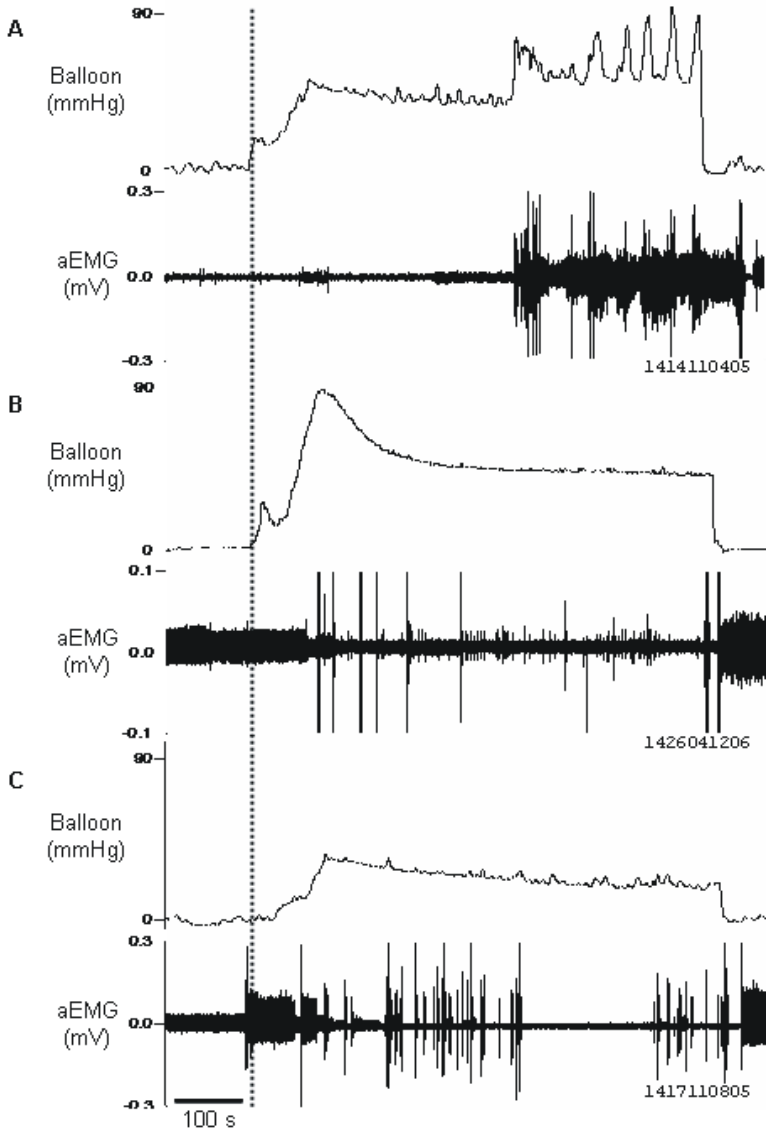

Figure 8. Abdominal EMG (aEMG) activity during a 9.5 min colorectal distension in 3 rabbits. In each example the top panel is colorectal balloon pressure (Balloon) and the bottom is raw aEMG activity (aEMG). The vertical dashed line

indicates the start of distension. A. Increased aEMG activity was present at the end of the distension. Presumably, the spikes in balloon pressure are due to increased intraabdominal pressure associated with increased abdominal muscle activity. B. Spontaneous aEMG activity at baseline shut off during distension, and then resumed after release of balloon pressure. The loss of aEMG activity during distension is not consistent with the visceromotor reflex. C. Variable changes in aEMG activity during colorectal distension.

Table 1. Stable response to repeated colorectal distension in conscious rabbits.

	Baseline				**Start**				**End**				**Release**			
Exp #	MAP	HR	RR	CRB	MAP	HR	RR	CRB	MAP	HR	RR	CRB	MAP	HR	RR	CRB
1	72	170	269	-4	85	174	167	46	81	173	198	37	71	168	220	-5
2	71	164	224	-4	84	173	147	49	83	174	157	35	72	166	172	-5
3	69	157	208	0	83	166	159	47	83	165	169	39	69	154	183	-1
4	69	155	212	-2	82	162	142	46	77	161	168	34	71	155	162	-2
SE	1	4	28	2	2	4	14	3	2	5	13	2	2	4	21	1
P	0.28	0.10	0.43	0.30	0.73	0.15	0.63	0.92	0.16	0.23	0.21	0.35	0.95	0.08	0.28	0.11

Mean values for 4 separate experiments performed in 5 male rabbits during a 2-week period. Values represent a 30 sec average before colorectal distension (Baseline), at the Start and End of a 9.5 min colorectal distension experiment, and during Release (following release of pressure within the colorectal balloon). MAP, mean arterial pressure (mmHg); HR, heart rate (bpm); RR, respiratory rate (bpm); CRB, colorectal balloon pressure (mmHg). Variability is represented by the SE calculated using the pooled estimate of the population variance from the 1-way repeated-measure ANOVA performed for each time period. *P*-values for each analysis are reported in the last row. There were no significant differences between experiments.

CHAPTER 6

The respiratory and cardiovascular effects of buprenorphine in conscious rabbits

Buprenorphine is an opioid analgesic that is used extensively in laboratory animals. The analgesic efficacy of buprenorphine has been well documented in a variety of species (105), and the physiological effects of this drug have been reported in conscious rats (24;31;94), dogs (52), sheep (147) and horses (138). In conscious dogs and horses, intravenous buprenorphine was associated with increases in respiratory rate and minute ventilation, and decreases in tidal volume (52;138). In contrast, intravenous buprenorphine did not change respiratory rate in conscious rats (94). Some have reported an increase in arterial carbon dioxide tensions following intraarterial buprenorphine administration (24;31), while others have reported no change in this measure of alveolar ventilation after intravenous buprenorphine (138;147). Changes in cardiovascular parameters associated with buprenorphine administration are not consistent. For example, arterial pressure increases in horses (138) while blood pressure and heart rate both appear to decrease in dogs (52) given buprenorphine. The apparent discrepancies in the reported physiological effects

of buprenorphine may be due in part to differences in species studied, drug dose and route of administration.

While the analgesic effects of buprenorphine in rabbits have been reported (39;151), the physiological effects of this drug in rabbits are not well documented. Intravenous buprenorphine administration to anesthetized rabbits was associated with decreases in arterial pressure and respiratory rate, and increases in arterial carbon dioxide tension (28). Flecknell and Liles (39) reported that buprenorphine reduced respiratory rate in conscious rabbits by approximately 50 percent, yet other details regarding the respiratory and cardiovascular effects of buprenorphine were not described in this publication. Thus, the objective of this study was to quantify the respiratory and cardiovascular effects of buprenorphine in conscious rabbits. We hypothesized that intravenous and subcutaneous buprenorphine would decreased respiratory rate but not change heart rate or mean arterial pressure.

METHODS

Animals and instrumentation. All procedures were approved by the University of Missouri's institutional Animal Care and Use Committee, and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (50). Animals were allowed at least one week to acclimatize to their new environment before undergoing surgical instrumentation. Eight New Zealand white rabbits (4 female) weighing 3.4 $\pm$ 0.1 kg (mean $\pm$ SE) were chronically instrumented with indwelling abdominal catheters and diaphragmatic

electromyographic (dEMG) electrodes. Antibiotics (22.7 mg enrofloxacin, sc; Baytril, Bayer) were administered the day prior to surgery. Food but not water was withheld 15 – 20 hr prior to surgery. Anesthesia was induced and maintained with halothane in oxygen via face mask (5% for induction, 0.5 – 3.5% for maintenance). The analgesic buprenorphine hydrochloride (Buprenex, Reckitt Benckiser, 0.06 mg, sc) was administered to all rabbits once prior to, and twice after surgery at 8 – 12 hr intervals.

Non-occlusive venous and arterial catheters were implanted in the abdominal vena cava and aorta, respectively, via a midline laparotomy (44). The venous catheter was used for intravenous administration of buprenorphine, and the arterial catheter was used to record pulsatile arterial pressure and obtain serial blood samples for blood gas analyses. EMG electrodes (126) were implanted in the diaphragm during the laparotomy, and a ground electrode was secured to the abdominal wall. Recording of dEMG activity allowed monitoring of respiratory rate. Catheters and EMG leads were tunneled under the skin and exteriorized at the base of the neck. Rabbits were allowed at least 2 weeks to recover from surgery before the start of experiments.

Husbandry and feeding. Rabbits were individually housed in standard cages (24” X 30” X 19.25”) and provided with polypropylene toys (Bio-Serv, Frenchtown, NJ). Water was available at all times. Pelleted rabbit chow (135 g; Purina, St. Louis, MO) was provided daily between the hours of 9:00 am and 10:00 am. Food but not water was withheld for 15 – 20 hours prior to

experiments. After each experiment, rabbits were returned to their home cages and offered food.

Experimental procedures. All experiments were performed on conscious rabbits accustomed to sitting quietly in an opaque plexiglass box (33 X 15 X 18 cm). On the day of experiments, rabbits were heparinized (sodium heparin, Elkins-Sinn, 2000 units, iv), the arterial catheter was connected to a pressure transducer, and dEMG leads were connected to differential amplifiers. Pulsatile arterial pressure, heart rate, and dEMG were monitored continuously throughout each experiment. Blood pressure and heart rate were allowed to stabilize for at least 10 min prior to the experiment. A white noise generator was used to mask background noise.

Eight rabbits (4 female) received buprenorphine intravenously (0.06 mg qs to 0.5 ml with 0.9% saline) followed by 2 ml saline flush. Respiratory rate, mean arterial pressure, heart rate and arterial blood gas data were evaluated at baseline, 10 and 22 min after intravenous buprenorphine administration. These times were chosen to match data gathered in a separate study.

Six rabbits (4 female) received subcutaneous buprenorphine (0.06 mg; 0.2 ml) in the right dorsal flank region. Respiratory rate, mean arterial pressure, heart rate and arterial oxygen and carbon dioxide tensions were evaluated at baseline, 30, 60 and 90 min after subcutaneous buprenorphine administration.

The volume of arterial blood withdrawn at each sampling time was 3.5 ml (2.5 ml dead space followed by 1.0 ml sample for analysis). The dead space blood was reinfused via a venous catheter at the end of the experiment. Arterial

blood samples were stored on ice and analyses were performed within 30 min of sampling (Osmetech OPTI, AVL Medical Corp., Roswell, GA). The alveolar-arterial oxygen gradient was calculated using the alveolar gas equation (149) and measured barometric pressure.

RESULTS

In conscious rabbits breathing room air, buprenorphine significantly altered respiratory but not cardiovascular parameters. The change in respiratory pattern and frequency following subcutaneous buprenorphine in a male rabbit is illustrated in Figure 1. Summary results for intravenous buprenorphine administration are shown in Table 1, and the results for subcutaneous buprenorphine are shown in Table 2. Intravenous and subcutaneous buprenorphine administration maximally reduced respiratory rate by 85 and 71 percent of baseline, respectively, within the time frame of these experiments. Respiratory rate was significantly less than baseline at all time points after intravenous or subcutaneous buprenorphine administration.

The changes in arterial carbon dioxide and oxygen tension observed at the first measurement after buprenorphine were similar at subsequent measurements for both routes of administration. Within ten min of intravenous buprenorphine administration, arterial carbon dioxide tension was slightly but significantly higher than baseline, arterial oxygen tension was moderately decreased and the A-a gradient was increased (Table 1). These differences from baseline persisted at 22 min post-buprenorphine. There was a significant

decrease from baseline in arterial pH at 10 min but not at 22 min after intravenous buprenorphine. Despite slight yet significant increases in carbon dioxide tensions, there was no change in arterial pH following subcutaneous buprenorphine. After subcutaneous buprenorphine the A-a gradient was increased at 30 min, but not at 60 or 90 min. For both routes of administration, buprenorphine did not appear to change mean arterial pressure or heart rate.

DISCUSSION

Buprenorphine is routinely administered for perioperative analgesia in rabbits. Understanding the physiological effects of this analgesic is important for optimizing respiratory and cardiovascular function. In previous studies, buprenorphine reduced respiratory rate by at least 50 percent (39). To our knowledge, the effects of buprenorphine on oxygenation, alveolar ventilation and cardiovascular parameters in rabbits have not been reported. Further evaluation of the respiratory and cardiovascular effects of intravenous and subcutaneous buprenorphine administration in conscious rabbits was undertaken in this study.

It is common practice for rabbits in our laboratory within a specified weight range to receive a fixed amount of buprenorphine. The total dose of buprenorphine administered to rabbits in this study was 0.06 mg. At the time of the experiments rabbits weighed between 3.1 and 3.8 kg, thus the dose of buprenorphine administered was 0.016 – 0.02 mg/kg. This dose is within the recommended analgesic dose range for rabbits (0.01 – 0.05 mg/kg) (30).

The rapid respiratory rates observed at baseline in this study reflect both the pattern and frequency of respiration in resting rabbits. At rest, rabbits are often sniffing, and demonstrate irregular breathing patterns (see Figure 1 A). These rabbits were purchased as specific-pathogen free (e.g. free of *Pasturella spp.*) and showed no clinical signs of respiratory disease during or after experiments performed in this study. Others have reported respiratory rates of 100 – 200 bpm in conscious rabbits (38;40).

Hypoxemia is traditionally defined as an arterial oxygen tension of less than 80 mmHg (128). In this study, buprenorphine given via the intravenous or subcutaneous route was associated with hypoxemia. Qualitatively, the decline in oxygen tension was greater than the increase in carbon dioxide tension following both intravenous and subcutaneous buprenorphine, suggesting that alveolar hypoventilation did not contribute substantially to the reduction in oxygen tension (127). The difference between the partial pressure of oxygen in the alveolus (P_AO_2) and the artery (PaO_2) is one clinically useful measure of the efficiency of gas exchange (150). The increase in A-a gradient observed after buprenorphine administration suggests that the concurrent hypoxemia resulted from a reduction in ventilation-perfusion matching within the lung (i.e. decreased efficiency of oxygenation) (150).

In summary, buprenorphine administration was associated with a decrease in respiratory rate and arterial oxygen tension in healthy conscious rabbits. The marked reduction in respiratory rate and mild hypoxemia observed following intravenous or subcutaneous buprenorphine may be clinically

significant in animals at risk of developing respiratory depression (i.e. thoracic disease or surgery). However, the degree of hypoxemia reported here is likely to be well tolerated by healthy animals not at risk for hypoventilation.

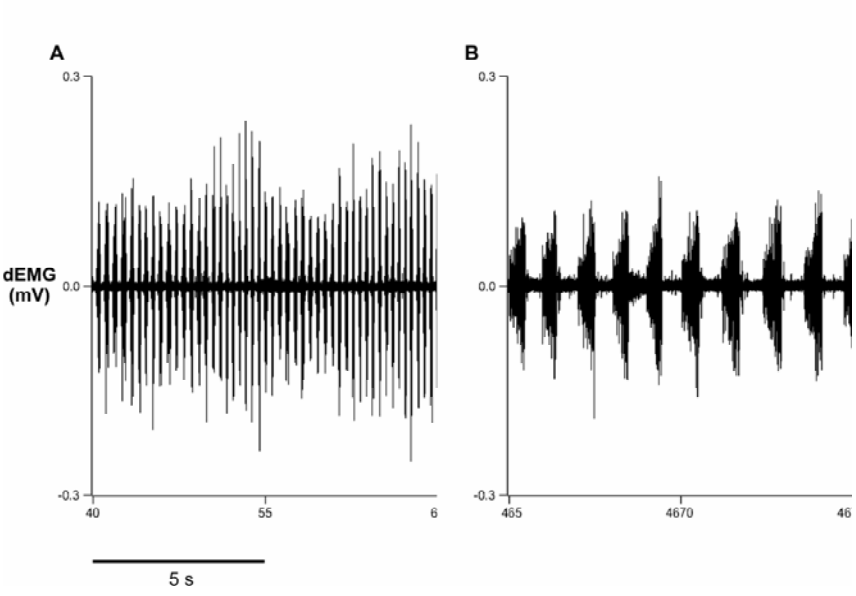

Figure 1. Recording of diaphragmatic EMG (dEMG) signal in a conscious male rabbit prior to (A) and 75 min after (B) subcutaneous administration of buprenorphine. Bursts of dEMG signal represent inspiration. Each panel is 10 s. Respiratory rate is approximately 275 bpm in A, and 66 bpm in B.

Table 1. Respiratory and cardiovascular effects of intravenous buprenorphine (0.06 mg) in conscious rabbits breathing room air.

	Baseline	**10 min**	**22 min**
RR	252 ± 9	61 ± 9 *	39 ± 9 *
pH	7.43 ± 0.01	7.42 ± 0.01 *	7.43 ± 0.01
$PaCO_2$	36 ± 1	40 ± 1 *	41 ± 1 *
PaO_2	88 ± 1	72 ± 1 *	75 ± 1 *
A-a gradient	15 ± 1	25 ± 1 *	22 ± 1 *
MAP	72 ± 2	74 ± 2	75 ± 2
HR	155 ± 3	146 ± 3	148 ± 3

Values (mean ± SE) at Baseline, and 10 and 22 min following intravenous buprenorphine. N=8 (4 female). RR, respiratory rate (bpm); $PaCO_2$ (mmHg); PaO_2 (mmHg); A-a gradient (mmHg); MAP, mean arterial pressure (mmHg); HR, heart rate (bpm). * Significantly different from Baseline ($P<0.05$).

Table 2. Respiratory and cardiovascular effects of subcutaneous buprenorphine (0.06 mg) in conscious rabbits breathing room air.

	Baseline	**30 min**	**60 min**	**90 min**
RR	306 ± 16	181 ± 16 *	93 ± 16 *	90 ± 16 *
pH	7.45 ± 0.01	7.45 ± 0.01	7.46 ± 0.01	7.46 ± 0.01
$PaCO_2$	36 ± 1	39 ± 1 *	40 ± 1 *	41 ± 1 *
PaO_2	87 ± 1	78 ± 1 *	81 ± 1 *	77 ± 1 *
A-a gradient	15 ± 1	20 ± 1 *	16 ± 1	17 ± 1
MAP	69 ± 2	72 ± 2	72 ± 2	73 ± 2
HR	149 ± 4	147 ± 4	140 ± 4	133 ± 4

Values (mean ± SE) at Baseline and 30, 60 and 90 min following subcutaneous buprenorphine. N=6 (4 female). Abbreviations as in Table 1. * Significantly different from Baseline (P<0.05).

CHAPTER 7

The effect of buprenorphine on the response to colorectal distension in conscious rabbits

Buprenorphine is an effective opioid analgesic in a wide variety of laboratory animal species (105). Several studies have demonstrated benefits from provision of buprenorphine analgesia in models of visceral pain in rats and cats, specifically laparotomy with visceral manipulation (66-68), ovariohysterectomy (132) and acute pancreatitis (63). In rabbits, buprenorphine effectively blunts the reflex response to a noxious thermal stimulus applied to the skin (39). However, little data exists on the analgesic efficacy of buprenorphine for visceral pain in rabbits.

We have adapted the model of colorectal distension for use in conscious, chronically instrumented rabbits to evaluate cardiovascular control during noxious visceral stimulation. We tested the hypotheses that administration of buprenorphine: 1) prior to colorectal distension; or 2) after initiation of colorectal distension would blunt the cardiorespiratory changes associated with colorectal distension. Two experimental series were performed to evaluate the cardiovascular and respiratory effects of colorectal distension in conscious,

chronically instrumented male and female rabbits with pre-distension or post-distension buprenorphine.

METHODS

Animals and instrumentation. All procedures were approved by the University of Missouri Animal Care and Use Committee, and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (50). Six male and eight female New Zealand white rabbits weighing 3.2 ± 0.1 kg and 3.3 ± 0.1 kg (mean ± SE), respectively, were chronically instrumented with indwelling catheters and electromyographic (EMG) electrodes. Antibiotics (22.7 mg enrofloxacin, sc; Baytril, Bayer) were administered the day prior to surgery. Food but not water was withheld 15 – 20 hr prior to surgery. Anesthesia was induced and maintained with halothane in oxygen via face mask (5% for induction, 0.5 – 3.5% for maintenance). The analgesic buprenorphine hydrochloride (Buprenex, Reckitt Benckiser, 0.06 mg, sc) was administered to all rabbits prior to, and after each surgery.

A midline laparotomy was performed for implantation of non-occlusive venous (vena cava) and arterial (aorta) catheters (44). The venous and arterial catheters were used for injection of drugs and recording of arterial pressure, respectively. EMG electrodes (126) were implanted in the diaphragm and internal abdominal musculature, and a ground electrode was secured to the abdominal wall. Diaphragmatic EMG (dEMG) activity allowed monitoring of respiratory rate. Abdominal EMG (aEMG) activity was used to assess the

visceromotor response to colorectal distension (91). Catheters and EMG leads were tunneled under the skin and exteriorized at the base of the neck. Rabbits were allowed at least 2 weeks to recover from surgery before the start of experiments.

Colorectal distension. Distension of the colon was performed by inflating a 5 cm balloon with water. The double-lumen balloon catheter (Figure 1) allowed for simultaneous inflation of the balloon and monitoring of intra-balloon pressure. The balloon was checked for leaks prior to each experiment. For acute placement of the colorectal balloon catheter, rabbits were placed on their backs with their eyes covered until they become relaxed (1 – 3 min). A well-lubricated colorectal balloon catheter was then inserted 9 cm into the rectum. The balloon catheter was secured to the tail with a loop of umbilical tape and a cord-lock (see Figure 1). Post-mortem examination has confirmed that, in this position, the balloon is within the pelvic canal (unpublished observations).

During colorectal distension experiments, a 30 sec test inflation (i.e. $\leq$ 20 mmHg) was performed to confirm the ability to register changes in intra-balloon pressure. Noxious colorectal distension was performed by manually inflating the balloon until mean arterial pressure increased by 10 – 15 mmHg, or colorectal balloon pressure reached 90 mmHg. This 90 mmHg limit was used to avoid tissue damage (55).

All rabbits underwent training to accustom them to colorectal balloon catheter placement, and being in a rabbit box (33 X 15 X 18 cm) in the laboratory setting. Training sessions were performed several times during the week prior to

beginning colorectal distension experiments, and subsequently on each day preceding an experiment. Initial duration of colorectal distension was 1 – 3 min and progressed to 25 min over a three week period. Colorectal distension experiments were performed 3 days per week if the distension was less than 15 min, and twice a week if the duration of distension was greater than 15 min. During Pre-distension Buprenorphine experiments, we sought to limit the total duration of colorectal distension to no more than 10 min. Because of the need for a short test inflation (30 sec), the duration of the noxious colorectal distension was limited to 9.5 min. The duration of colorectal distension during Post-distension Buprenorphine experiments was 25 min to allow for evaluation of the effects of saline or buprenorphine 10 min after administration.

Experiments were performed on conscious rabbits that had been fasted for 15 – 20 hr prior to each experiment. On the day of an experiment, the colorectal balloon was placed acutely and rabbits were returned to the rabbit box. Rabbits were then heparinized (sodium heparin, Elkins-Sinn, 2000 units, iv), arterial and colorectal balloon catheters were connected to pressure transducers, and EMG leads were connected to differential amplifiers. Pulsatile arterial pressure, heart rate, colorectal balloon pressure, dEMG and aEMG activity were monitored continuously throughout each experiment. Blood pressure and heart rate were allowed to stabilize for at least 10 min prior to the experimental control period. A white noise generator was used to mask background noise.

Pre-distension buprenorphine. The timeline for these experiments is illustrated in Figure 2A. Following a 1 min control period, saline (0.2 ml/kg) or

buprenorphine (Buprenex, Reckitt Benckiser; 0.06 mg) was administered intravenously (iv). After 10 min, a test inflation of the colorectal balloon was performed. Two min later, noxious colorectal distension was performed and sustained for 9.5 min. A 2 min recovery period followed the release of balloon pressure. The effect of buprenorphine was determined by comparing measured parameters between treatments 10 min after injection of buprenorphine or saline (see Figure 2A). For buprenorphine experiments, the colorectal balloon catheter was inflated to a pressure that matched that of a previous saline experiment where a 10 – 15 mmHg increase in mean arterial pressure was achieved. Thus, the order of these experiments was fixed: the pre-distension saline experiment preceded the pre-distension buprenorphine experiment by at least two days. Thirteen rabbits underwent both treatments.

Post-distension buprenorphine. The timeline for these experiments is illustrated in Figure 2B. Following a 1 min control period, saline (0.2 ml/kg) was administered iv. Ten min later, a test inflation of the colorectal balloon was performed. Following a 2 min delay, noxious colorectal distension was performed. Saline (0.2 ml/kg, iv) or buprenorphine (0.06 mg, iv) was administered 2 minutes after colorectal distension was initiated. Total duration of the distension was 25 min (e.g. 23 min after injection), and a 2 min recovery period followed release of the balloon pressure. Eight rabbits underwent both treatments in this experimental series. The order of experiments was balanced, with experiments separated by at least two days. Some rabbits underwent both Pre-distension and Post-distension experiments.

Two animals did not complete either experimental series. One female developed abnormally high arterial blood pressure (> 25 mmHg increase) in response to very low colorectal balloon pressures (< 30 mmHg). A second female was euthanized after developing rectal bleeding following an apparently normal colorectal distension experiment (e.g. peak colorectal balloon pressure was 80 mmHg and remained < 70 mmHg throughout the 9.5 min distension). Histological evaluation of the distal colon showed acute mucosal hemorrhage.

Data collection and analysis. Data were acquired at 4 kHz using a PC-based data acquisition system (Power Lab, AD Instruments, Colorado Springs, CO), and analyzed using commercially available software (Spike2, CED, Cambridge, UK). Heart rate was determined from the pulsatile arterial pressure signal. The dEMG signal was high-pass filtered (200 Hz) to minimize ECG artifact. The signal baseline was set to zero, then rectified and smoothed (time constant = 50 – 100 msec). Respiratory rate was determined from the inspiratory bursts in the dEMG signal. The aEMG signal was evaluated subjectively.

Repeated measures analysis of variance (ANOVA) was used to compare mean arterial pressure, heart rate and respiratory rate between treatments with sex and treatment as independent variables. Measured parameters were compared 10 min after saline or buprenorphine administration to address the effects of buprenorphine in the absence of colorectal distension. The effect of Pre-distension saline or buprenorphine on the response to colorectal distension was determined by comparing the change in measured parameters from before distension to after distension (open and closed arrow heads Figure 2 A).

Similarly, the change in measured parameters from before colorectal distension to after distension were compared to address the effect of Post-distension saline or buprenorphine on the response to colorectal distension (Figure 2 B). Results from males and females were combined if there was not a significant interaction between sex and treatment, or a significant main effect of sex. Bonferroni's multiple comparison test was used to compare individual values. Significance was set at $P < 0.05$. Thirty sec averages of measured parameters were used for all analyses and data are presented as mean $\pm$ SE. The SE was calculated from the pooled estimate of the variance from associated ANOVA.

RESULTS

Figure 3 is a typical 9.5 min colorectal distension experiment illustrating the changes in mean arterial pressure, heart rate, respiratory rate, and colorectal balloon pressure. Noxious colorectal distension was associated with an increase in arterial pressure and heart rate, and a decrease in respiratory rate. In this example, aEMG activity increased approximately 2 min after distension was initiated. The increase in aEMG was brief and the onset of activity was not associated with the onset of colorectal distension. Subjective evaluation of aEMG in all colorectal distension experiments revealed that aEMG at baseline was variable, and changes in aEMG activity during colorectal distension were not predictable. In most experiments, like the one shown in Figure 3, there was no change in aEMG temporally associated with colorectal distension. Thus, aEMG

was not used to assess the behavioral response to noxious visceral stimulation in this study.

Effect of pre-distension buprenorphine. In the absence of colorectal distension, intravenous buprenorphine increased mean arterial pressure, decreased respiratory rate and did not change heart rate (Table 1). The values for arterial pressure, heart rate and respiratory rate in Table 1 represent the pre-distension values for the two treatments, saline or buprenorphine. Inflation of the colorectal balloon increased mean arterial pressure and heart rate in both saline and buprenorphine treatments (Figure 4). However, compared with the buprenorphine treatment, the increase in mean arterial pressure was significantly larger in the saline treatment ($P<0.001$). There was a larger decline in respiratory rate associated with colorectal distension in the saline treatment ($P=0.01$) compared to the buprenorphine treatment. The absolute values for respiratory rate following colorectal distension were 131 $\pm$ 8 and 45 $\pm$ 8 bpm in the saline and buprenorphine treatments, respectively. The increase in heart rate following colorectal distension was not different between saline and buprenorphine treatments ($P=0.74$). Colorectal balloon pressure was significantly higher in the buprenorphine treatment (61 $\pm$ 1 mmHg) compared with the saline treatment (52 $\pm$ 1 mmHg; $P<0.001$).

There were no significant interactions between sex and treatment for any measured parameter, nor was there a significant main effect of sex for mean arterial pressure, heart rate or respiratory rate. Significant differences between males and females were seen in colorectal balloon pressure during distension.

Colorectal balloon pressure was higher in females (66 ± 3 mmHg) than males (48 ± 3 mmHg) (P=0.003). The volume of water used to inflate the colorectal balloon was compared between males and females. Females required a significantly larger distension volume (8 ± 0.3 ml) than males (5 ± 0.3 ml; P<0.001) to reach a similar mean arterial pressure (P=0.65).

Effect of post-distension buprenorphine. The were no differences between the two treatments for mean arterial pressure, heart rate and respiratory rate prior to colorectal distension (Table 2). Arterial pressure and heart rate increased, and respiratory rate decreased during colorectal distension in both treatments (Figure 5). There was no difference between the two post-distension treatments for changes in mean arterial pressure (P=0.98) or heart rate (P=0.73) associated with colorectal distension. Respiratory rate decreased to a much greater extent following post-distension buprenorphine compared with saline (P=0.004).

With the exception of colorectal balloon pressure, there was not a significant main effect of sex on measured parameters. Similar to the results reported above, colorectal balloon pressure was significantly higher in females than males (P=0.02). In addition, the volume of water used to distend the colorectal balloon during these experiments was significantly greater in females (9 ± 0.3 ml) compared to males (6 ± 0.3 ml; P<0.001).

DISCUSSION

Colorectal distension is a noxious visceral stimulus that is associated with reproducible increases in arterial pressure in conscious rabbits (55;125), rats (91) and human volunteers (90). The purpose of this study was to evaluate the effect of the opioid analgesic, buprenorphine, on the physiological responses to colorectal distension in conscious, chronically instrumented rabbits. Distension of the colon was associated with increases in mean arterial pressure and heart rate, and decreases in respiratory rate. Two experimental series were performed to evaluate the effect of timing of buprenorphine administration on the cardiovascular and respiratory response to colorectal distension. Specifically, buprenorphine was administered prior to initiation of noxious colorectal distension (e.g. pre-distension) or after initiation of distension (e.g. post-distension). Male and female rabbits were used in these studies to assess the effect of sex on the physiological response to colorectal distension and/or buprenorphine administration.

Colorectal distension is a model of visceral pain that has been used in conscious rabbits (25;54;55;95), rats (57;91), mice (58) and horses (107;130). We chose to use colorectal distension as a model of noxious visceral stimulation in rabbits because it is associated with short-lived, reproducible physiological changes, and causes no lasting tissue damage (55;124).

We instrumented animals with aEMG electrodes and recorded aEMG activity during noxious colorectal distension. Prior to beginning experiments, we verified that all aEMG electrodes were functional by gently palpating or stroking

the abdomen to elicit increases in aEMG activity. Increases in aEMG activity associated with the visceromotor response to noxious visceral stimulation has been used as an objective means of assessing the intensity of visceral pain (91). However, in our model, aEMG activity was variable at baseline, and increases in aEMG activity were not predictably associated with noxious colorectal distension (see Figure 3). Consequently, we did not use the visceromotor response as an index of visceral pain in this study. The lack of a predictable response in aEMG activity may have been due to the small amount of abdominal muscle from which we were recording. It is also possible that the relatively slow increase in colorectal balloon pressure may not have triggered the visceromotor response.

Response to buprenorphine. In our laboratory, it is common practice for animals within a specified weight range to receive a fixed amount of buprenorphine. The total dose of buprenorphine administered to rabbits in this study was 0.06 mg. At the time of the experiments rabbits weighed between 3.1 and 3.9 kg, thus the dose of buprenorphine administered was 0.015 – 0.02 mg/kg. This dose is within the recommended analgesic dose range for rabbits (0.01 – 0.05 mg/kg) (30).

This study suggests that timing of analgesic administration affects the cardiovascular response to colorectal distension. We have demonstrated that pre-distension buprenorphine blunted the increase in arterial pressure associated with colorectal distension by 64 percent. However, when given after initiation of the noxious visceral stimulus, buprenorphine did not alter the pressor response to colorectal distension. Others have demonstrated that prior administration of

morphine, the prototypical opioid analgesic, reduced the behavioral response to colorectal distension (e.g. pelvic withdrawal) in rabbits (55). In human subjects, the increase in arterial pressure associated with noxious colorectal distension is positively correlated with pain scores, as well as with colorectal balloon pressures (90). If we assume that the pressor response to colorectal distension reflected the degree of discomfort experienced by rabbits in this study, then we would conclude that buprenorphine, when given preemptively, is an effective visceral analgesic in rabbits.

Sex differences. Males required lower colorectal balloon pressures and smaller volumes of balloon inflation compared with females to achieve the same increase in mean arterial pressure associated with colorectal distension. Thus, in this study, males may be more sensitive to this noxious visceral stimulus than females. Another study of colorectal distension in rabbits reported that male rabbits did not exhibit behavioral signs of discomfort until colorectal balloon pressures reached higher pressures (~85 mmHg) compared with females (~67 mmHg) (55). Likewise, many published reports suggest that in humans, females are more sensitive to visceral pain than males (3;80). Estrous cycle may be a factor contributing to alterations in visceral pain threshold in women (80). We did not control for estrous cycle in this study because rabbits are classified as induced ovulators that do not have a spontaneous estrous cycle (22). However, based upon our results, further evaluation of sex differences in response to noxious stimuli in rabbits is warranted.

In conclusion, colorectal distension is a noxious visceral stimulus that predictably alters cardiovascular and respiratory control in conscious, chronically instrumented rabbits. Pre-treatment with buprenorphine blunts the increase in arterial pressure and the decrease in respiratory rate associated with colorectal distension. In contrast, administration of buprenorphine after initiation of the noxious visceral stimulus does not alter the cardiovascular response to colorectal distension but enhances the decrease in respiratory rate.

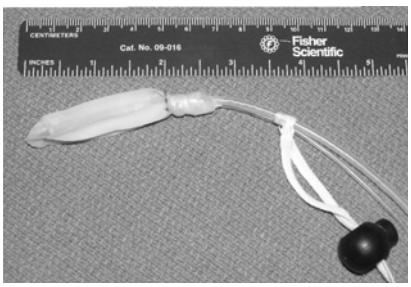

Figure 1. Double lumen colorectal balloon catheter allows monitoring of balloon pressure while inflating. The 5 cm balloon is inserted 9 cm and secured to the rabbit's tail with a loop of umbilical tape and a cord lock.

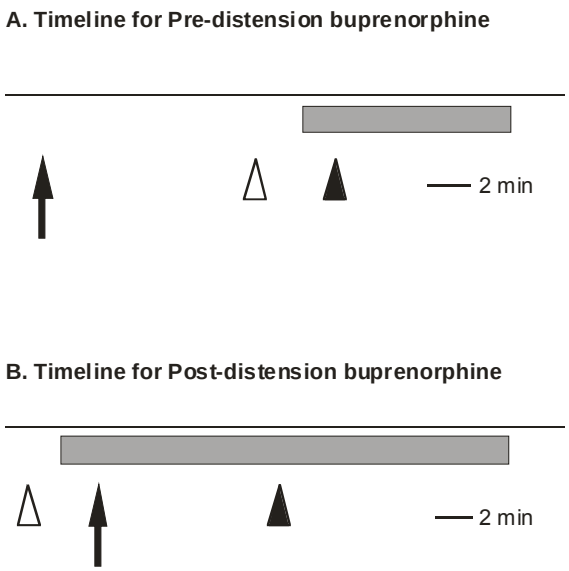

Figure 2. Timeline for colorectal distension experiments. Gray bars indicate the period of colorectal distension. Arrows indicate the time of injection (buprenorphine or saline). A) The effect of Pre-distension saline or buprenorphine was addressed by comparing changes in mean arterial pressure, heart rate and respiratory rate from before (open arrowhead) to after (filled arrowhead) colorectal distension. B) The change in mean arterial pressure, heart rate and respiratory rate between the open and filled arrowheads (prior to, and after distension, respectively) were compared to determine the effect of Post-distension saline or buprenorphine.

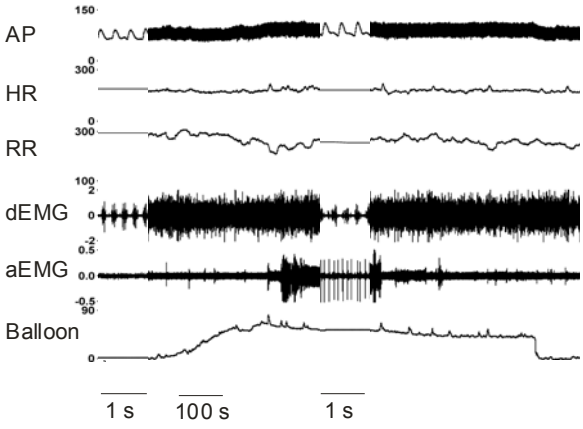

Figure 3. An experimental record of a colorectal distension experiment in a female rabbit. There are two 1 sec insets on an expanded time scale illustrating pulsatile arterial pressure, inspiratory bursts of diaphragmatic EMG (dEMG) activity, and abdominal EMG (aEMG) activity. There is a short burst of aEMG activity during the colorectal distension. The total duration of colorectal distension in this experiment was 9.5 min. AP, arterial pressure (mmHg); HR, heart rate (bpm); RR, respiratory rate (bpm); Balloon, colorectal balloon pressure (mmHg).

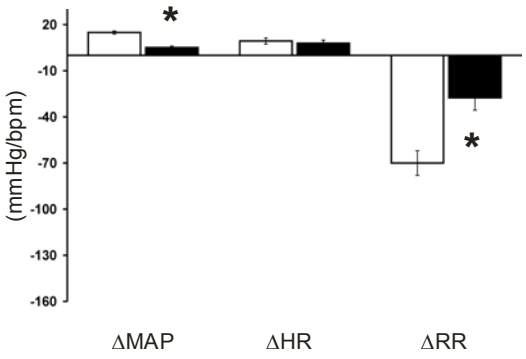

Figure 4. Values (mean ± SE) for changes in mean arterial pressure (ΔMAP), heart rate (ΔHR), and respiratory rate (ΔRR) associated with colorectal distension approximately 10 min after pre-distension administration of saline or buprenorphine. Bars represent the two pre-distension treatments: saline (open) and buprenorphine (black). * Significantly different from Saline (P<0.05).

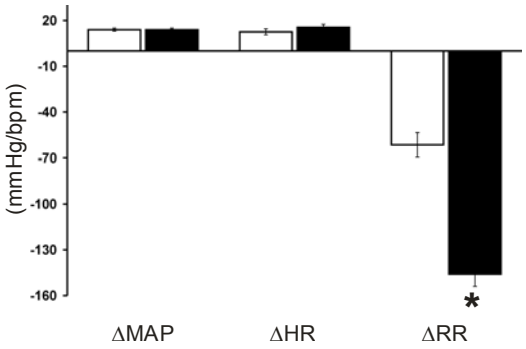

Figure 5. Values (mean ± SE) for changes in mean arterial pressure (ΔMAP), heart rate (ΔHR), and respiratory rate (ΔRR) associated with colorectal distension 10 min after post-distension administration of saline or buprenorphine. Bars

represent the two post-distension treatments: saline (open) and buprenorphine (black). * Significantly different from Saline (*P*<0.05).

Table 1. Effect of intravenous saline or buprenorphine (0.06 mg) on cardiovascular and respiratory parameters in conscious rabbits.

	Saline	**Buprenorphine**
MAP (mmHg)	68 ± 1	76 ± 1 *
HR (bpm)	158 ± 2	152 ± 2
RR (bpm)	201 ± 8	73 ± 8 *

Values (mean ± SE) for mean arterial pressure (MAP), heart rate (HR) and respiratory rate (RR) 10 min after intravenous saline or buprenorphine. N=13 (6 female). * Significantly different from Saline (*P*<0.05).

Table 2. Pre-distension values for saline and buprenorphine treatments in the experimental series evaluating the effect of post-distension buprenorphine.

	Saline	**Buprenorphine**
MAP (mmHg)	73 ± 1	74 ± 1
HR (bpm)	148 ± 4	147 ± 4
RR (bpm)	235 ± 9	194 ± 9

Values (mean ± SE) prior to colorectal distension in the post-distension saline or buprenorphine treatments. Abbreviations as in Table 1. N=8 (4 female).

CHAPTER 8

The effect of noxious visceral stimulation on the response to blood loss in conscious rabbits

ABSTRACT

Pain is a component of traumatic blood loss, yet little is known about how pain alters the response to blood loss in conscious animals. We evaluated the effects of colorectal distension on the cardiorespiratory response to blood loss in 12 conscious, chronically instrumented New Zealand white rabbits. The goal of these experiments was to test the hypothesis that colorectal distension would increase blood loss required to produce hypotension (mean arterial pressure $\leq$ 40 mmHg) in males and females. For hemorrhage, venous blood was withdrawn until mean arterial pressure decreased to $\leq$ 40 mmHg. Conscious rabbits underwent three treatments: a control hemorrhage (Control); hemorrhage with a colorectal balloon present but not inflated (Sham CRD); and hemorrhage in the presence of noxious colorectal distension (CRD). Colorectal distension reproducibly increased mean arterial pressure, decreased respiratory rate, and did not change heart rate. There was no difference in Control blood loss between males (21.8 $\pm$ 0.3 ml/kg) and females (21.6 $\pm$ 0.3 ml/kg). However, while CRD blood loss did not change in males (22.8 $\pm$ 0.3 ml/kg), it was

significantly less than Control in females (19.1 ± 0.3 ml/kg). Thus, in conscious rabbits, colorectal distension alters cardiovascular control during hemorrhage. Inflation of the colorectal balloon increased blood pressure in both sexes, but was associated with decreased tolerance to blood loss in females.

INTRODUCTION

Blood loss is the second leading cause of death following traumatic injury (109;123). The cardiovascular response to blood loss in conscious animals, including humans, can be divided into two phases (118). Initially, during Phase 1, mean arterial pressure is well maintained. Continued blood loss leads to an abrupt transition from Phase 1 maintenance of arterial pressure to Phase 2 hypotension. At least two lines of evidence suggest that the precipitous drop in blood pressure at Phase 2 is an active process, and does not reflect a lack of volume reserve or exhaustion of compensatory mechanisms. First, exposure of conscious animals to stressful stimuli during hemorrhage extends Phase 1 maintenance of arterial pressure in the face of a larger volume loss (114;117). Second, Phase 2 hypotension can be reversed or blocked by a variety of neurally active substances (e.g. opioid antagonists (37)) indicating that, indeed, the animal has compensatory reserve at the time of the transition from Phase 1 to Phase 2 (45).

Although pain invariably accompanies traumatic blood loss, little is known about how noxious stimuli alter the response to blood loss in conscious animals. Interestingly, there is close interaction between central nervous system

regulation of cardiovascular function and nociceptive inputs (71;101;152), and numerous studies have demonstrated that noxious stimuli alter cardiovascular (1;11;12;97) and respiratory control (23;98).

We feel that evaluating the cardiovascular and respiratory responses to blood loss in a conscious animal in the presence of pain may begin to approximate the response to blood loss in a clinical setting. Thus, the aim of this study was to evaluate the effect of a noxious visceral stimulus, colorectal distension, on the cardiovascular and respiratory responses to blood loss in conscious, chronically instrumented rabbits. We hypothesized that colorectal distension would increase the blood loss required to achieve hypotension. We tested this hypothesis in conscious, chronically prepared male and female rabbits and predicted there would be no difference between males and females in their response to colorectal distension plus hemorrhage.

METHODS

Animals and instrumentation. All procedures were approved by the University of Missouri Animal Care and Use Committee, and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (50). Six female and six male New Zealand white rabbits weighing 3.2 ± 0.1 kg and 3.3 ± 0.1 kg (mean ± SE), respectively, were chronically instrumented with indwelling catheters and diaphragmatic electromyographic (dEMG) electrodes. Antibiotics (22.7 mg enrofloxacin, sc; Baytril, Bayer) were administered the day prior to surgery. Food but not water was withheld 15 – 20 hr prior to surgery.

Anesthesia was induced and maintained with halothane in oxygen via face mask (5% for induction, 0.5 – 3.5% for maintenance). The analgesic buprenorphine hydrochloride (Buprenex, Reckitt Benckiser, 0.06 mg, sc) was administered to all rabbits prior to, and after each surgery. Rabbits were allowed to recover from surgery a minimum of 2 weeks before the start of experiments.

Catheters and EMG electrodes. A midline laparotomy was performed for implantation of non-occlusive abdominal venous and arterial catheters (44). The arterial catheter allowed for recording of pulsatile arterial pressure and the venous catheter was used for blood withdrawal and administration of drugs. EMG electrodes (126) were implanted in the diaphragm to record diaphragmatic muscle activity, and allow monitoring of respiratory rate. A ground electrode was secured to the abdominal wall. Catheters and EMG wires were tunneled under the skin and exteriorized at the base of the neck.

Colorectal distension. Mechanical distension of the distal colon and rectum was performed by manually inflating a 5 cm balloon with water. A double-lumen balloon catheter allowed monitoring of balloon pressure during inflation. Acute colorectal balloon placement was achieved by placing the rabbits on their backs with their eyes covered until they became relaxed (1 – 3 min). The well-lubricated balloon was inserted 9 cm into the rectum and gently secured to the tail and the rabbit was returned to the rabbit box. In the course of a colorectal distension experiment, we first performed a 30 sec test inflation (i.e. $\leq$ 20 mmHg) to check for the ability to register changes in balloon pressure. Noxious colorectal distension was then performed by manually inflating the balloon until

mean arterial pressure increased by 10 – 15 mmHg, or colorectal balloon pressure reached 90 mmHg. This 90 mmHg limit was used to avoid tissue damage (55).

All rabbits underwent training to accustom them to placement of the colorectal balloon catheter (≤ 10 min), and being in a rabbit box (33 X 15 X 18 cm) in the laboratory setting. Training sessions were performed several times during the week prior to initiating experiments, and subsequently on each day preceding an experiment. Over a three-week period rabbits underwent progressively longer colorectal distensions. The initial duration of the distension was 1 – 3 min and progressed up to 25 min before colorectal distension plus hemorrhage experiments were performed. Colorectal distension experiments were done 3 times per week if the distension duration was less than 15 min, and no more than twice per week if the duration of distension was greater than 15 min.

To evaluate reproducibility of the cardiovascular and respiratory response to colorectal distension, preliminary experiments were performed in 5 male rabbits. Four 9.5 min colorectal distension experiments were performed in each rabbit over a two week period. Results demonstrated that there were no differences in cardiovascular or respiratory parameters between the four experiments (Table 1). To assess stability of the cardiovascular and respiratory changes associated with colorectal distension, we evaluated the response of 8 rabbits (2 female) to 25 min colorectal distension.

Colorectal distension plus hemorrhage. All experiments were performed on conscious rabbits accustomed to sitting quietly in a rabbit box that restricted their movement. Animals were fasted for 15 – 20 hr prior to each experiment. On the day of the experiment, rabbits were heparinized (sodium heparin, Elkins-Sinn, 2000 units, iv), the arterial catheter was connected to a pressure transducer, and the EMG electrode wires were connected to custom-built, differential amplifiers (10X) placed in close proximity to the rabbit. Heart rate, mean arterial pressure and dEMG activity were monitored throughout each experiment. Time was allowed for the animal to reach a steady baseline in terms of heart rate and arterial pressure (e.g. equilibration period).

Three treatments (Control, Sham CRD, and CRD) were performed with hemorrhage in each rabbit in a balanced design. In Control experiments, hemorrhage was performed in the absence of a colorectal balloon catheter. For Sham CRD and CRD treatments, acute placement of the colorectal balloon catheter was performed. The colorectal balloon was connected to a pressure transducer and intra-balloon pressure was monitored throughout the experiment. The colorectal balloon catheter was present but was not inflated during hemorrhage in the Sham CRD treatment. During CRD, the colorectal balloon catheter was inflated 4 minutes prior to blood loss (Figure 1).

For hemorrhage, venous blood was withdrawn into sterile syringes at a fixed rate of 8 – 9 ml/min until 5 ml after mean arterial pressure reached 40 mmHg. No more than 45 percent of the animal's calculated blood volume, or 27 ml/kg, was withdrawn. All animals in this study reached hypotension within this

limit. In preparation for future experiments evaluating pharmacologic interventions aimed to improve recovery from hypotensive blood loss, a control injection (0.2 ml/kg of 0.9% saline, iv, followed by a 2 ml saline flush) was performed 1 min after mean arterial pressure reached 40 mmHg.

Colorectal distension was maintained throughout the hemorrhage and the 5 min recovery (see Figure 1). Pressure was released from the balloon after the recovery period by withdrawing water from the catheter. We reinfused the shed blood at the end of each experiment. Experiments involving hemorrhage were separated by at least 4 days in individual rabbits.

Data acquisition and analysis. Data were acquired at 4 kHz using a PC-based data acquisition system (Power Lab, AD Instruments, Colorado Springs, CO). Offline analysis of all records was performed using Spike2 software (CED, Cambridge, UK). The pulsatile arterial pressure signal was used to determine heart rate. Diaphragmatic EMG activity was high-pass filtered (200 Hz) to minimize ECG artifact. The signal baseline was set to zero, then rectified and smoothed (time constant = 50 – 100 msec). Inspiratory bursts of dEMG activity were used to measure respiratory rate.

Repeated measures analysis of variance (ANOVA) (SigmaStat v3.1, Systat Software, Inc., Richmond, CA) was used to compare mean arterial pressure, heart rate, respiratory rate and colorectal balloon pressure during: 1) 25 min colorectal distension; 2) hemorrhage; and 3) recovery from hemorrhage. The Bonferroni multiple comparison test was used to compare individual values. For all analyses, significance was set at $P < 0.05$. Data are presented as mean $\pm$

SE. The pooled estimate of the variance from the associated ANOVA was used to calculate the SE.

The response to a 25 min colorectal distension was evaluated by comparing 30 sec averages of the four measured parameters at four times. The times were: 1) during a stable period prior to the test inflation (Baseline); 2) during the first 2 min of the distension (Start); 3) during the last 2 min of the distension (End); and 4) within 2 min of release of the pressure in the balloon (Release).

The amount of blood withdrawn to achieve hypotension is variable in conscious rabbits. Thus, hemorrhage data were normalized to the percent of total blood loss (111). Total blood loss (100%) was defined as the amount of blood withdrawn when mean arterial pressure reached 40 mmHg. Because an additional 5 ml of blood was withdrawn after mean arterial pressure reached 40 mmHg, it was possible to quantify cardiorespiratory changes from 0 – 105 percent of total blood loss. The difference among treatments at pre-hemorrhage (0%), Phase 1 (80%) and Phase 2 (105%) were compared statistically (61). The independent variables for the 2-way ANOVA evaluating the response to hemorrhage and recovery from hemorrhage were treatment (i.e. Control, Sham CRD, and CRD) and percent of total blood loss. Sex differences were evaluated within each treatment using a 2-way ANOVA with sex and time as independent variables. The results for males and females were pooled if there was not a significant interaction between sex and time, or a significant main effect of sex. Total blood loss (ml/kg) for each of the three treatments was compared, and sex

differences were evaluated using a 2-way ANOVA for repeated measures with sex and treatment as independent factors. Post-hoc analyses were performed to evaluate differences between treatments for each sex, and differences between sexes within each treatment. The effect of treatment on recovery from hypotensive hemorrhage was assessed by comparing the difference among treatments for arterial pressure at the end of the 5 min recovery period.

RESULTS

Response to colorectal distension. Figure 2 is an experimental record illustrating the cardiovascular and respiratory response to a 25 min colorectal distension in a conscious rabbit. Table 2 contains summary data from similar experiments in 8 rabbits. In the experiment shown, colorectal balloon pressure remained at approximately 50 mmHg for 25 min. Colorectal distension produced significant increases in mean arterial pressure and heart rate, and decreases in respiratory rate (Table 2). The elevation in mean arterial pressure (approximately 20%) was sustained throughout the 25 min distension, and returned to baseline within 2 min following release of the pressure in the colorectal balloon. Heart rate increased (10%) at the onset of colorectal distension, but was not different from baseline at the end of the 25 min distension, nor during release. Respiratory rate decreased dramatically (40%) at the start of colorectal distension and remained below baseline throughout the distension and release. Thus, colorectal distension was associated with stable

changes in arterial pressure for as long as 25 min. The duration of distension during hemorrhage experiments never exceeded 25 min.

Effects of colorectal distension on the response to hemorrhage. Baseline values for the hemorrhage experiments with Control, Sham CRD, and CRD treatments are shown in Table 3. At baseline there was no significant interaction for sex and treatment for mean arterial pressure (P=0.62), heart rate (P=0.83), or respiratory rate (P=0.35). Nor was there a significant main effect of sex at baseline for arterial pressure (P=0.78), heart rate (P=0.13) or respiratory rate (P=0.53). Therefore, grouped (i.e. male and female) data are presented in Table 3. There were no significant differences among treatments at baseline.

The response to Control hemorrhage in a conscious rabbit is illustrated in Figure 3A. Maintenance of arterial pressure and increases in heart rate during Phase 1 were followed by an abrupt transition to hypotension accompanied by a relative bradycardia during Phase 2. Figure 3B illustrates the cardiovascular and respiratory response to hemorrhage in the presence of colorectal distension (e.g. CRD) in the same rabbit. Colorectal distension increased arterial pressure and decreased respiratory rate prior to blood withdrawal. However, the biphasic nature of the changes in arterial pressure and heart rate associated with hemorrhage was not altered by noxious colorectal distension.

The cardiovascular responses of males and females during hemorrhage are plotted against total blood loss in Figure 4. The patterns of change in arterial pressure, heart rate and respiratory rate were qualitatively similar for all three treatments in males and females. Hemorrhage produced a biphasic response in

arterial pressure with maintenance early in hemorrhage followed by an abrupt drop in pressure. Heart rate increased steadily during Phase 1 before falling during Phase 2. Compared to Control, Sham CRD and CRD treatments appeared to decrease the ability of females to maintain blood pressure in the face of hemorrhage. The blood loss required to produce hypotension (mean arterial pressure $\leq$ 40 mmHg) in males and females is shown in Figure 5.

Previous work in our laboratory has shown that the decrease in arterial pressure at the transition from Phase 1 to Phase 2 of hemorrhage in conscious rabbits predictably occurs between 80 – 85 percent of the total blood loss (119). Therefore, we chose to statistically compare cardiorespiratory parameters among the three treatments at 0, 80 and 105 percent of total blood loss. These three points represent pre-hemorrhage, the end of Phase 1, and during Phase 2, respectively (Figure 6). Arterial pressure was 15 – 20 mmHg higher in the CRD treatment compared with Sham CRD and Control at 0 and 80 percent of total blood loss. Pre-hemorrhage arterial pressure was 5 mmHg higher in the Sham CRD treatment compared to Control, but was not different from Control at 80 percent total blood loss. Mean arterial pressure was statistically similar for all treatments at 105 percent of total blood loss (i.e. during Phase 2). Heart rate during CRD was 10 – 15 percent higher than the other two treatments at pre-hemorrhage, the end of Phase 1 and during Phase 2. Heart rate does not appear to differ between 80 percent and 105 percent in Control or Sham CRD. However, this is somewhat misleading because the points graphed in Figure 6 do not reflect the fact that heart rate peaks at approximately 90 – 95 percent of total

blood loss (i.e. during the transition to phase 2) and then falls (see Figure 4). While respiratory rate appeared to be lower during CRD before hemorrhage, there were no significant differences among the treatments at 0, 80 or 105 percent total blood loss. Colorectal balloon pressure was 53 ± 3, 44 ± 3, and 47 ± 3 mmHg at 0, 80, and 105 percent total blood loss, respectively. There was no significant interaction of sex and time for colorectal balloon pressure (P=0.86), nor was there a significant main effect of sex (P=0.06) or time (P=0.11).

Sex differences in response to hemorrhage plus colorectal distension. There were no differences between males and females in each treatment at baseline (see above). Nor were there differences between males and females with respect to mean arterial pressure or respiratory rate pre-hemorrhage, during Phase 1 or Phase 2 (0, 80 and 105% total blood loss; Figure 6). However, there was a significant interaction of sex and time for heart rate during the CRD treatment (P<0.001). While males (166 ± 2 bpm) and females (167 ± 2 bpm; P=0.88) were similar pre-hemorrhage in the CRD treatment, heart rates were higher in males (290 ± 2 and 276 ± 2 bpm) compared with females (250 ± 2 and 236 ± 2 bpm) at 80 (P<0.001) and 105 (P<0.001) percent of total blood loss, respectively.

When the sexes were pooled, there was no difference in the blood loss required to produce hypotension in Control, Sham CRD and CRD treatments (21.7 ± 0.3, 21.0 ± 0.3, and 20.9 ± 0.3 ml/kg, respectively). However, the 2-way ANOVA for repeated measures indicated that there was a significant interaction of sex and treatment (P=0.006). Post-hoc analysis revealed no difference in

blood loss between males (21.8 ± 0.3 ml/kg) and females (21.6 ± 0.3 ml/kg) in the Control treatment (Figure 5). The difference in blood loss between males (22.1 ± 0.3 ml/kg) and females (20.0 ± 0.3 ml/kg) during Sham CRD did not reach significance (*P*=0.08). However, during CRD, females (19.1 ± 0.3 ml/kg) required significantly less blood removal than males (22.8 ± 0.3 ml/kg; *P*=0.005) to become hypotensive. Within each sex, differences among treatments were compared. In females, total blood loss tended to decrease during Sham CRD (*P*=0.07), and was significantly less than Control during CRD (*P*=0.004). By contrast, in males there was no difference from Control blood loss during Sham CRD (*P*=0.90) or CRD (*P*=0.33).

Recovery. There was no difference in mean arterial pressure among the three treatments during Phase 2 hypotension (see Figure 6). However, at the end of recovery, mean arterial pressure in the CRD treatment (64 ± 2 mmHg) was significantly higher than Control (56 ± 2 mmHg; *P*=0.02), and tended to be higher than Sham CRD (57 ± 2 mmHg; *P*=0.05). There was not a significant interaction of sex and treatment (*P*=0.39), nor a significant main effect of sex (*P*=0.81) for mean arterial pressure at the end of the recovery period.

DISCUSSION

Traumatic blood loss is the leading cause of death in battlefield conditions (9), and contributes significantly to mortality in the civilian population as well (96;109;123). Since pain modifies cardiovascular (1;11;12;97) and respiratory

control (23;98), it seems likely that pain may alter the response to blood loss. Improving the management of trauma patients suffering from blood loss requires a better understanding of how the cardiovascular and respiratory response to hemorrhage is altered in the presence of painful stimuli.

The purpose of this study was to assess the effect of a noxious visceral stimulus on the response to blood loss. Conscious, chronically instrumented rabbits were used to avoid the effects of anesthesia and acute surgical preparation. We hypothesized that colorectal distension would increase the total blood loss necessary to produce hypotension. If the response to the noxious stimulus was “protective” as expected, then a greater volume of blood loss would be required to achieve hypotension. The important new finding of this study is that while noxious visceral stimulation increased blood pressure, it did not increase tolerance to blood loss as predicted. In fact, in females, colorectal distension decreased the blood loss required to produce hypotension.

Noxious stimulation and blood loss. Several earlier studies have tried to assess the impact of noxious stimuli and/or injury on the response to blood loss. Kirkman, Little and their colleagues (41;77;99;100) have used afferent nerve stimulation as a model of noxious somatic stimulation and/or tissue injury. In acutely prepared, anesthetized pigs, brachial nerve stimulation was associated with lower blood pressure following a fixed-volume hemorrhage (40% blood volume) compared with control (100). The authors concluded that painful stimuli compromised maintenance of blood pressure. However, details related to cardiovascular changes during blood loss (e.g. the blood loss required to

produce hypotension) were not provided. Thus, we are unable to directly compare their results with our own. In another study, this same group (99) compared the effects of skeletal muscle injury or nerve stimulation on the response to blood loss in anesthetized pigs. All animals were acutely prepared for invasive cardiovascular measurements. A captive bolt was used to inflict bilateral hindlimb trauma in one group, and bilateral brachial nerve stimulation was performed in a second group. Muscle injury had no effect on baseline blood pressure, whereas brachial nerve stimulation increased arterial pressure by 20 percent. Mean arterial pressure at the end of hemorrhage was higher than control in the nerve stimulation group, and less than control in the muscle injury group. The authors concluded that muscle injury had a greater deleterious effect on the maintenance of arterial pressure during blood loss compared to nerve stimulation. In another investigation, Kirkman and colleagues demonstrated that thoracic blast injury in anesthetized rats blunted the tachycardia associated with Phase 1 of hemorrhage, and decreased the blood loss required to reduce arterial pressure to 40 mmHg (110). From this study, it appears that traumatic injury alters the cardiovascular response to hemorrhage and reduces the animals' tolerance to blood loss.

On the other hand, according to additional studies by Kirkman and colleagues, injury may have a protective effect on maintenance of arterial pressure during hemorrhage. For example, during brachial nerve stimulation, anesthetized pigs were better able to maintain blood pressure in the face of 30 percent blood volume loss compared with control (41;77). In contrast to control

animals in these studies, “injured” animals did not become hypotensive (MAP < 65 mmHg) suggesting that injury conferred some degree of cardiovascular protection during hemorrhage. In an investigation of ischemic muscle injury plus hemorrhage in acutely instrumented rats, tourniquets were placed around both hindlimbs under brief anesthesia and animals were allowed to recover (69). Hemorrhage was then performed in conscious animals. Tourniquet application increased blood loss required to produce hypotension. Thus, in this study, muscle injury provided protection against hemorrhagic hypotension.

Considering this earlier work, while most studies suggest that noxious stimulation and/or injury are deleterious to maintenance of arterial pressure during hemorrhage (99;100;110), some have reported that injury enhances maintenance of blood pressure during blood loss (41;69;77). Interpretation of the results from these studies, as well as direct comparisons with the work reported here, is complicated by anesthesia (41;77;99;100;110), or by limited time for recovery (69).

Colorectal distension and the response to blood loss. Colorectal distension is a model of visceral pain that has been used in human volunteers (102), as well as conscious rabbits (25;54;55;95), rodents (57;58;91) and horses (130). We have recently evaluated the physiological effects of colorectal distension in chronically instrumented, conscious rabbits (124) and have found that this model of pain causes short-lived physiological responses, and no lasting tissue damage.

Traumatic injury is often associated with damage to skin, muscle or bone, and such injuries are typically accompanied by somatic pain. Though injury accompanies traumatic blood loss, the fact that we chose a model of visceral pain that was not associated with overt tissue damage may be considered a limitation of this study. However, while noxious colorectal distension may not be an ideal model for traumatic blood loss, it is a clinically relevant model of pain during blood loss. For example, it is not uncommon for hemorrhage to occur in combination with obstetrical pain or gastrointestinal distress.

Some consider distension of the colon to be a psychological stressor (75). We know that noxious colorectal distension increases stress hormones in humans [e.g. adrenocorticotropic hormone (ACTH) and cortisol (75)], and conscious rats [e.g. corticosterone (35)]. In addition, colorectal distension activates brain regions also stimulated by other psychological stressors (139). Thus, we predicted that colorectal distension would alter the response to blood loss like other psychological stressors. However, the effects of colorectal distension during hemorrhage differ from those of acute psychological stressors such as air jet and oscillation. The biphasic pattern of change in arterial pressure during hemorrhage appeared to be similar during exposure to air jet, oscillation or colorectal distension, yet the blood loss required to produce hypotension was different. Air jet stress and oscillation significantly increase tolerance to hemorrhage in conscious rabbits (114;117). In fact, it is not uncommon for animals to reach maximum blood loss (i.e. 45% of blood volume) without developing hypotension during oscillation stress (117). For this reason, it was

surprising to find that colorectal distension did not increase blood loss required to produce hypotension.

While colorectal distension, air jet, and oscillation may all be considered psychological stressors (75;113), their cardiorespiratory effects differ. Air jet stress produces cardiovascular changes consistent with the defense reaction in conscious rabbits (e.g. increases in arterial pressure and heart rate) (113). Like the freezing response seen in some animals (133), oscillation stress is associated with moderate increases in arterial pressure and little change in heart rate (113). The increase in arterial pressure following colorectal distension in this study was comparable to the increase seen following air jet stress. However, in contrast to air stress, colorectal distension produced only slight, short-lived increases in heart rate (e.g. more like oscillation stress (113)). In our laboratory, air jet (134) and oscillation (unpublished observations) stress both increase respiratory rate in conscious rabbits, whereas colorectal distension decreases respiratory rate (124) (Table 1 & 2). Differences in the physiological response to air jet, oscillation and colorectal distension suggest differential alterations in cardiorespiratory control in response to these three stressors.

As mentioned earlier, colorectal distension increased arterial pressure similar to air jet and oscillation stress. We did not perform experiments controlling for the elevation in blood pressure in this study. However, in earlier work, we have used an infusion of phenylephrine to increase pre-hemorrhage blood pressure. Phenylephrine did not qualitatively alter the biphasic response to hemorrhage, but it did significantly increase total blood loss (61). While both

phenylephrine and colorectal distension increase pre-hemorrhage blood pressure, phenylephrine increases total blood loss and colorectal distension does not. Consequently, it appears that colorectal distension alters cardiovascular control during blood loss in a way different than air jet, oscillation or phenylephrine infusion.

We assumed that the cardiovascular response to colorectal distension did not change over the course of this study. Prior exposure to stress has been associated with increased sensitivity to distension of the colon in conscious rats (148). Hemorrhage and noxious stimuli are considered stressors (2), therefore, it is possible that rabbits developed sensitization to colorectal distension during this study. If rabbits became more (or less) sensitive to the noxious visceral stimulus, we would have expected the response to repeated colorectal distension to change. Others have reported that colorectal distension is associated with reproducible cardiovascular responses in conscious rats (91). Likewise, our own experiments demonstrate that the cardiorespiratory response to colorectal distension appears similar following repeated experiments (see Table 1). In addition, we performed the three treatments in a balanced design to minimize any effect of time on the response to the different treatments.

Sex differences. One of our important new findings was that sex differences existed in the effects of noxious visceral stimulation on the response to hemorrhage. Specifically, colorectal distension decreased tolerance to blood loss in females, but not in males. This is in contrast to the results from the Control experiments reported here, and also previous studies in our laboratory

(unpublished observations) where males and females had similar blood loss under control conditions. The occurrence of sex differences during CRD points to the potential involvement of sex hormones. Rabbits are classified as induced ovulators (also referred to as “reflex ovulators”) and do not have a spontaneous estrous cycle (22). For this reason we did not control for estrous cycle in our female rabbits.

The fact that, in females, Sham CRD and CRD altered total blood loss in a similar manner (see Figure 5) suggests that colorectal balloon catheter placement and not inflation accounted for at least part of the decreased tolerance to hemorrhage. The 10 percent increase in pre-hemorrhage arterial pressure associated with Sham CRD (see Figure 6) is also consistent with some effect of placement of the colorectal balloon catheter. Since mechanical stimulation of the vagina, mounting by other rabbits, and excitement are all reported to induce ovulation in rabbits (22), the possibility exists that placement of the colorectal balloon catheter stimulated ovulation and subsequent neurohumoral changes. In rabbits, ovulation occurs 10 - 12 hr after stimulation. In the absence of fertilization, a state of pseudo-pregnancy ensues, during which the ovary secretes progesterone for approximately 2 weeks (22). Thus, hormonal changes associated with ovulation in rabbits occur on the order of hours to days, in other words, on a time scale that might be expected to alter cardiovascular function over the course of multiple hemorrhage experiments performed in this study. As part of the training for this study, rabbits underwent brief colorectal balloon placement the day prior to experiments regardless of treatment (see Methods),

and importantly, a colorectal distension experiment was performed within 4 days of all but one of the Control experiments. If neurohumoral changes associated with ovulation accounted for the observed sex differences, we would have expected to see that blood loss in females was decreased during Control experiments as well. Others have reported that tolerance to blood loss decreases in conscious, term-pregnant rabbits (13). While the mechanisms are unknown, they may be related to those responsible for decreased tolerance to blood loss seen in female rabbits in this study. Further evaluation of sex hormone levels is warranted to investigate their potential role in acutely altering the response to blood loss in female rabbits during colorectal distension.

It is possible that an acute neurohumoral change, not related to sex hormones, was associated with placement of the colorectal balloon catheter, and that this change decreased tolerance to blood loss in females. Because these animals had undergone noxious colorectal distension experiments prior to the hemorrhage series, the presence of the balloon catheter may have been associated with anticipation of noxious stimulation. Consequently, placement of the balloon catheter may have been an acute anticipatory stressor that differentially altered the response to blood loss in males and females. One piece of evidence suggests that anticipation of a noxious visceral stimulus is a stressor that may be capable of acutely altering neurohumoral function. Specifically, anticipation of noxious colorectal distension is associated with increased plasma ACTH and cortisol in human subjects (75). A second piece of evidence suggests that sex differences may exist in the response to anticipation stress. In a recent

study, women had increased neuronal activity compared with men in regions of the brain associated with fear and anxiety during anticipation of an electrical shock (15). Potential sex differences in the neurohumoral response to anticipation of noxious visceral stimulation might account, in part, for the decreased tolerance to blood loss seen in females in the current study.

Recovery from blood loss. Noxious colorectal distension was associated with increased recovery of arterial blood pressure after hypotensive hemorrhage. Distension of the colon may have resulted in release of neurohumoral agents that aid in recovery of blood pressure and/or plasma volume after blood loss. This appears to be the case in anesthetized rats where vasopressin levels increased following repeated colorectal distension (49). Thus, improved recovery of arterial pressure following CRD may have been due to noxious stimulus-induced release of vasopressin or some other vasoactive compound.

In conclusion, noxious visceral stimulation alters cardiovascular control during hemorrhage. Colorectal distension increases pre-hemorrhage blood pressure but does not change the biphasic nature of the response to blood loss. We hypothesized that, like other psychological stressors, colorectal distension would increase blood loss required to produce hypotension in conscious male and female rabbits. In contrast, our results suggest that colorectal distension decreases tolerance to blood loss in females and does not change blood loss in males.

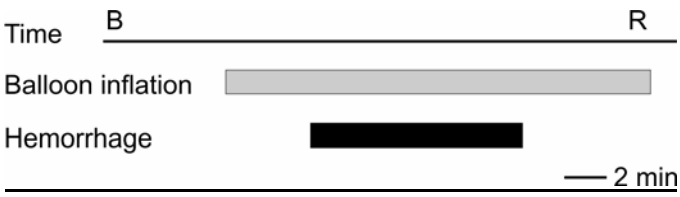

Figure 1. Timeline for hemorrhage experiments. The duration of hemorrhage was approximately 10 min. Measurements were compared at baseline (B), during hemorrhage, and at the end of a 5 min recovery period (R).

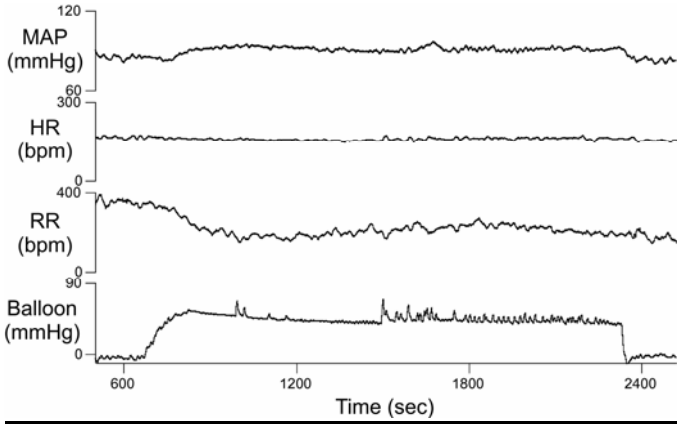

Figure 2. Cardiovascular and respiratory changes during a 25 min colorectal distension experiment in a male rabbit. The colorectal balloon was inflated until mean arterial pressure (MAP) increased by 10 – 15 mmHg. This distension was sustained for 25 min. HR, heart rate; RR, respiratory rate; Balloon, colorectal balloon pressure.

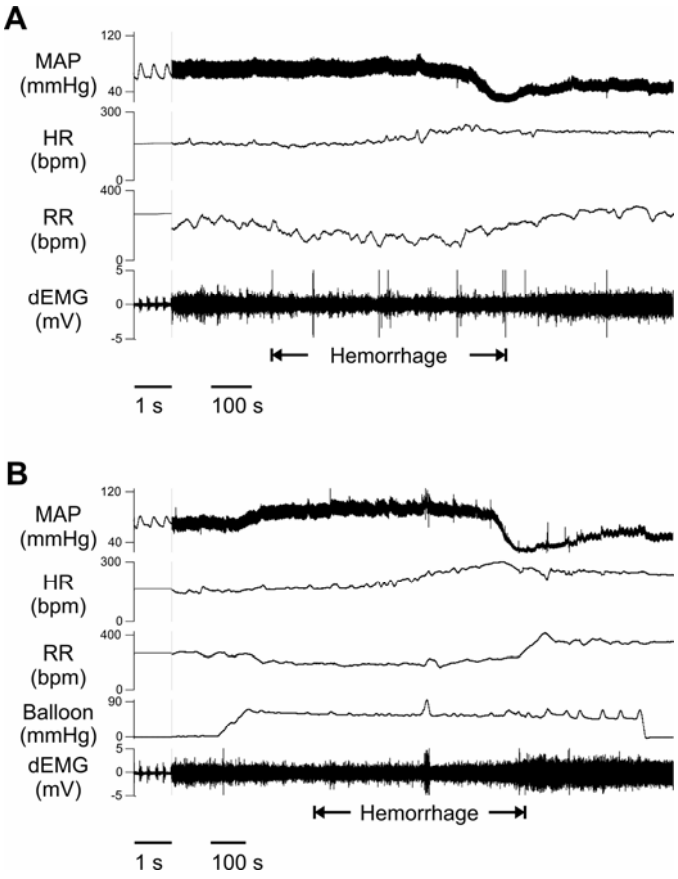

Figure 3. Cardiovascular and respiratory response to hemorrhage during Control (A) and CRD (B) treatments in the same male rabbit. At the beginning of each record is a 1-sec inset on an expanded time scale illustrating pulsatile arterial pressure and inspiratory bursts of diaphragmatic EMG (dEMG) activity. Abbreviations as in Figure 2.

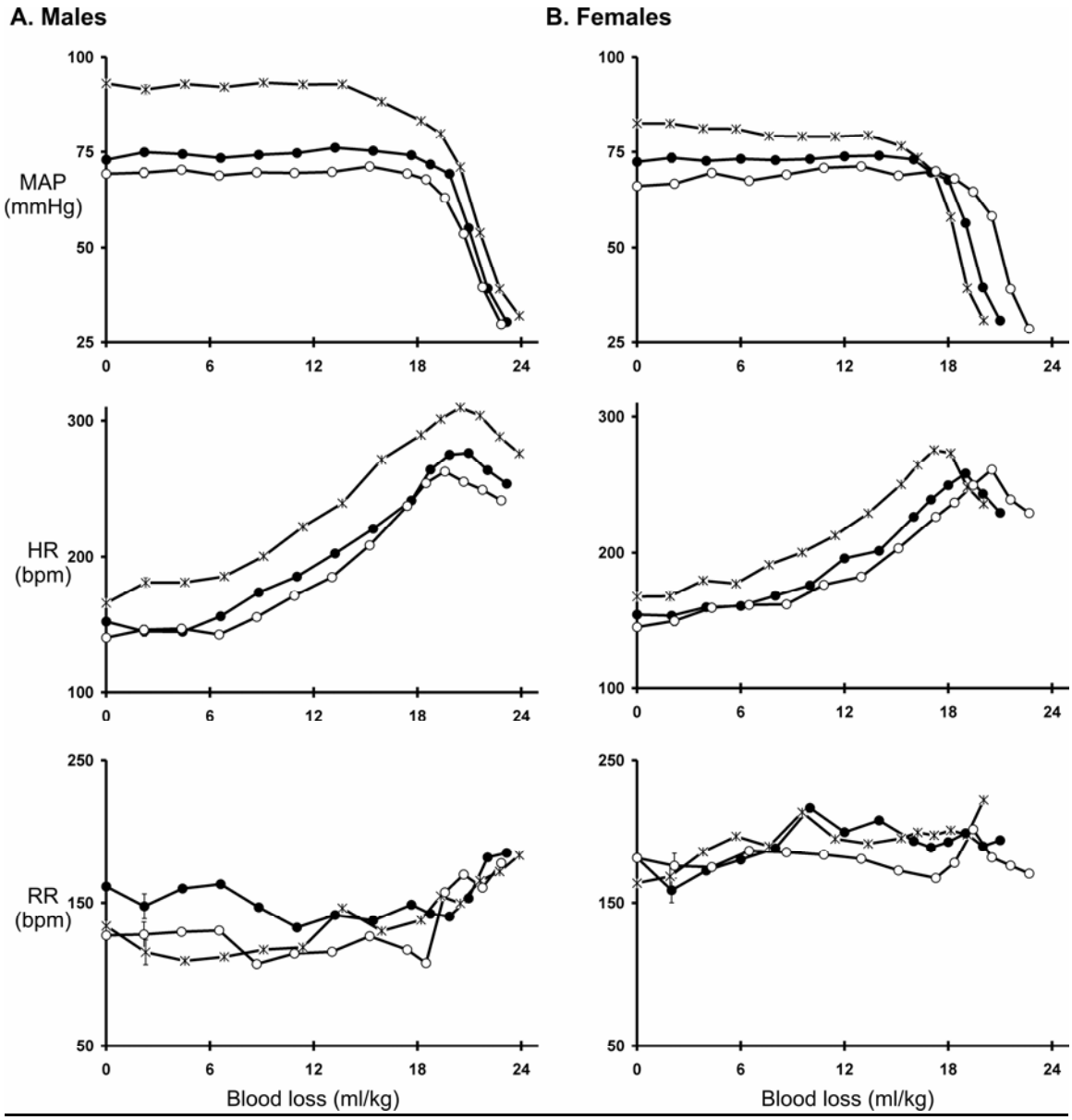

Figure 4. Average changes in cardiovascular and respiratory parameters for males (A) and females (B) during hemorrhage with three treatments: Control (open symbols), Sham CRD (filled symbols), and CRD (asterisks). Blood loss adjusted to body weight is on the abscissa. Abbreviations as in Figure 2. Error bars (SE) are shown on the second data point (in some cases obscured by

symbol). At the start of hemorrhage, colorectal balloon pressure was 55 ± 3 mmHg in males and 50 ± 3 mmHg in females, whereas at the end of hemorrhage, colorectal balloon pressure was 50 ± 3 and 42 ± 3 mmHg in males and females, respectively.

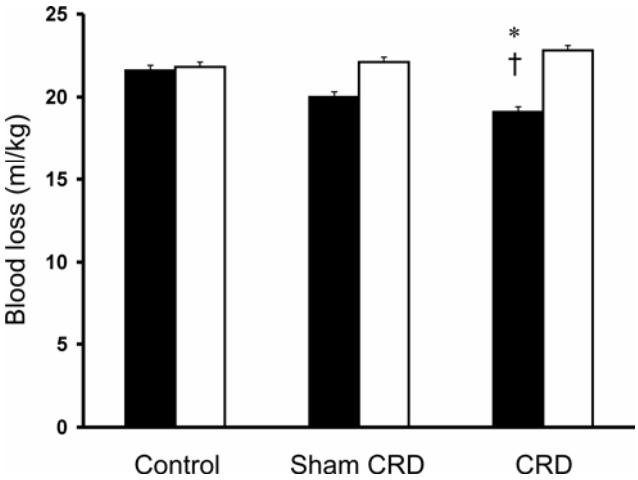

Figure 5. Blood loss required to produce hypotension in females (filled bars) and males (open bars) in 3 treatments: Control, Sham CRD and CRD. There was no difference between females and males during Control (*P*=0.89). Placement of the colorectal balloon catheter (Sham CRD) tended to reduce tolerance to blood loss in females compared to Control (*P*=0.07). Females tolerated significantly less blood loss during CRD compared with Control (*P*=0.004). Within the CRD treatment, blood loss was significantly less in females compared to males (*P*=0.005). * Significantly different from Control (*P*<0.05). † Significantly different from males within CRD (*P*<0.05).

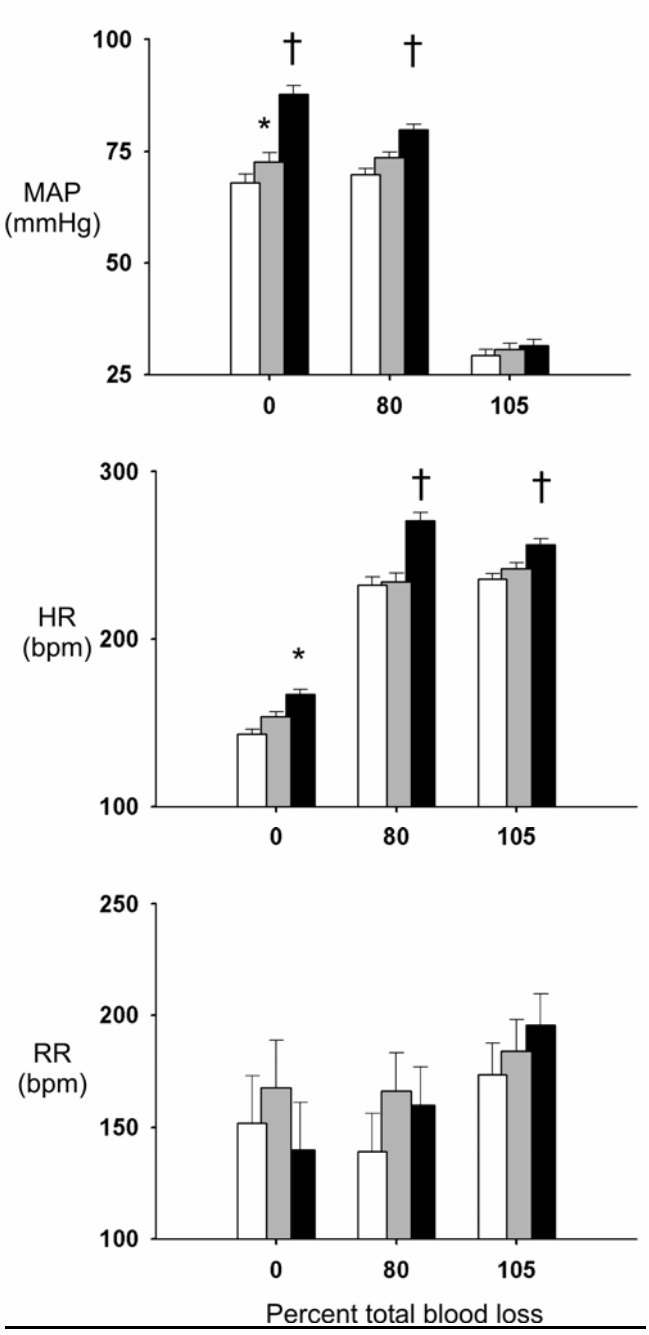

Figure 6. Values (mean ± SE) for mean arterial pressure (MAP), heart rate (HR), and respiratory rate (RR) at 0, 80, and 105 percent of total blood loss. Bars represent Control (open), Sham CRD (gray), and CRD (black) treatments. Pre-

hemorrhage MAP was higher than Control in Sham CRD (P=0.04) and CRD treatments (P<0.001). At 80 percent total blood loss, MAP remained significantly higher in the CRD treatment compared to Sham CRD (P<0.001) and Control (P<0.001). There was no difference in MAP among the treatments at 105 percent (P=0.19). HR was significantly higher in the CRD treatment compared with Sham CRD and Control at all three times (P<0.001). There were no differences in RR among the treatments at any time point. * Significantly different from Control (P<0.05). † Significantly different from Sham CRD and Control (P<0.05).

Table 1. *Cardiorespiratory response to repeated 9.5 min colorectal distension*

	Baseline				**Start**				**End**				**Release**			
Exp #	MAP	HR	RR	CRB	MAP	HR	RR	CRB	MAP	HR	RR	CRB	MAP	HR	RR	CRB
1	72	170	269	-4	85	174	167	46	81	173	198	37	71	168	220	-5
2	71	164	224	-4	84	173	147	49	83	174	157	35	72	166	172	-5
3	69	157	208	0	83	166	159	47	83	165	169	39	69	154	183	-1
4	69	155	212	-2	82	162	142	46	77	161	168	34	71	155	162	-2
SE	1	4	28	2	2	4	14	3	2	5	13	2	2	4	21	1
P	0.28	0.10	0.43	0.30	0.73	0.15	0.63	0.92	0.16	0.23	0.21	0.35	0.95	0.08	0.28	0.11

Mean values for 4 separate 9.5 min colorectal distension experiments performed in 5 male rabbits during a 2 week period. Values are 30 sec averages taken at 4 time points: before colorectal distension (Baseline); at the Start of the colorectal distension experiment; at the End of the distension; and after Release (following release of pressure within the colorectal balloon). MAP, mean arterial pressure (mmHg); HR, heart rate (bpm); RR, respiratory rate (bpm); CRB, colorectal balloon pressure (mmHg). SE was calculated using the pooled estimate of the variance from the ANOVA performed for each time period. *P* values for each analysis are reported in the last row.

Table 2. *Stability of cardiorespiratory changes during a 25 min colorectal distension*

	Baseline	**Start**	**End**	**Release**
MAP (mmHg)	72 ± 1	86 ± 1 *	83 ± 1 *	75 ± 1
HR (bpm)	152 ± 4	167 ± 4	163 ± 4	153 ± 4
RR (bpm)	207 ± 15	141 ± 15 *	134 ± 15 *	136 ± 15 *
Balloon (mmHg)	0 ± 3	50 ± 3 *	38 ± 3 *	0 ± 3

Values (mean ± SE) for mean arterial pressure (MAP), heart rate (HR), respiratory rate (RR) and colorectal balloon pressure (Balloon) at 4 times (see Table 1) during a 25 min colorectal distension experiment in 8 rabbits (2 female). There was a significant main effect of time for MAP ($P<0.001$), HR ($P=0.02$), RR ($P=0.006$) and Balloon pressure ($P<0.001$), however there were no differences between individual points for HR. There was no significant difference in MAP between the Start and End of the distension ($P=0.18$). * Statistically different from baseline ($P<0.05$).

Table 3. *Baseline values for Control, Sham CRD and CRD treatments*

Treatment:	**Control**	**Sham CRD**	**CRD**
MAP (mmHg)	69 ± 1	72 ± 1	72 ± 1
HR (bpm)	148 ± 4	149 ± 4	156 ± 4
RR (bpm)	187 ± 27	185 ± 27	199 ± 27
Balloon (mmHg)	NA	0 ± 1	1 ± 1

Values (mean ± SE) are 20 sec averages at baseline during hemorrhage experiments. SE from pooled estimates by ANOVA as in Table 1. There were no differences among treatments for mean arterial pressure (MAP; P=0.10), heart rate (HR; P=0.27), respiratory rate (RR; P=0.78) and colorectal balloon pressure (Balloon; P=0.38). N=12

CHAPTER 9

The effect of noxious stimulation plus acute stress on the response to blood loss in conscious rabbits.

INTRODUCTION

Traumatic blood loss is frequently accompanied by psychological stress and pain. Evaluation of these stimuli independently has shown that acute psychological stress (114) or noxious visceral stimulation (125) alters the response to blood loss. However, how these two stimuli interact to influence the response to blood loss remains uncertain. We feel that evaluating cardiovascular and respiratory responses to blood loss in a conscious animal in the presence of simultaneous stress and pain may best approximate the response to blood loss in a clinical setting.

The biphasic cardiovascular response to blood loss in conscious animals is characterized by maintenance of arterial pressure during Phase 1, followed by a precipitous drop in pressure leading to Phase 2 hypotension (118). Previous work in our laboratory has demonstrated that air jet, a psychological stressor, increased pre-hemorrhage arterial pressure and increased blood loss required to achieve hypotension (i.e. hypotensive blood loss) (114). Like air jet stress, colorectal distension, a noxious visceral stimulus, increased pre-hemorrhage

arterial pressure in conscious rabbits. Unlike air jet stress, however, colorectal distension decreased hypotensive blood loss in females by approximately 10% and did not change hypotensive blood loss in males (125). There were no differences between males and females in hypotensive blood loss in the absence of colorectal distension. Similarly, there do not appear to be any sex differences with regard to hypotensive blood loss during air jet stress in our laboratory (unpublished observations).

The aim of this study was to evaluate the effect of colorectal distension plus air jet stress on the cardiovascular and respiratory responses to blood loss in conscious, chronically instrumented rabbits. We hypothesized that acute air jet stress plus noxious visceral stimulation would increase total blood loss required to produce hypotension. We predicted that there would be no difference between males and females for the hemodynamic response to hemorrhage, or hypotensive blood loss.

METHODS

Animals and instrumentation. The procedures performed in this study were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (50) and were approved by the University of Missouri Animal Care and Use Committee. Four male and six female New Zealand white rabbits weighing 3.4 $\pm$ 0.1 kg and 3.5 $\pm$ 0.1 kg (mean $\pm$ SE), respectively, were instrumented with indwelling catheters and diaphragmatic electromyographic (dEMG) electrodes. Antibiotics (22.7 mg enrofloxacin, sc; Baytril, Bayer) were administered the day

prior to surgery. Food but not water was withheld 15 – 20 hr prior to surgery. The analgesic buprenorphine hydrochloride (Buprenex, Reckitt Benckiser, 0.06 mg, sc) was administered to all rabbits prior to, and after each surgery. Anesthesia was induced and maintained with halothane in oxygen via face mask (5% for induction, 0.5-3.5% for maintenance) and a midline laparotomy was performed. Non-occlusive venous and arterial catheters were implanted in the abdominal vena cava and aorta (44). The arterial catheter allowed for recording of pulsatile arterial pressure and the venous catheter was used for blood withdrawal and administration of drugs. EMG electrodes (126) were secured in the diaphragm to record inspiratory bursts in diaphragmatic muscle activity and allow monitoring of respiratory rate. A ground electrode was sutured to the abdominal wall. Catheters and EMG wires were tunneled subcutaneously and exteriorized at the base of the neck. A minimum of 2 weeks recovery was allowed before rabbits started experiments.

Colorectal distension. Mechanical distension of the distal colon and rectum was performed by manually inflating a 5 cm balloon with water. A double-lumen balloon catheter allowed monitoring of balloon pressure during inflation. Acute balloon placement was achieved by placing the rabbits on their backs with their eyes covered until they became relaxed (1 – 3 min). The well-lubricated balloon was inserted 9 cm into the rectum and gently secured to the tail, and then rabbits were returned to the rabbit box (33 X 15 X 18 cm) for the experiment. Noxious colorectal distension was performed by manually inflating the balloon until mean arterial pressure increased by 10 – 15 mmHg, or colorectal balloon

pressure reached 90 mmHg. This 90 mmHg limit has been used by others as a means of avoiding tissue damage in conscious rabbits (55). Over a three-week period rabbits underwent progressively longer colorectal distension experiments. Initial duration of the distension was 1 – 2 min and progressed up to 25 min before hemorrhage experiments were performed.

Air jet stress. Pressurized room air was delivered through plastic tubing (Tygon, ID 0.25 in, OD 0.375 in) to a 2 mm opening that was positioned 3 cm from the front of the rabbit box. For air jet stress, a continuous stream of air was directed at the rabbit's nose through a 6 cm diameter hole in the front of the rabbit box. Exposure to air jet stress was limited to once per week.

Colorectal distension, air jet stress and hemorrhage. Experiments were performed on conscious rabbits accustomed to sitting quietly in a rabbit box that restricted their movement. All rabbits underwent training to accustom them to placement of the colorectal balloon catheter ($\leq$ 10 min), and to being in a rabbit box in the laboratory setting. Training sessions were performed several times during the week prior to initiating experiments, and subsequently on each day preceding an experiment. Animals were fasted for 15 – 20 hr prior to each experiment. Acute placement of the colorectal balloon catheter was performed in all treatments. Following colorectal balloon placement, rabbits were heparinized (sodium heparin, 2000 units, iv, Elkins-Sinn), the arterial catheter was connected to a pressure transducer, and the EMG electrode wires were connected to custom-built, differential amplifiers (10X) placed in close proximity to the rabbit.

Mean arterial pressure, heart rate and dEMG activity were monitored continuously throughout each experiment.

The four treatments performed in each rabbit were sham colorectal distension plus hemorrhage (Sham CRD), sham colorectal distension plus air jet stress during hemorrhage (Sham CRD + AIR), colorectal distension plus hemorrhage (CRD) and colorectal distension plus air jet stress and hemorrhage (CRD + AIR). Experiments not involving air jet (e.g. Sham CRD and CRD) were performed before those involving air jet stress (e.g. Sham CRD + AIR and CRD + AIR). This experimental series was performed concurrently with those reported earlier (125) and some of the animals were used in both studies.

The colorectal balloon catheter was present but was not inflated in the Sham CRD, and Sham CRD + AIR treatments. The timeline for experiments is illustrated in Figure 1. During CRD and CRD + AIR experiments, the colorectal balloon catheter was inflated 4 minutes prior to hemorrhage. During air jet stress experiments (e.g. Sham CRD + AIR and CRD + AIR), the air jet was turned on 2 min prior to hemorrhage. Noxious colorectal distension and/or air jet stress were maintained throughout the duration of hemorrhage.

Time was allowed for each rabbit to reach a steady baseline in terms of heart rate and arterial pressure before beginning hemorrhage. Venous blood was withdrawn into sterile syringes at a fixed rate of 8 – 9 ml/min until 5 ml after mean arterial pressure reached 40 mmHg. The limit for blood withdrawal was 45 percent of the animal's calculated blood volume, or 27 ml/kg. None of the animals reached this limit. At the end of the hemorrhage, pressure was released

from within the colorectal balloon by withdrawing water from the catheter, the air jet stress was turned off, and the shed blood was reinfused. Hemorrhage experiments were separated by at least 4 days in individual rabbits.

Data acquisition and analysis. Data were acquired at 4 kHz using a PC-based data acquisition system (Power Lab, AD Instruments, Colorado Springs, CO), and offline analysis of all records was performed using commercially available software (Spike2, CED, Cambridge, UK). Heart rate was derived from the pulsatile arterial pressure signal. The raw dEMG signal was high-pass filtered (200 Hz) to minimize ECG artifact. The DC offset was removed and then the signal was rectified and smoothed (time constant = 50 – 100 msec). Peaks in dEMG activity were used to calculate respiratory rate.

Hemorrhage data were normalized to the percentage of total hypotensive blood loss because the amount of blood withdrawn to produce hypotension varied among rabbits. Hypotensive blood loss (100%) was defined as the amount of blood withdrawn when mean arterial pressure fell to 40 mmHg. Total hypotensive blood loss was compared between treatments. Because an additional 5 ml of blood was withdrawn after mean arterial pressure reached 40 mmHg, it was possible to evaluate the effects of each treatment from 0 – 105 percent of hypotensive blood loss. Previous work in our laboratory has shown that the transition from Phase 1 to Phase 2 of hemorrhage in conscious rabbits predictably occurs between 80 – 85 percent of hypotensive blood loss (119). Thus, we evaluated the cardiorespiratory differences between treatments prior to hemorrhage (pre-hemorrhage), at the end of Phase 1, and during Phase 2.

These time points corresponded to 0, 80 and 105 percent of hypotensive blood loss.

The effect of colorectal distension on the response to blood loss has been reported elsewhere (125). In the current study we were interested in the effect of air jet stress plus colorectal distension on the cardiorespiratory response to hemorrhage. Thus, for analyses, we evaluated the effect of air jet stress on the response to blood loss in the absence of colorectal distension by comparing Sham CRD and Sham CRD + AIR treatments. Similarly, the effect of air jet stress on the response to blood loss in the presence of colorectal distension was evaluated by comparing CRD with CRD + AIR treatments. We used repeated measures analysis of variance (ANOVA) to compare mean arterial pressure, heart rate, respiratory rate and colorectal balloon pressure among treatments (SigmaStat v3.1, Systat Software, Inc., Richmond, CA). The independent variables for the ANOVA were treatment and sex. Data are presented as mean $\pm$ SE. The SE was calculated from the pooled estimate of the variance from the associated ANOVA. The Bonferroni multiple comparison test was used for post-hoc analyses. Significance for all analyses was set at $P < 0.05$.

RESULTS

There were no differences among the four treatments prior to colorectal distension or air jet stress (data not shown). The changes in mean arterial pressure, heart rate, respiratory rate and colorectal balloon pressure associated with each of the four treatments is summarized in Table 1. Exposure to air jet

stress or inflation of the colorectal balloon increased mean arterial pressure by 26 and 21 percent, respectively. The combination of colorectal distension plus air jet stress increased arterial pressure by 29 percent. Air jet stress, colorectal distension and the combination of the two increased heart rate by 14 – 17 percent. Respiratory rate was approximately doubled in the presence of air jet stress.

Examples of the cardiovascular and respiratory response to hemorrhage during the four treatments in a conscious female rabbit are shown in Figure 2. The average changes in cardiovascular and respiratory parameters for males and females over the course of progressive blood withdrawal are plotted in Figure 3. None of the treatments altered the biphasic nature of the cardiovascular response to blood loss in males or females. Apparent differences between the sexes are addressed in analyses below.

The graphs of mean arterial pressure for males and females during hemorrhage (Figure 3) illustrate that during treatments with air jet stress animals were able to maintain arterial pressure in the face of greater blood loss before becoming hypotensive. While colorectal distension and air jet stress both increased pre-hemorrhage arterial pressure, hypotension occurred after withdrawal of less blood during the CRD treatment compared to the Sham CRD + AIR treatment. In these experiments, sex (P=0.02) and treatment (P<0.001) affected hypotensive blood loss (Figure 4), but there was not a significant interaction between sex and treatment (P=0.96). For all treatments, females required approximately 3 ml/kg (14%) less blood loss to produce hypotension

compared with males. Exposure to air jet stress in the absence (Sham CRD + AIR) and presence of colorectal distension (CRD + AIR) increased hypotensive blood loss in both males and females by approximately 2 ml/kg, or 10 percent.

We evaluated the nature of the interaction between air jet and colorectal distension with regard to blood loss. We predicted that the change in hypotensive blood loss attributable to air jet stress [(Sham CRD + AIR) – (Sham CRD)] plus the change associated with colorectal distension [(CRD) – (Sham CRD)] would be equal to the change in blood loss seen with colorectal distension plus air jet stress [(CRD + AIR) – (Sham CRD)]. In other words, we predicted that the effects of the two stimuli on hypotensive blood loss would be additive. The increase in hypotensive blood loss associated with air jet stress was not different between males (2 $\pm$ 1 ml/kg) and females (2 $\pm$ 1 ml/kg; P=0.89). The change in blood loss associated with colorectal distension was slight, and not significantly different between males (0 $\pm$ 1 ml/kg) and females (-1 $\pm$ 1; P=0.6). Thus we predicted that colorectal distension plus air jet stress would increase hypotensive blood loss by approximately 2 ml/kg in males and 1 ml/kg in females. The increase in blood loss observed in the CRD + AIR treatment compared with the Sham CRD treatment was 2 $\pm$ 1 ml/kg in males and 2 $\pm$ 1 ml/kg in females. There was no difference between the predicted and observed changes (P=0.68).

The effect of treatment and sex was evaluated for arterial pressure, heart rate and respiratory rate prior to hemorrhage, at the end of Phase 1 and during Phase 2. Differences attributable to air stress in the absence and presence of

colorectal distension are illustrated in Figure 5 A and B, respectively. Sham CRD + AIR was associated with higher arterial pressure, heart rate and respiratory rate compared with Sham CRD, except during Phase 2 when arterial pressures were similar. Before hemorrhage there was a significant interaction between sex and treatment where arterial pressure was higher in males in the CRD treatment, and higher in females in the CRD + AIR treatment. There was not a significant main effect of treatment (e.g. CRD or CRD + AIR) for arterial pressure at any time point. At the end of Phase 1 heart rate was higher in the CRD + AIR treatment compared with CRD. Respiratory rates were significantly higher in the CRD + AIR treatment throughout hemorrhage.

DISCUSSION

Stress and pain frequently accompany traumatic blood loss. A better understanding of the effects of psychological stress and noxious stimulation on the cardiovascular and respiratory response to hemorrhage may lead to improvement in the management of trauma patients. Thus, the purpose of this study was to evaluate the effect of simultaneous stress and noxious stimulation on the response to blood loss in conscious rabbits. We evaluated the response to blood loss in the presence of both a noxious visceral stimulus and an acute psychological stressor. Colorectal distension is a model of visceral pain in rabbits (25;54;55;95) and human volunteers (10;33;75) that reproducibly increases arterial pressure (90;125). Air jet stress is an acute environmental stressor that produces cardiovascular changes consistent with the defense

response in conscious rabbits (e.g. increased blood pressure and heart rate) (113). To our knowledge this is the first study to evaluate the effect of a noxious stimulus in the presence of a psychological stressor on the response to blood loss in conscious animals. Previous studies have demonstrated that noxious stimuli and psychological stressors alter cardiovascular function. For example, arterial baroreflex function is altered by noxious visceral stimulation in rats (93;98) and human volunteers (142), and in conscious rats (46) and rabbits (113) exposed to air jet. The independent effects of a psychological stressor and noxious visceral stimulation on the cardiovascular response to hemorrhage have been reported (114;125). As seen in this study, both air jet and colorectal distension increase pre-hemorrhage arterial pressure. However, their effects on the response to hemorrhage are distinctly different. Air jet stress increased hypotensive blood loss (114) while noxious colorectal distension did not change hypotensive blood loss in males and reduced tolerance to blood loss in females (125). Based upon these earlier studies, we hypothesized that colorectal distension plus air jet stress would increase the blood loss required to reduce mean arterial pressure to 40 mmHg. Conscious, chronically instrumented rabbits were used to avoid the effects of anesthesia and acute surgical preparation. Each rabbit underwent four treatments. The treatment of primary interest was CRD + AIR, during which colorectal distension and air jet stress were initiated prior to hemorrhage. The other treatments served as controls for the effects of colorectal distension (Sham CRD and Sham CRD + AIR) and air jet stress (Sham CRD and CRD) during hemorrhage. The important new finding of this study was

that colorectal distension and air jet stress had an additive effect on total blood loss required to produce hypotension.

Many studies have evaluated the response to a variety of stressors independently, yet few have studied the physiological responses to concurrent stressors. In the real world we are often exposed to a mixture physical and psychological stressors simultaneously. In the present study, we report the physiological effects of two psychological stressors (e.g. air jet and noxious stimulation) on the response to a physical stressor (e.g. hemorrhage). Two hypotheses regarding the physiological response to concurrent stressors have been proposed (106). The *masking hypothesis* states that concurrent physical and psychological stressors induce a response that is no greater than the maximal response to a single stressor (i.e. the response to one stressor is masked by that to another). The *synergistic hypothesis* states that the physiological response to concurrent physical and psychological stressors is greater than the response induced by each stressor independently (i.e. the sum of the responses is greater than that to a single stressor). We interpret the synergistic hypothesis to include physiological responses to concurrent stressors that are additive. The results from this study support both hypotheses. First, the respiratory response appeared to fit the masking hypothesis because the increase in respiratory rate associated with air jet stress was not different in the presence or absence of colorectal distension (see Table 1 & Figure 5). Second, the effect of air jet stress plus colorectal distension on total hypotensive blood loss appeared to be additive, that is to say that the increase in hypotensive blood

loss seen in CRD + AIR was similar to the predicted increase based upon the changes in blood loss associated with CRD and with Sham CRD + AIR. Given the complexity of the neurohumoral response to blood loss in conscious animals (118), it is not surprising that concurrent stressors would differentially affect cardiovascular and respiratory control during hemorrhage. However, evaluating of the specific interactions among each of the three stressors presented in this study remains difficult because the physical stressor, hemorrhage, does not produce steady state changes in cardiovascular or respiratory parameters.

Females tolerated significantly less blood loss in all four treatments. Unpublished results from our laboratory failed to reveal sex differences in the effect of air jet stress alone on the response to blood loss. However, previous work suggested that simple placement of a colorectal balloon catheter tended to reduce tolerance to blood loss in females (125). Because of training and previous experiments, placement of the balloon catheter may have been associated with anticipation of noxious colorectal distension. It is possible that females are more sensitive to anticipation stress compared to males. Although we did not test the effect of air jet stress on the response to hemorrhage in the absence of a colorectal balloon catheter in this study, the results of this study suggest that increases in hypotensive blood loss associated with air jet are similar for male and female rabbits.

Prior exposure to acute stressors has been associated with visceral analgesia (4). While initiation of the noxious visceral stimulus preceded the onset of the air jet stress in all experiments, it is possible that air jet stress

modified the response to the noxious stimulus. The experiments performed in this study were designed to test the effects of a combination of stressful and painful stimuli on the response to hemorrhage. Further evaluation of the effects of stress on visceral noxious stimulation, and vice versa, are warranted.

Typically hemorrhage experiments are performed in the quiet, controlled laboratory setting in the absence of stressful sensory stimuli. In this study, experiments evaluating the response to hemorrhage were performed with concurrent psychological stress and a noxious stimulus to better approximate the clinical setting where stress and pain accompany traumatic blood loss. We focused our analyses on the effect of air jet stress 1) in the absence of colorectal distension, and 2) in the presence of colorectal distension to determine if the two stimuli presented concurrently would have an additive effect on blood loss . The cardiovascular and respiratory effects of air jet stress were similar in the absence and presence of noxious colorectal distension, yet the tolerance to blood loss increased in an additive fashion when the two stressors were combined prior to hemorrhage.

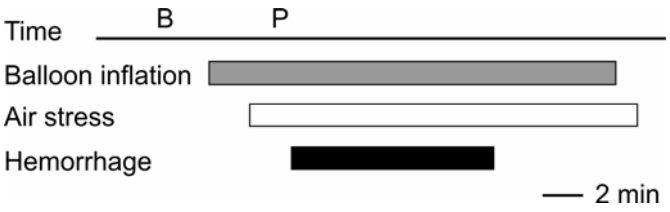

Figure 1. Timeline illustrating the relationship and duration of colorectal balloon inflation, air jet stress and hemorrhage. The duration of hemorrhage in these experiments was approximately 10 min. Values for mean arterial pressure, heart rate, respiratory rate and colorectal balloon pressure were compared at Baseline (B) and after initiation of air jet and/or colorectal distension (Post-stimulus; P) but before hemorrhage.

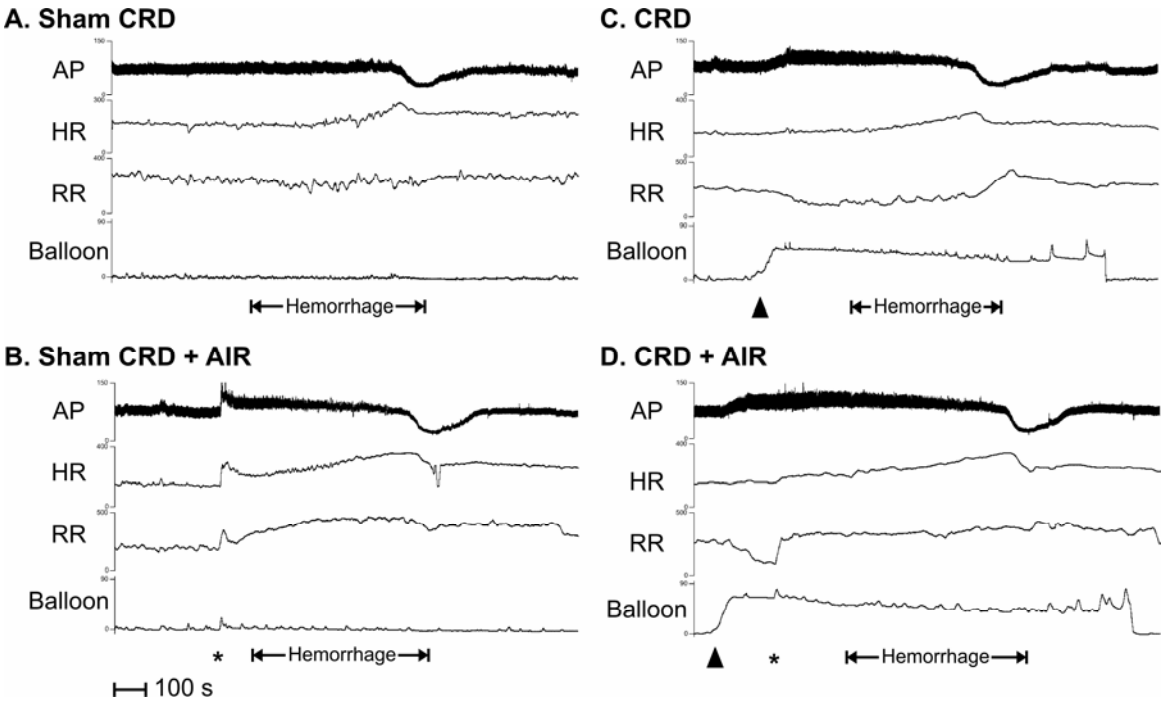

Figure 2. Cardiovascular and respiratory changes during hemorrhage in the same female rabbit during Sham CRD (A), Sham CRD + AIR (B), CRD (C) and CRD + AIR (D). AP, arterial pressure (mmHg); HR, heart rate (bpm); RR, respiratory rate (bpm); Balloon, colorectal balloon pressure (mmHg). Arrow heads represent the initiation of colorectal distension and asterisks indicate the onset of air jet. The period of hemorrhage is indicated for each experiment.

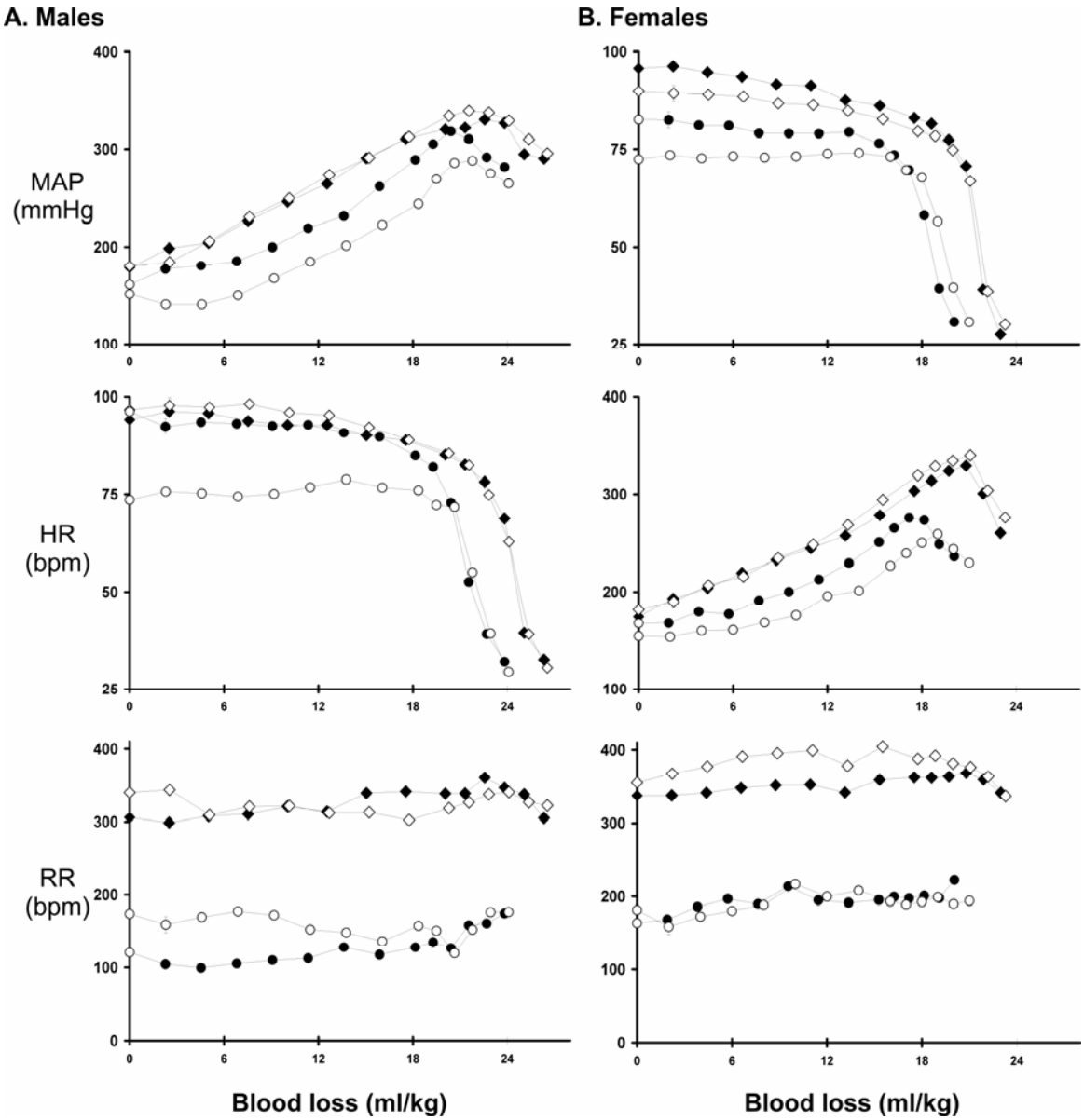

Figure 3. Average changes in cardiovascular and respiratory parameters associated with progressive blood withdrawal in four males (A) and six females (B) during four treatments: Sham CRD (open circles), Sham CRD + AIR (open diamonds), CRD (filled circles), and CRD + AIR (filled diamonds). Blood loss adjusted to body weight is on the abscissa. Error bars (SE) are shown on the

second data point (in some cases obscured by symbol). MAP, mean arterial pressure; HR, heart rate; RR, respiratory rate.

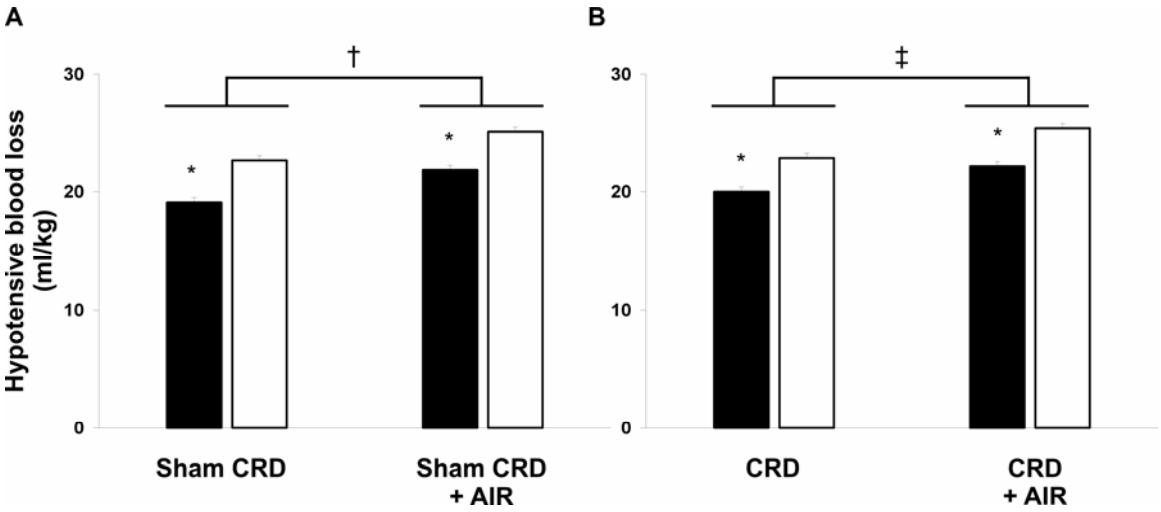

Figure 4. Hypotensive blood loss in females (black bars) and males (open bars). A) The effect of air jet stress in the absence of colorectal distension. Air jet stress increased hypotensive blood loss in both sexes (P=0.02). Females tolerated less blood loss (P=0.01) compared to males. B) The effect of air jet stress in the presence of colorectal distension. Air jet increased total blood loss in males and females (P=0.002). Females tolerated less blood loss (P=0.02) than males in both CRD and CRD + AIR treatments. There was not a significant interaction between sex and treatment in A (P=0.87) or B (P=0.73).

* Significantly different from males (P<0.05). † Significantly different from Sham CRD (P<0.05). ‡ Significantly different from CRD (P<0.05).

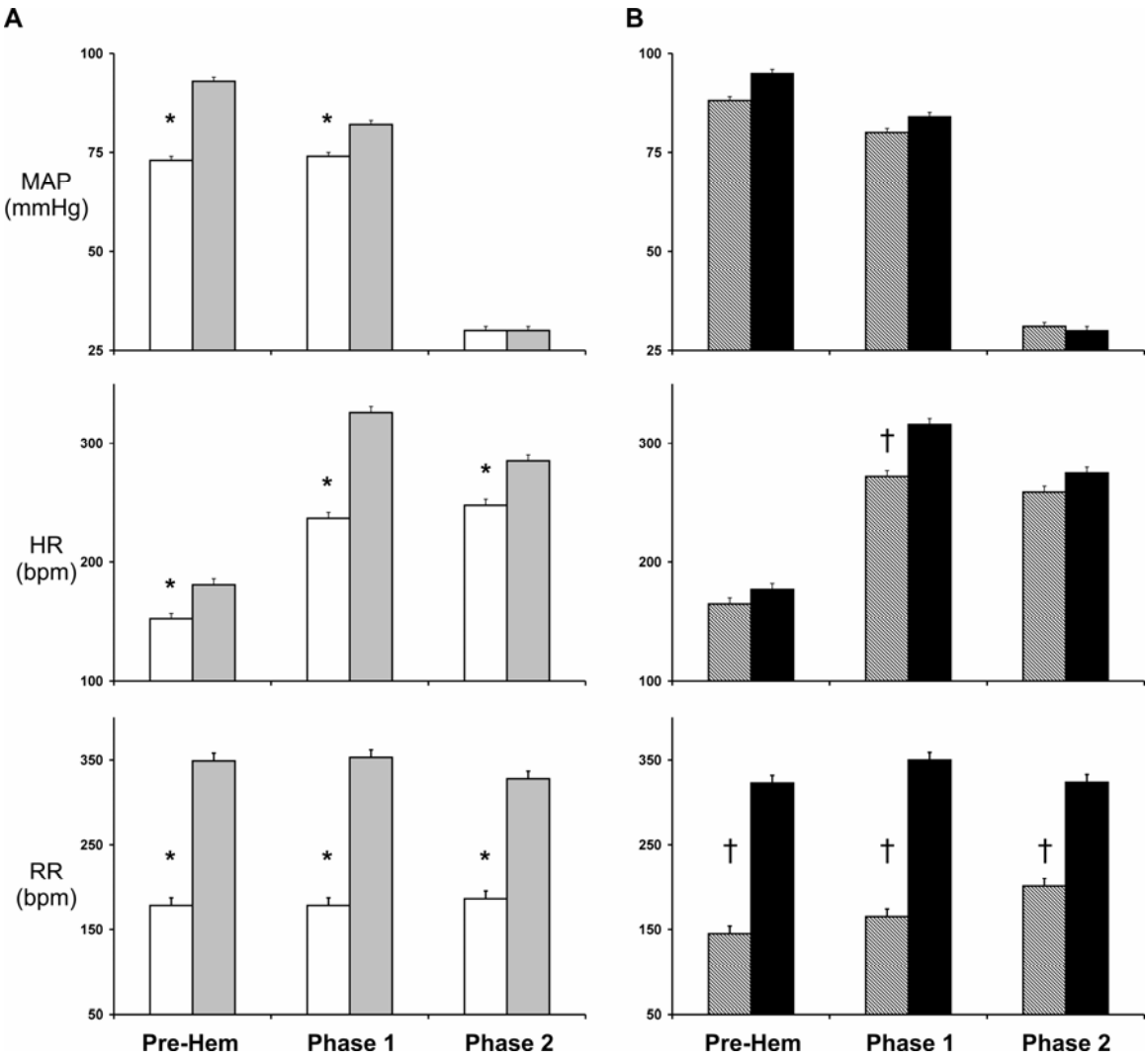

Figure 5. The effect of air jet stress and colorectal distension on the cardiovascular and respiratory response to hemorrhage. A) Sham CRD (open bars) versus Sham CRD + AIR (gray bars). B) CRD (cross hatched bars) versus CRD + AIR (black bars). Values (mean ± SE) for mean arterial pressure (MAP), heart rate (HR) and respiratory rate (RR) prior to hemorrhage (Pre-Hem), at the end of Phase 1, and during Phase 2 hypotension. These time points correspond

to 0, 80, and 105 percent of hypotensive blood loss, respectively. In the absence of colorectal distension, air jet stress significantly increased HR and RR. MAP was higher in the Sham CRD + AIR treatment pre-hemorrhage ($P<0.001$) and at the end of Phase 1 ($P=0.007$) compared to Sham CRD but the treatments were not different during Phase 2 hypotension (105%; $P=0.89$). In the presence of colorectal distension, air jet stress increased RR throughout the experiment and significantly increased HR at the end of Phase 1 ($P=0.01$). Average colorectal balloon pressure in during Sham CRD and Sham CRD + AIR was -1 $\pm$ 1 and 1 $\pm$ 1 mmHg, respectively. On average, colorectal balloon pressure was 57 $\pm$ 2, 48 $\pm$ 2, and 49 $\pm$ 2 mmHg at 0, 80 and 105 percent of hypotensive blood loss during CRD and CRD + AIR. There was no difference between treatments for balloon pressure in B at 0 ($P=0.18$), 80 ($P=0.06$) or 105 ($P=0.61$) percent of hypotensive blood loss. * Significantly different from Sham CRD ($P<0.05$). † Significantly different from CRD ($P<0.05$).

Table 1. Post-stimulus parameters.

	Sham CRD	Sham CRD + AIR	CRD	CRD + AIR
MAP (mmHg)	73 ± 2	92 ± 2 *	88 ± 2 *	94 ± 2 *
HR (bpm)	150 ± 6	176 ± 6 *	171 ± 6 *	175 ± 6 *
RR (bpm)	167 ± 15	336 ± 15 *	142 ± 15	313 ± 15 †
Balloon (mmHg)	-1 ± 2	3 ± 3	55 ± 2	60 ± 3

Values (mean ± SE) for mean arterial pressure (MAP), heart rate (HR), respiratory rate (RR) and colorectal balloon pressure (Balloon) after initiation of air jet and/or colorectal distension but prior to blood loss. Colorectal distension and air jet stress were associated with increases in MAP and HR. Air jet stress increased RR. Balloon pressure was not different between the CRD and CRD + AIR treatments. * Significantly different from Sham CRD ($P<0.05$). † Significantly different from CRD ($P<0.05$).

CHAPTER 10

Summary

The central theme of this thesis was investigation of the physiological consequences of sensory stimulation. Each chapter demonstrated cardiovascular, respiratory or central nervous system responses to sensory stimuli, or, in the case of Chapter 4, developed a technique for evaluating such responses. The two most intriguing pieces of information that I learned from these experiments were: 1) concurrent stress and pain did not alter the nature of the response to hemorrhage but revealed sex differences in tolerance to blood loss; and 2) individual neurons in the PAG respond to multiple internal and external sensory inputs.

I fully expected that a painful stimulus would dramatically change the response to blood loss in a conscious rabbit, or, at the very least, would change the response in a manner similar to other stressors. For example, air jet and oscillation stress are two psychological stressors that have been used in our laboratory. Exposure to air jet or oscillation increased tolerance to blood loss in conscious rabbits, and did not appear to affect males or females differently. Therefore, I predicted that colorectal distension would also increase blood loss required to produce hypotension in male and female rabbits. Thus, it was

surprising that hypotensive blood loss was decreased in females, and not changed in males. With regard to cardiovascular control, it would appear that pain may be a unique stressor that differentially affects males and females, unlike air jet or oscillation stress.

Another interesting finding was the discovery of differences between males and females with regard to sensitivity to colorectal distension (Chapter 7), and tolerance to blood loss (Chapters 8 & 9). The human literature consistently reports that females exhibit lower pain thresholds and higher pain scores for experimental and clinical visceral pain (3;21;80). In rats, females appear to be more sensitive to colorectal distension (49) and estrogen may play a role in the increased sensitivity to noxious visceral stimuli (56). In an early study detailing colorectal distension in rabbits (55), data reported from a pilot study suggested that male rabbits had a significantly higher threshold for pelvic withdrawal following inflation of the colorectal balloon compared with males. Because the pressure needed to induce a behavioral response to colorectal distension in males (~85 mmHg) approached the pressure limit (90 mmHg), the investigators chose to use females in subsequent studies (25;55). By contrast, in our studies, males routinely required a smaller distension volume to achieve the same change in arterial pressure as females, or exhibited a larger increase in arterial pressure for a given increase in colorectal balloon pressure. Both of these findings suggest that our male rabbits may have been more sensitive than females to noxious visceral stimulation. To my knowledge, no other studies have evaluated sex differences in the response to colorectal distension in rabbits. It is

possible that prior exposure to colorectal distension altered the sensitivity to the noxious stimulus. A follow up study investigating the effects of acute colorectal balloon placement and distension on the response to blood loss in males and females may yield different results.

A recent publication provides evidence for sexually dimorphic anatomical and functional activation of the periaqueductal gray that may contribute to sex differences in morphine analgesia (74). I would like to speculate that the periaqueductal gray may be an important substrate for sex differences in sensitivity to visceral pain. Though I did not compare the changes in neuronal activity between males and females, perhaps sex is a variable that should be considered in future analyses. The response of neurons in the periaqueductal gray to a variety of intero- and exteroceptive sensory stimuli has piqued my curiosity about how these neurons would respond to noxious stimuli and concurrent sensory stimulation.

Opioid analgesic administration is part of the standard therapy for human and veterinary patients suffering from traumatic blood loss. Thus, a logical next step for studies evaluating the effect of pain and stress on the response to blood loss is the addition of an analgesic. The model developed here may be useful for evaluating the influence of opioid administration on the response to blood loss in the presence of stress and pain in conscious animals.

In conclusion, these studies demonstrate that colorectal distension is a noxious visceral stimulus that predictably alters cardiovascular and respiratory control in conscious, chronically instrumented rabbits. Early studies presented in

this thesis suggest that the midbrain periaqueductal gray may well be involved in integrating noxious and non-noxious sensory information and altering cardiovascular control. Future studies are warranted to investigate the intriguing interaction between sex, cardiorespiratory control and response to concurrent stressors. Likewise, the mechanisms underlying the observed differences between males and females, and between stress treatments, remain to be investigated.

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VITA

Heidi Lyn Shafford was born in Kodiak, Alaska and attended public school in Homer. Heidi graduated as valedictorian of her high school class in 1991 and then spent a year in Belgium as an exchange student where she became fluent in French. Heidi began pursuing a career in veterinary medicine by studying biology at Middlebury College in Vermont. In 1996, Heidi graduated cum laude from Middlebury with a BA in biology and highest departmental honors. She entered veterinary school intent on becoming a dairy vet, but soon discovered a passion for pain management. After graduating from Colorado State University with a DVM degree in 2000, Heidi completed an unconventional internship in Anesthesia and Food Animal Medicine. Intent on improving the understanding and treatment of pain in animals, Heidi moved east to embark on a clinical residency and a basic science graduate program at the University of Missouri's College of Veterinary Medicine. Heidi completed her residency in 2004 and became board certified in veterinary anesthesia and pain management in 2005. Following graduation from the University of Missouri with a PhD in Biomedical Sciences, she will move east again with her husband, Nick Bezzerides, to begin a post-doctoral fellowship at the University of Pittsburgh.