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Activation of $GABA_A$ and $5HT_{1A}$ receptors in the raphe pallidus abolish the cardiovascular responses to stress in conscious rats

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Activation of $GABA_A$ and $5HT_{1A}$ Receptors in the Raphe Pallidus Abolish the Cardiovascular Responses to Stress In Conscious Rats

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Abstract

Serotonergic neurons of the raphe pallidus area of the brainstem are involved in the cardiovascular responses to stress. However, the manner in which they mediate these responses is not well understood and severe abnormalities in this system have been associated with Sudden Infant Death Syndrome. In the current study, microinjections of muscimol and 8-OH-DPAT ($GABA_A$ and $5HT_{1A}$ receptor agonists, respectively) into the raphe pallidus abolished the increase in mean arterial pressure and the tachycardiac responses following air jet, handing, and restraint stress that were observed following microinjections of artificial cerebrospinal fluid. The stress responses following muscimol microinjections suggest that this region of the brainstem was responsible for mediating cardiovascular responses to stress. The result of DPAT microinjections suggests that within this region of the brainstem, the serotonin system is responsible for these responses.

Introduction

Overview

Serotonin (5-hydroxytrytamine; 5HT) is used as an intercellular messenger both in the central nervous system (CNS) and the periphery (Bijl 2004). The 5HT system includes seven know families of receptors, with at least 14 distinct subtypes (Barnes & Sharp 1999). Abnormalities in the 5HT system, particularly the brainstem, have been implicated in several pathologies including sudden infant death syndrome (SIDS) (Sahni *et al.* 2007; Paterson *et al.* 2006; Marzano *et al.* 2008; Sawaguchi *et al.* 2003), congenital central hypoventilation syndrome (Cutz *el al.* 1997; Saito *et al.* 1999; Weese-Mayer *et al.* 2008), migraines (Hamel 2007), and several psychiatric disorders (Moore *et al* 2000). Additionally, serotonergic pathways in the brainstem have been found to be involved in coordination of autonomic functions including the regulation of the cardiovascular system (Karlsson *et al.* 2006; Gong *et al.* 2006), respiratory system (Doi *et al.* 2008), and nociception (Suzuki *et al* 2004; Bartsch *et al.* 2004).

The brainstem is situated between the spinal cord and higher cortical regions. Due to its location, numerous ascending and descending neural tracts must traverse the brainstem, and are subject to modulation by various brainstem nuclei. Many of the nuclei in the brainstem contain populations of 5HT neurons such as the midline raphe nuclei (MR) (Marco *et al.* 1999), rostral ventral medulla (RVM) (Curran *et al.* 2007), and the nucleus tract solitarius (NTS) (Jeggo *et al.* 2005). A recent study by Paterson *et al.* (2006) found extensive abnormalities in this system in infants that have died of SIDS. Specifically, there was a significant increase in the concentration of 5HT neurons in the brainstem coupled with a decrease in the concentration of the $5HT_{1A}$ receptor, especially

in the MR. These abnormalities may lead to an insufficient stress response and an increased risk for SIDS.

Regulation of the Cardiovascular System

The body's regulation of the cardiovascular system involves both the CNS and peripheral systems. The factor ultimately regulated in the cardiovascular system is arterial pressure (AP), which is a function of cardiac output (a function of heart rate (HR) and stroke volume) and vascular resistance (Guyenet 2006). Short term control of the mean AP (MAP) is mediated by several regions of the brain (primarily the hypothalamus, rostral ventrolateral medulla (RVLM), and the nucleus of the solitary tract). The arterial baroreceptors help to sense rapid changes in MAP and make quick adjustment to enable short term stability of pressure around a set point. Long term regulation of MAP is maintained by these brainstem structures as well as other organs such as the kidneys and select endocrine processes like the Renin- angiotensin-aldosterone system (Guyenet 2006; Osborn 2005). This endocrine system helps regulates MAP via controlling serum Na^+ concentration. Changes in Na^+ concentration can lead to changes in blood volume and therefore changes in MAP. Studies in both human and animal models have shown that the kidneys are able to maintain and, for the most part, prevent an increase in MAP in response to large increases in salt consumption by increasing natriuresis (sodium excretion) (Hall *et al.*1980; DeClue *et al.* 1978; Pechere-Bertschi *et al.* 2000). Therefore, regulation of MAP requires coordination between various organs and the sympathetic (SNS) and parasympathetic (PNS) nervous systems. The brainstem is an essential component of these coordinated responses which enable survival of the organism.

Cardiovascular Responses to Stress

Although an organism's long term AP is regulated around a particular set point, short term changes in AP are normal. AP, HR and SVR can fluctuate drastically from moment to moment. These changes can be elicited by changes in posture, body movements, physical exertion or emotions (Guyenet 2006). These changes are primarily adaptive as they allow the body to redirect blood flow to areas of the body that need more nutrients at any given time. During stress, for example, MAP and HR can rise substantially above its basal levels to assure adequate blood flow to vital organs. These changes are quick and primarily mediated by the sympathetic nervous system (SNS). Additionally, these cardiovascular responses occur in conjunction with changes in the respiratory system in anticipation of, or in response to, an increased oxygen demand (Jian *et al.* 2005). Numerous regions of the brain have been implicated in these responses ranging from regions of the forebrain to those in the brainstem (Walker & Carrive 2003; Furlong & Carrive, 2007). Recent studies have shown that activation of $5HT1_A$ receptors in the MR area of the brainstem can attenuate cardiovascular responses elicited by activation of the hypothalamus (Horiuchi, Wakabayashi, & Hampney 2005; Horiuchi, McDowall, & Dampney, 2008). Therefore, pathways that help coordinate short term cardiovascular responses to stress appear to converge in the serotonin rich area of the MR.

The Role of Brainstem Serotonin in Cardiovascular Regulation

Serotonin (5-hydroxytrytamine; 5HT) is found in both the vasculature as well as the CNS. (Yuwiler *et al* 1977). In the vasculature, it regulates vasomotor tone (therefore affecting blood pressure) and platelet aggregation (Rapport *et al.* 1948;

Azmitia *et al.* 1996). Its role in the CNS involves the regulation of the cardiovascular system and other physiological processes, as well as cognitive functions including memory, emotions, and mood (Jacobs & Azmitia 1992). When $5HT_{1A}$, the serotonin receptor of interest, is activated in the vasculature by administration of 8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT; DPAT), a selective agonist, it generally has a negative effect on pressure. It also had bradycardic effects when administered systemically (Baire, Laubie, & Smith 1990, Di Francesco, Petty, & Fozard 1988; Gradin *et al.* 1985). Clement and McCall (1990) found that these effects were due to inhibition of presympathetic neurons in the rostral ventrolateral medulla (RVLM). These findings suggest a possible site in the brainstem of the depressive effects on the vasculature following global activation of the $5HT_{1A}$ receptor.

Control of the cardiovascular system, especially stress responses, by the CNS originates in higher cortical regions of the brain as well as regions that regulate homeostasis. Activation of the amygdala, insular cortex, and the hypothalamus have resulted in arrhythmias in rodent models (Nalivaiko 2006; Oppenheimer *et al*. 1991; Poisson, Christen, & Sannajust 2000). These cortical pathways appear to converge in the brainstem before they are relayed to the rest of the body (Cao & Morrison 2003; Samuels, Zaretsky, & Dimicco 2002; Zaretsky *et al.* 2003). The primary relay centers for cardiovascular responses in the brainstem are the RVLM and the MR, both of which are rich in serotonergic neurons. The MR is a collection of neurons near the ventral surface of the brainstem that consists of the raphe pallidus (NRP), raphe magnus (NRM), and raphe obscurus (NRO). These neurons have been implicated in the cardiovascular, thermoregulatory, pulmonary, and nociceptive responses to stress (Messier, Li, Nattie

2004; Cao & Morrison 2003; Fields & Anderson 1978; Morrison *et al.* 1999). The rostral and caudal domains of the MR projects to different sites in the nervous system and have different functions (Figure 1). The rostral domain projects to cortical regions of the brain and likely mediate afferent information to the cortex while the caudal domains projects to other brainstem regions and the spinal cord and likely mediate efferent responses from the brain (Kinney *et al.* 2009).

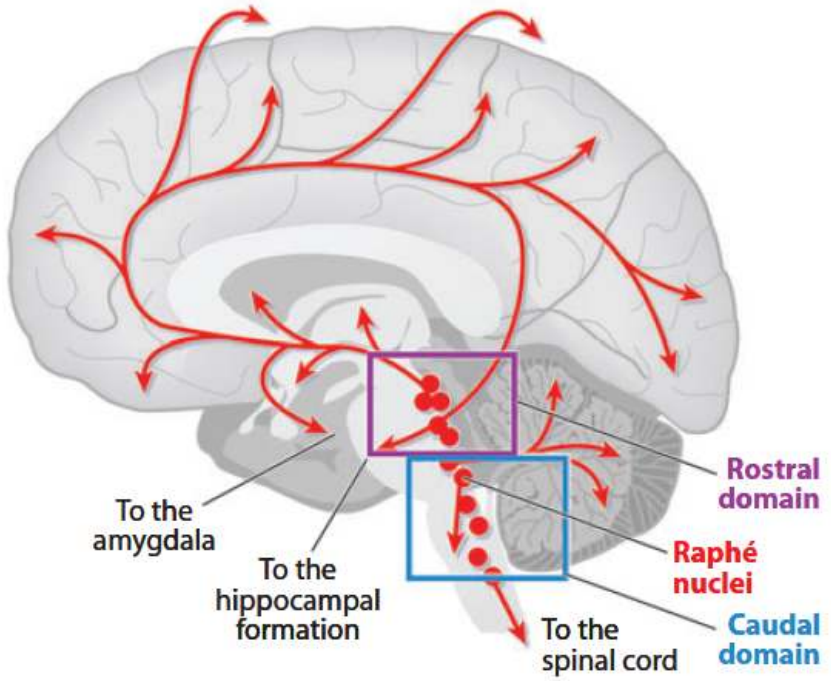

Figure 1. **Projections of the rostral and caudal domains of serotonergic cells of the MR.** Sagittal view of the human brain showing the projections of serotonergic neurons of the MR. Cells of the rostral domain of the raphe project towards cortical regions of the brain where as cells of the caudal domain project towards other brainstem regions and the spinal cord. Cell bodies are represented by the red circles and projections are represented by the red arrows. Adapted from Kinney *et al.* (2009).

During a stress response, there is a coordination in the body to increase the activity of the SNS and decrease the activity of the PNS. The role of brainstem 5HT in mediating stress responses has been documented in numerous studies and may enable this SNS activation and PNS inhibition. Chemical activation of raphe neurons leads to activation of sympathetic responses such as tachycardia (Cao & Morrison 2003). During a stress response, there is decreased activity of the Vagus nerve, indicating decreased PNS activity during a stress response (Nalivaiko & Sgoifo 2009). The response of the Vagus nerve to stress can be suppressed by activation of the $5HT_{1A}$ receptor in the MR(Ngampramuan *et al.* 2008). Recent studies that examined the effects of $5HT_{1A}$ receptors on psychological stress using the open field stress paradigm found that activation of the receptor reduces the cardiovascular response in four different strains of rats (van den Buuse & Wegener 2005). Similar results were found by Vianna, Allen, and Carrive (2008) who blocked the responses to conditioned fear by chemical inhibition of the MR. Studies using transgenic $5HT_{1A}$ knockout models of mice found cardiovascular responses to be exacerbated when exposed to stressful stimuli (Gross *et al* 2000; Pattjj *et al.* 2002). In the brainstem, the $5HT_{1A}$ receptor has been found to inhibit the activity of the serotonin system which likely help to mediate cardiovascular responses to exogenous stress (Lundberg *et al.* 2007; Nalivaiko *et al* 2005).

Clinical Implications

Understanding of the role of the brainstem 5HT system has large implications for human health. Knowing the role of these systems allows for a better understanding of the neural circuitry that controls the physiological processes necessary for life. This allows for a better understanding of what to expect when there are abnormalities in this system.

Numerous pathologies have been associated with abnormalities in the brainstem 5HT system. The most notable in recent years has been Sudden Infant Death Syndrome (SIDS). SIDS is the leading cause of infant mortality in the United States with an incidence rate of 0.67 per 1000 live births (Krous *et al.* 2004). While its exact causes are still unknown, the 5HT system in the brainstem has been heavily implicated. A study by Paterson *et al*. (2006) found numerous abnormalities with this system following autopsies of infants that have died of SIDS. Specifically, there was an increase in the density of serotonergic neurons (Figure 2) and a decreased density of $5HT_{1A}$ receptors in infants that died of SIDS compared to infants that died of other causes, especially in the MR (Figure 3). Recently, Duncan *et al.* (2010) found that the brainstems of infants that have died of SIDS had decreased concentrations of 5HT and trytophan hydroxylase, the enzyme responsible for the rate limiting step in the biosynthesis of serotonin. These findings are consistent with adults who have been diagnosed with multiple system atrophy (MSA). Among patients with (MSA), serotonin depletion, especially of structures in the medulla, have been associated with sudden death compared to MSA patients that have died of known causes (Tada *et al.* 2009).

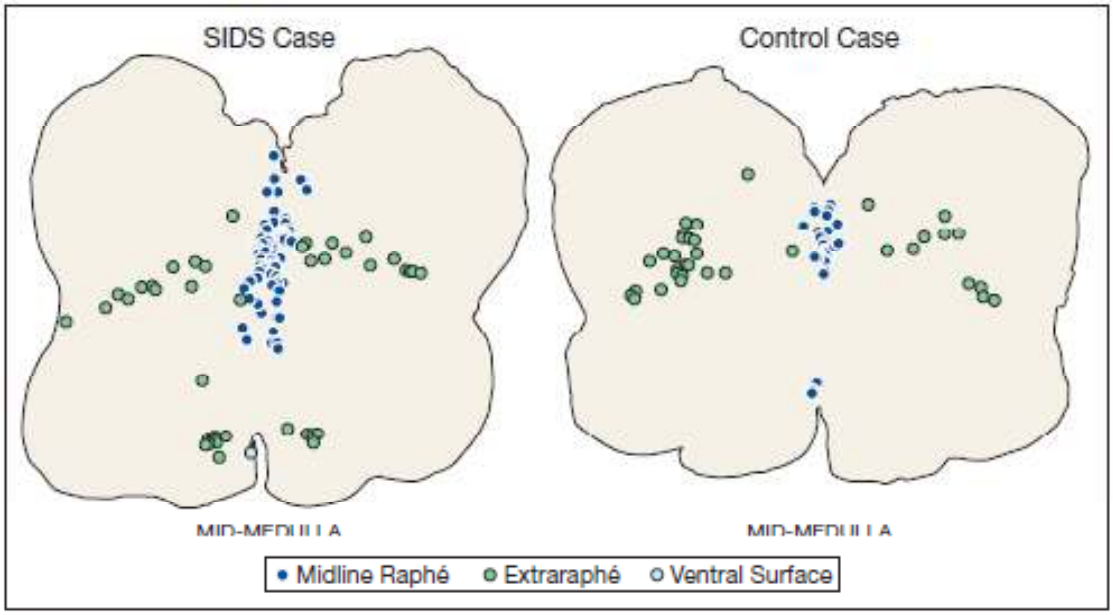

Figure 2. Changes in the density of serotonergic neurons associated with SIDS. The density and distribution of serotonergic neurons in infants that died of SIDS compared to those that died of other causes. Infants that have died of SIDS have a much higher density of serotonergic neurons, especially in the MR. Adapted from Paterson *et al* (2006).

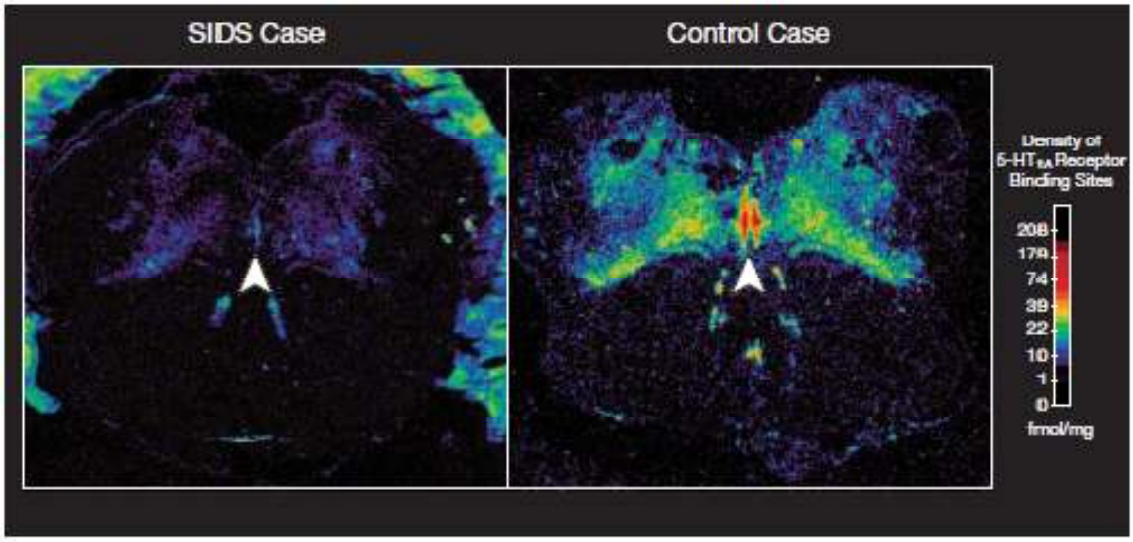

Figure 3. Changes in the distribution of the $5HT_{1A}$ receptor associated with SIDS. The density and distribution of $5HT_{1A}$ receptors in infants that died of SIDS compared to those that died of other causes. Receptor density was determined by its binding density to 8-OH-DPAT, a selective $5HT_{1A}$ receptor agonist. Infants that have died of SIDS have a reduced density of the $5HT_{1A}$ receptor. Adapted from Paterson *et al* (2006).

Due to its role in regulation of the cardiovascular system, the brainstem 5HT system has also been implicated in hypertension, a major public health problem in the United States. Although the mechanisms are not fully understood, the network of systems that regulate long term basal MAP appear to have an increase in its set point during hypertension (Guyenet 2006; Lechin & can der Dijs 2006). A component of this network, hyperactivity of the brainstem 5HT system can lead to increased sympathetic activation and contribute to the increase in MAP.

Serotonin has been heavily implicated in mental disorders, particularly depression and anxiety. A decrease in 5HT activity has been associated with depression and an increase in activity has been associated with anxiety. These psychological factors have been associated with cardiac mortality, sudden cardiac death, and coronary artery disease (Bunker *et al.* 2003; Jonnakuty & Gragnoli 2008; Nalivaiko 2006). Treatments of these disorders with drugs that affect the 5HT system are effective, but the complete mechanisms by which they are mediated have not been fully elucidated.

Current Study

The role of the brainstem 5HT system in regulation of autonomic function suggests that it this area of the nervous system is important in controlling the physiological functions necessary for life. Abnormalities in 5HT development in the brainstem which increase the risk for SIDS support this. However, its precise role in mediating physiological responses to stress has not been fully established. Many studies examining the role of the $5HT_{1A}$ receptor in the MR on the cardiovascular system have been attempted in the past however the use of anesthetized preparations have confounded the results. When the $5HT_{1A}$ receptor was activated in the nucleus of the raphé pallidus

(NRP) and raphé obscurus (NRO) of the brainstem, there were bradycardic responses and depressive effects on MAP in anesthetized animals (Coleman & Dampney 1995; McCall *et al.* 1987; Hof & Fozard 1989; Fozard *et al.* 1987). However, studies in conscious animals found that these depressive responses were either much weaker or absent (Di Francesco *et al.*1988; Kolbasa *et al.*1991). Thus the role of the MR $5HT_{1A}$ receptors in mediation of cardiovascular responses to stress remains unclear.

A recent study by Nalivaiko *et al. (*2005) found (using rabbits as a model animal) that the cardiovascular responses to several stressors were attenuated by the administration of 8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT), a selective agonist of the $5HT_{1A}$ receptor. However, the 8-OH-DPAT was microinjected into the fourth ventricle and $5HT_{1A}$ receptors in a wide area may have been affected. The MR was one of many areas of the brain affected, therefore the cardiovascular effects could not have been definitively attributed to the activation of $5HT_{1A}$ receptors in the MR. Among the nuclei of the MR, the NRP is an area of interest due to abnormalities associated with this region in SIDS (Paterson *et al.*2006). Previous studies has shown that the NRP is involved in regulation of the cardiovascular responses to stress (Alvarenga, Pires & Futuro Neto 2005; Coleman & Dampney 2006; Edwards & Paton 2000; Zaretsky *et al.* 2003). However, its role in mediating cardiovascular has to be fully elucidated. The NRP was targeted because it may serve as a model to study stress responses as it relates to SIDS.

The intent of the present study is to determine if the NRP is responsible for the mediation of cardiovascular responses to exogenous stress. The specific aims of this study are:

Specific Aim 1: To determine if the NRP is responsible in mediating the cardiovascular responses to exogenous stress. To this end, muscimol, a gamma amino-butyric acid (GABA) agonist, will be used to non-selectively inhibit neurons of the NRP during mild stress induction.

Specific Aim 2: To determine if these stress responses are mediated by the $5HT_{1A}$ receptor. The inhibitory $5HT_{1A}$ receptors of the NRP will be activated with 8-OH-DPATduring mild stress induction.

Materials and Methods

Animal Use Rational

Laboratory rats have been used extensively as an animal model in the study of mammalian physiology. The rat $5HT_{1A}$ receptor has 89% homology with the human analog (Albert *et al.* 1990) and its distribution in the brainstem is similar to that of a human (Burnet *et al.* 1995). A recent study (Charkoudian *et al.* 2010) found that the cardiovascular system and the mechanisms by which they are controlled were qualitatively similar between rats and human. This study will contribute both to the understanding of CNS regulation of the cardiovascular physiology and pathologies, such as SIDS and hypertension, that may develop when there are abnormalities within this system. All methods were performed with approval from James Madison University's animal care and use committee (IACUC).

Animal Care

Male Sprague-Dawley rats were used for all parts of the study. All rats were at least 9 weeks old and had a mass of 250g-375g. All surgeries performed on rats were conducted using antiseptic techniques. Specifically, incision sites were scrubbed with iodine, all surgical materials were sterilized, and the rat was placed on a sterile field. During surgeries (described below) animals were anesthetized with 75mg/kg ketamine and 15mg/kg xylazine with boosters as necessary. Following surgeries, all animals were given 1mg/kg indomethacin on rat treats for pain control and a rest period of at least 1 week before experimentation. Both surgeries were done at the same time (for experimental timeline, see Figure 4).

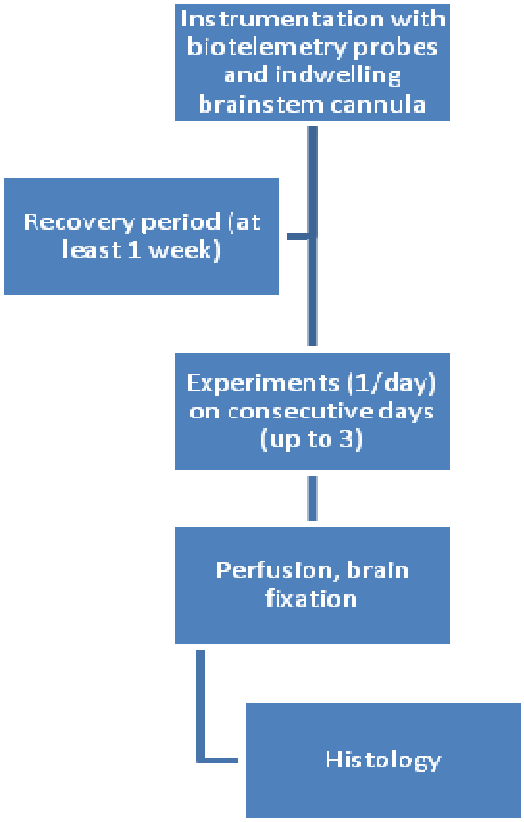

Figure 4. Experimental timeline. Rats were instrumented with both an indwelling cannula targeting the NRP and a telemetry probe that measured MAP and HR. Following a resting period, 3 experiments were done on consecutive days (1 experiment/day). Following experimentation, the brains of the rats were fixed with 10% formalin for histology.

Biotelemetry

A biotelemetry probe (PhysioTel PA-C40 small animal transmitter; DataSciences International; St. Paul, MN) was used to measure MAP and HR in the rats used for experiments. PA-C40 probes have 2 parts- a catheter and probe body. Rats were anesthetized and the abdominal region was shaved and scrubbed with iodine. An incision (~1.5 inch) was made in order to access the peritoneal cavity and the descending aorta was exposed. The descending aorta was separated from the inferior vena cava using gentle pressure from curved forceps and non-absorbable nylon sutures were inserted under the isolated aorta between the celiac and renal branches. These sutures were used to momentarily stop the blood flow through the artery.

In order to instrument the probe, a 23 gauge needle, bent ~90° at the tip, was used to pierce the occluded aorta in order to insert the catheter of the PA-C40 probes. The aorta was repaired with tissue adhesive (LiquidVet Rapid; MedRep Express; Prescott, AZ). A small piece of filter paper was used to increase surface area for adhesion and to hold the catheter in place. Sutures were then removed to restore blood flow. The probe body was sutured onto the abdominal muscles using non-absorbable silk sutures. The abdominal muscle layer was closed with non-absorbable silk sutures and the skin was held together by autoclips.

Stereotaxic Surgery

Stereotaxic surgery allowed for a cannula to be placed in the brainstem so that pharmacological manipulation of the NRP could later be performed using microinjection via a Hamilton syringe. Immediately following instrumentation of the biotelemetry

probes, rats were placed in a stereotaxic frame. The skin covering the skull was swabbed with iodine and an incision was made to expose the skull of the rat. A hole was drilled on the rat's skull -2.3mm caudal to lambda, where a 20 gauge stainless steel cannula, 26 mm long, was implanted -9.2 mm ventral to the skull surface where the hole was made. The cannula was held in place with at least 3 screws that were placed into the rat's skull and dental cement.

Pharmacology

On the day of experimentation, a 25gauge, 28 mm long, cannula was used to deliver pharmacologically active ingredients to the NRP before the rats were stressed. Artificial cerebrospinal fluid (ACSF; 128 mM NaCl, 3.0 mM KCl, 1.3mM $CaCl_2$, 1.0 Mg Cl_2, 21.0 mM Na_2HPO_4, 1.3mM NaH_2PO_4, 1.0 mM D-glucose, pH=7.4 titrated by bubbling in CO_2; McNay & Sherwin 2003) was used as a control and a basis for comparison to 8-OH-DPAT and muscimol. Muscimol is an agonist for gamma-amino butyric acid (GABA) receptors, an inhibitory neurotransmitter. For all experiments, 300nL of ACSF, 30mM muscimol, or 30nM 8-OH-DPAT was injected into the NRP at a rate of 100nL/minute. Immediately following microjections of these drugs, 100nL of air was injected and the injection cannula was left in the brain for 1 minute to allow the drugs to diffuse into the brain tissue and to prevent reflux of drug back through the injection cannula.

Data Collection

Biotelemetry probes that had been instrumented into the rats allowed for continuous data collection without disturbing the animal. The rat's cage was placed directly above receiver plates (RPC-1 receiver plates; DataSciences International; St.

Paul, MN) and data was collected every 30 seconds. Each time data was collected, HR and MAP data was sampled for 10 seconds and automatically averaged by the software (Dataquest A.R.T.; DataSciences International; St. Paul, MN). Data was recorded for at least 15 minutes prior to the rat's cage being opened for microinjections and at least 15 minutes after the end of a stress. The 15 minutes prior to microinjections were averaged and used as a baseline measure. The rat's baseline HR and MAP were subtracted from values after they were stressed to obtain the change in the respective parameters. Data from the point the rat's cage was opened until the end of the stress was not used for analysis due to the effect of the microinjection protocol on the animal.

Experiments

Prior to microinjections, all rats were allowed to acclimate in its cage until its MAP (remained within ± 5 mmHg) and HR (remained within ± 25 beats per minute (bpm)) has stabilized for at least 15 minutes. All experiments were conducted in the rat's home cage. No more than 3 stress experiments were conducted on each rat. Only one experiment was conducted per day, and experiments were conducted on consecutive days. The order in which drug or stress was done each experiment was randomized. Immediately following microinjections, rats were stressed as described below:

Handling: Rats are handled on a regular basis - transfer to a new cage, experiments, etc. These experiments tested the rat's responses to a stress that they have been familiar with. The rats were held at approximately the midpoint of the tail for 30 seconds. After 30 seconds, they were released and the lid of the cage was replaced.

Air Jet: The air jet stress have been used in previous studies (Nalivaiko 2005) as an "acute psychological" stress. This stress is novel to the animal and it may be difficult for

them to interpret what is happening. During the air jet stress, the rats were allowed to freely move around the cage and filter tops were kept in place. A nozzle attached to a canister of compressed air was inserted into the cage and a continuous jet of air was injected into the cage for 60 seconds. The nozzle was aimed at the rat during the course of the stress.

Restraint: Rats in a laboratory setting are rarely restrained, except in circumstances where it may be deemed necessary for experimental protocols or surgical preparations. In nature, restraint usually means that it had been captured by a predator. Such instances would elicit full activation of the "fight or flight" response. This stress was meant to achieve such a response. Specifically, these rats were held (with handlers wearing a leather glove) in the thoracic region behind the front limbs. While the rats were able to move their limbs, they were not able to move their bodies, and were held in place for 60 seconds.

Histologic Verification

After experiments were completed, the rats were sacrificed to verify that the cannula was placed in the NRP during the stereotaxic surgery. Prior to sacrificing with isofluorane, 300 nL of a 1% solution of potassium permanganate was microinjected into the brainstem of the rats (in the same manner as the pharmacologically active drugs). Potassium permanganate stains the brain tissue where the pharmacologically active agents were microinjected. The heart will be exposed and the jugular vein cut. A needle was inserted into the left ventricle of the heart and perfused with 200 mL saline or until the fluid coming out of the jugular vein is clear. Following perfusion by saline, the rats were fixed with 200mL 4% formalin. The brains were extracted and placed into a jar

with 10% formalin for at least 4 days. Once the brains were fully fixed, they were placed into a jar containing approximately 30% sucrose until it is saturated with sucrose, evidenced by the brain sinking to the bottom of the solution (approximately 4-5 days). The brain tissue will then be mounted onto a cryostat and cut into 50 μm sections and set onto glass slides. Slides were dried, stained with cresyl violet, and covered with a cover slip using Permount® adhesive. Stain on the tissue will be compared to areas of the brain identified as the NRP in the sterotaxic atlas (Paxinos & Watson 1998). Stereotaxic targeting of the NRP will be deemed successful if there is staining by potassium permanganate within 1.0 mm of the ventral surface, 1.5 mm rostral/caudal to the caudal aspect of the facial nucleus, and within 0.5 mm of the midline as indicated by comparison to the stereotaxic atlas (Figure 5). Only rats with injection marks within these parameters were used for statistical analysis.

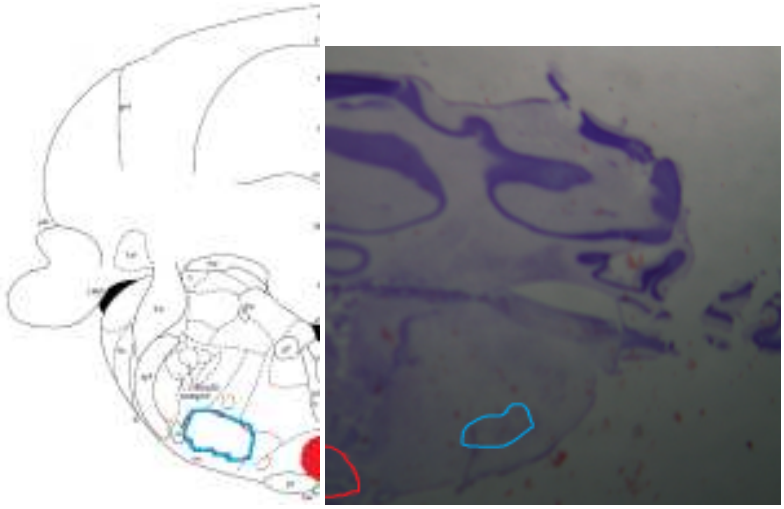

Figure 5. Coronal section of the rat medulla at -2.00 mm relative to the interaural point. A histological section (right) is compared to a stereotaxic map of the rat brain (left). The facial nuclei are outlined in blue. On the left, the NRP is represented by the red mark (Adapted from Paxinos & Watson 1998). On the right, the injection site is outlined in red.

Staining of the brain sections with cresyl violet were done according to the following conventional protocol:

1. Tissue dehydration: Brain sections on glass slides were placed into solutions of increasing concentrations of ethanol (50%, 70%, 90%, and 100%.) for 5 minutes in each solution.

2. Dehydrated brain sections were placed into 100 % xylene for 5 minutes.

3. The brain sections were returned to 100% ethanol for 5 minutes after soaking in xylene.

4. Brain sections were stained in a 1% cresyl violet solution at ~60ºC for 6 minutes.

5. Stained brain sections were dipped into dH_2O.

6. Brain sections were then destained for visualization of brain structures:

    a. Sections were soaked in 50% ethanol for 5 minutes.

    b. Destaining was done in 70% ethanol with 5 drops of acetic acid.

    c. Destained brain sections were dehydrated in 90% and 100% ethanol as stated above.

7. Destained brain sections were fixed in xylene overnight. The following day, permount and a coverslip was placed over slides.

Statistical Analysis

Raw MAP and HR data were averaged into 3 minute bins for each rat. An analysis of variance (ANOVA) was conducted at each 3 minute bin between each treatment to determine if there was a statistical difference between each treatment group for each time segment. Tukey-HSD was conducted as a post-hoc test to determine if

there were differences between individual groups at each time point. All reported data are means ± standard error with a level of significance standard of $p<0.05$.

Results

In most instances, statistical analysis of the data revealed that the change in the MAP and HR of rats in the DPAT and muscimol treatment groups were not statistically different from pre handling base line. There was also no significance in the post handling treatment effect on HR or MAP between DPAT and Muscimol injected rats. Most importantly, changes in MAP and HR of rats microinjected with ACSF were usually statistically different ($p<0.05$) than both pre stress base line and those responses from rats microinjected with DPAT or muscimol. Please assume this was the case unless otherwise noted.

Baseline MAP and HR

Rats that were handled had MAP of 107±2.99 (range 95.8-117), 114±4.07 (range 102-130), and 110±7.55 (range 92.9-141) mmHg and HR of 325±13.9 (range 295-388), 327±13.3 (range 305-391), and 330±8.30 (range 305-354) bpm for the ACSF, DPAT, and muscimol microinjected treatments, respectively (Figure 6). ANOVA analysis of pre treatment values revealed that neither MAP ($p=0.69$) nor HR ($p=0.96$) were significantly different in rats exposed to handling stress.

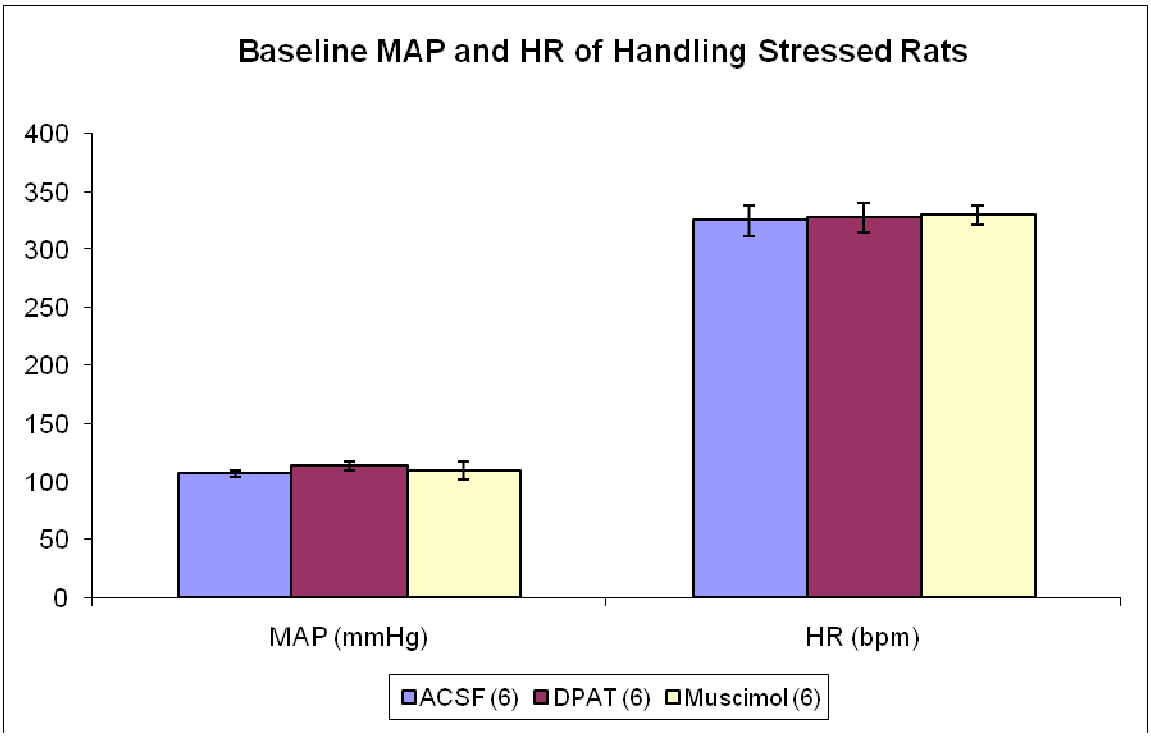

Figure 6. Baseline measures of MAP and HR prior to handling. Baseline measures of MAP and HR in rats that were stressed by handling. There were no significant differences.

Rats that were stressed using an air jet had MAP of 110±3.67 (range 96.3-119), 113±6.76 (range 93.2-141), and 109±3.06 (range 103-119) mmHg and HR of 304±22.7 (range 199-354), 298±26.6 (range 199-350), and 310±16.2 (range 244-368) bpm for the ACSF, DPAT, and muscimol microinjected treatments, respectively (Figure 7). ANOVA analysis of pre treatment values revealed that neither MAP ($p=0.84$) nor HR ($p=0.08$) were significantly different in rats exposed to air jet stress.

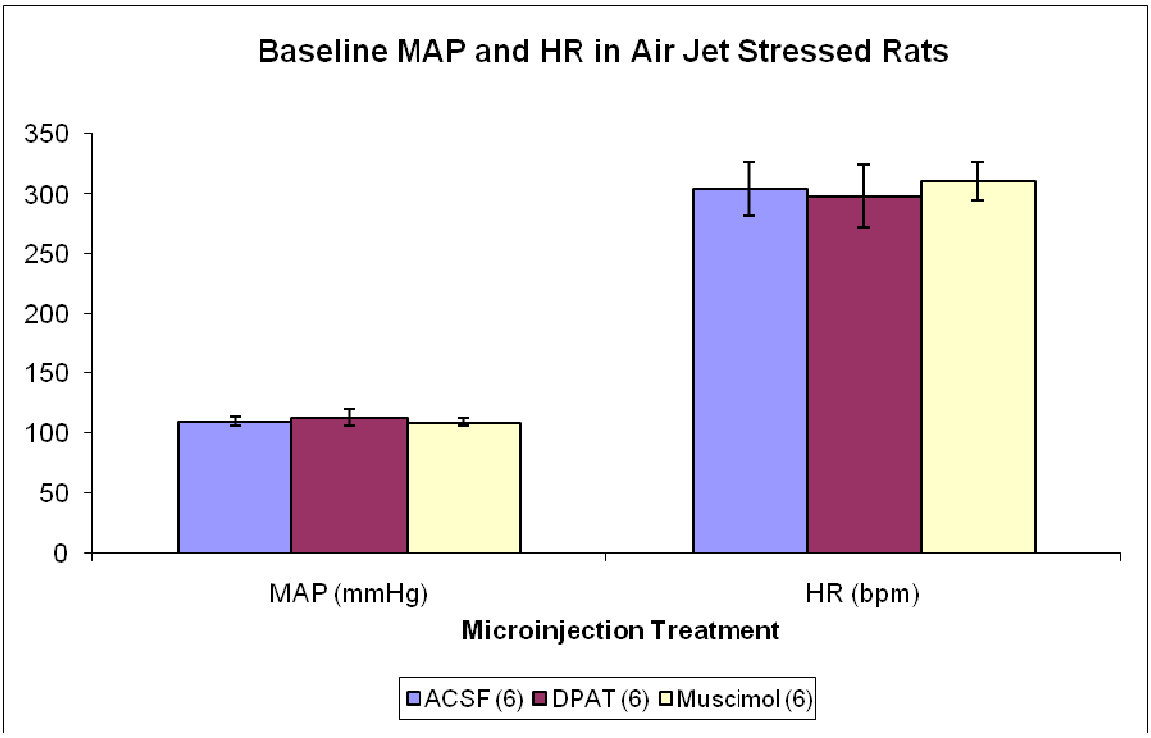

Figure 7. Baseline measures of MAP and HR prior to air jet stress. Baseline measures of MAP and HR in rats that were stressed by air jet. There were no significant differences.

Rats that were restrained had MAP of 116±6.25 (range 96.8-138), 114±9.40 (range 92.9-143), and 108±3.66 (range 98.6-121) mmHg and HR of 314±4.37 (range 306-330), 322±10.2 (range 294-361), and 312±22.0 (range 243-361) bpm for the ACSF, DPAT, and muscimol microinjected treatments, respectively (Figure 8). ANOVA analysis of pre treatment values determined that neither MAP ($p=0.70$) nor HR ($p=0.88$) were significantly different in rats exposed to air jet stress.

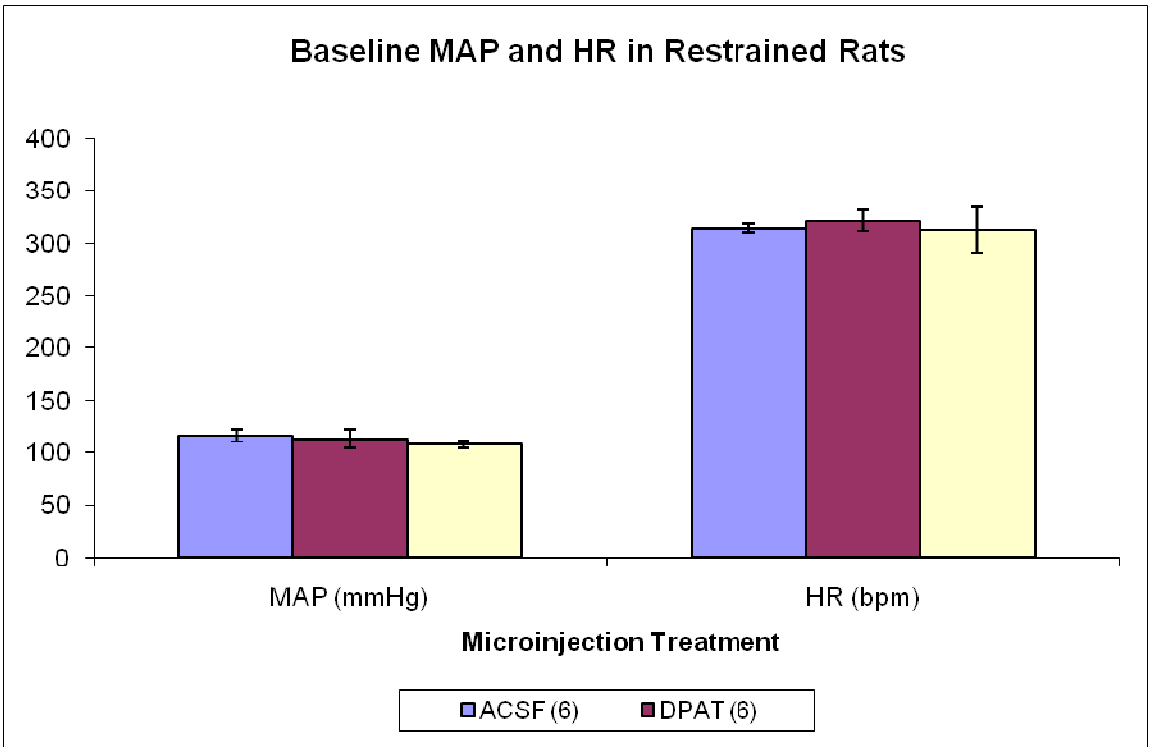

Figure 8. Baseline measures of MAP and HR prior to restraint stress. Baseline measures of MAP and HR in rats that were restrained. There were no significant differences.

The Effect of Microinjections on Cardiovascular Responses to stress

Handling Stress

Rats were handled in order to assess their cardiovascular response to a familiar stress. The change in MAP at 3, 6, 9, 12, and 15 minutes after handling was 14.8±2.88, 12.9±4.36, 12.6±3.62, 11.3±3.80, and 12.0±3.82 mmHg for ACSF microinjected rats, 2.60±2.19, 0.81±2.36, 0.39±3.03, -1.53±2.95, and -2.84±3.68 mmHg following DPAT microinjected rats, and -0.03±2.43, -1.19±2.40, -1.83±3.24, 1.73±4.60, and -0.33±4.11 mmHg for muscimol microinjected rats respectively (Figure 9). ANOVA with post hoc analysis revealed significant differences between ACSF and both muscimol and DPAT microinjections at 3 ($p=0.002$), 6 ($p=0.014$), 9 ($p=0.016$), and 15 ($p=0.036$) minute averages, but not at the 12 minute average ($p=0.079$). At 15 minutes, the difference between ACSF and muscimol microinjected groups were not statistically significant ($p=0.096$).

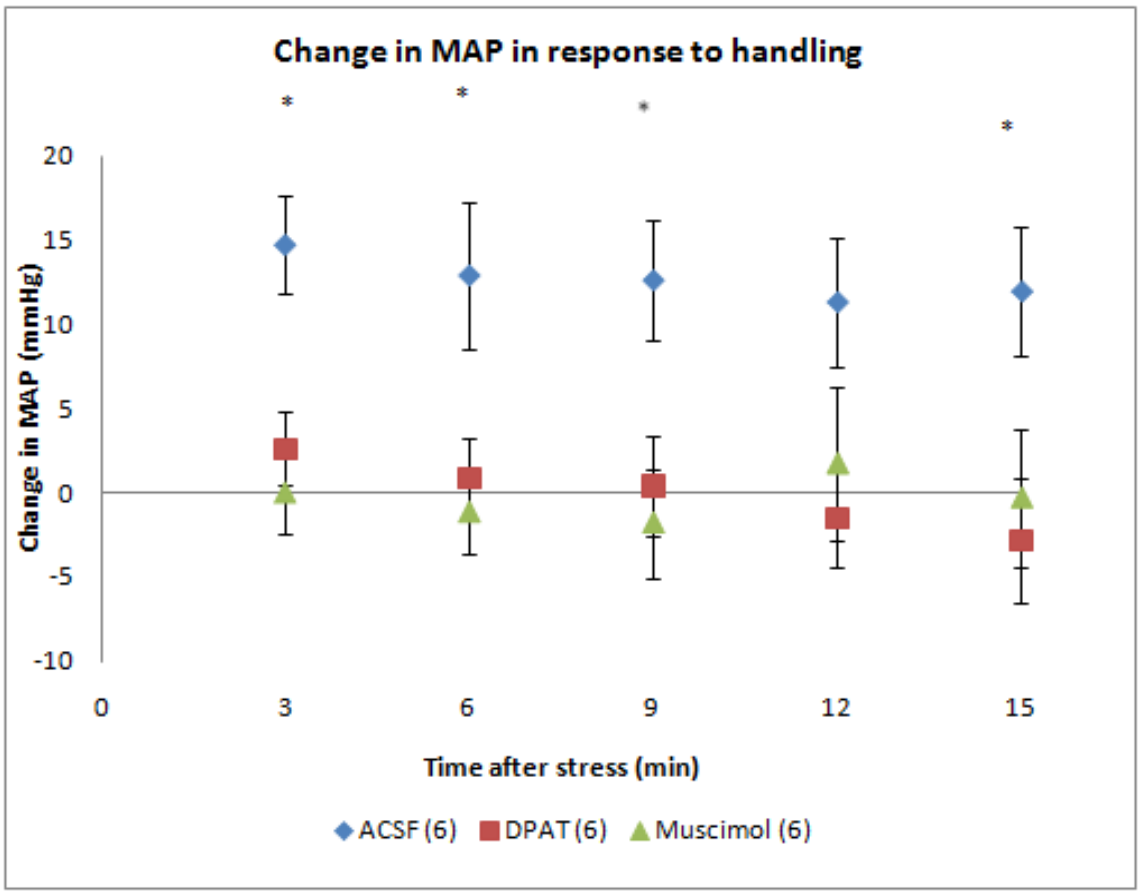

Figure 9. Change in MAP in response to handling. The change in MAP in response to handling stress. * Denotes statistical difference.

The changes in HR at 3, 6, 9, 12, and 15 minutes following handling were 76.9±13.7, 52.8±8.47, 44.6±6.85, 47.2±11.8, and 52.4±11.4 bpm for ACSF microinjected rats, -5.68±9.42, -10.9±9.67, -4.05±12.3, -10.5±9.99, and -11.2±11.0 bpm for DPAT microinjected rats, and -2.63±7.17, -17.9±8.78, -18.62±13.8, -17.6±15.7, and -12.2±15.13 mmHg for muscimol microinjected rats, respectively (Figure 10). ANOVA analysis with post hoc analysis revealed that these differences were statistically different at 3 ($p<0.001$), 6 ($p<0.001$), 9 ($p=0.003$), 12 ($p=0.005$), and 15 ($p=0.003$) minute averages.

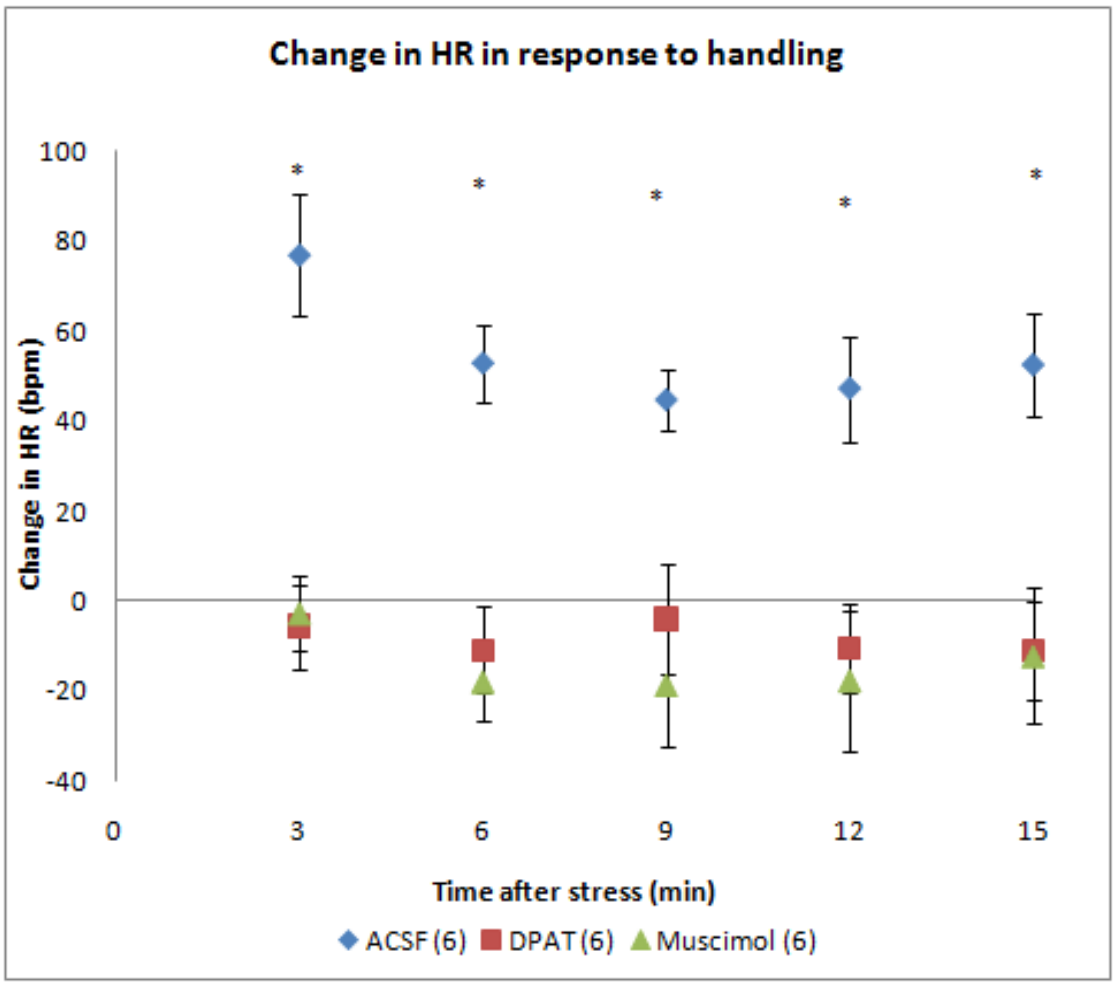

Figure 10. Change in HR in response to handling. The change in HR in response to handling stress. * Denotes statistical difference.

Air Jet Stress

The air jet stress was used to assess the rats' response to a novel stress. The change in MAP at 3, 6, 9, 12, and 15 minutes following the air jet stress were 14.0±2.88, 11.0±2.93, 10.0±2.66, 10.3±3.45, and 8.90±3.43 mmHg for ACSF microinjected rats, -5.62±1.68, -8.35±1.02, -7.30±1.43, -7.93±1.54, and -7.18±1.38 mmHg for DPAT microinjected rats, and -2.81±4.38, -6.53±4.89, -7.51±4.51, -9.16±5.27, and -11.3±6.31 mmHg for muscimol microinjected rats, respectively (Figure 11). ANOVA analysis with post hoc analysis revealed that these differences were statistically different at 3 ($p=0.001$), 6 ($p=0.002$), 9 ($p=0.002$), 12 ($p=0.003$), and 15 ($p=0.010$) minute averages.

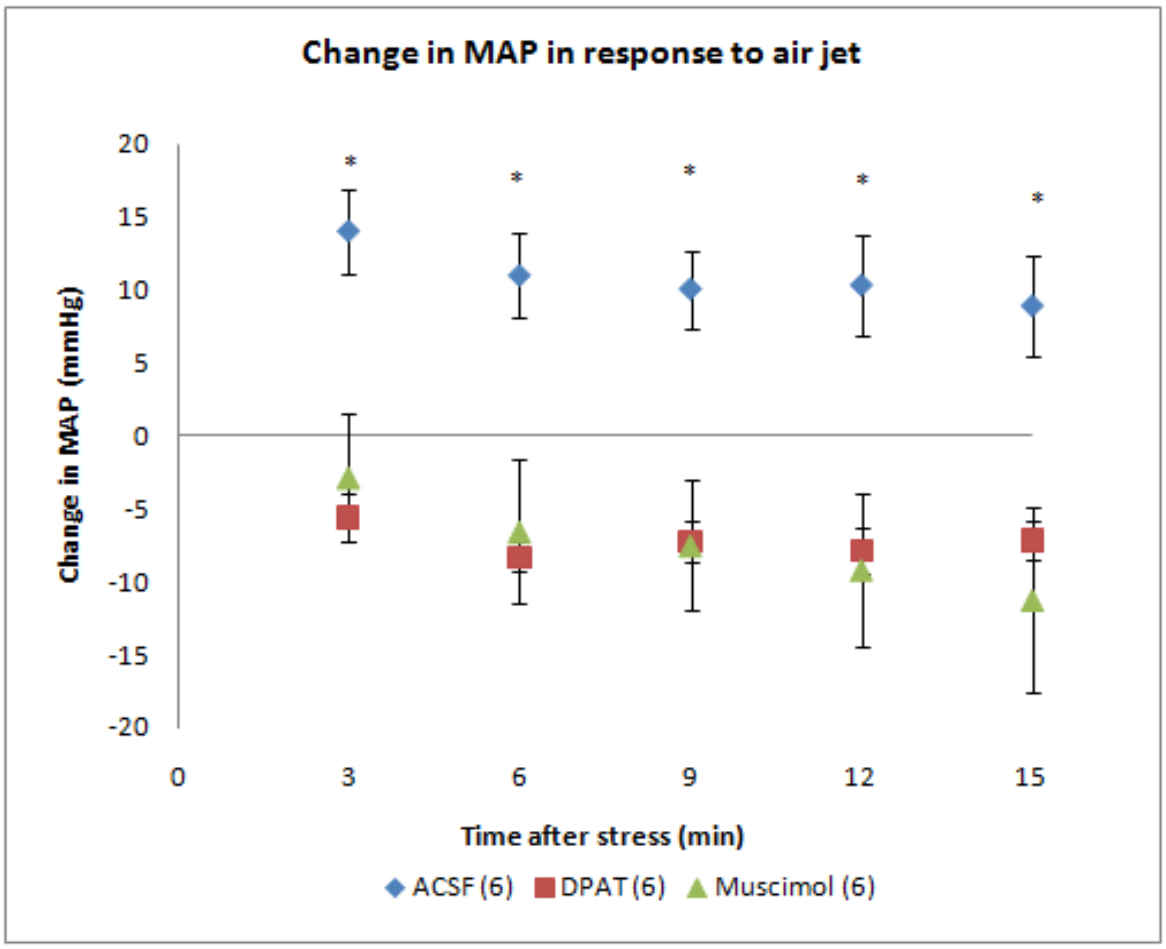

Figure 11. Change in MAP in response to air jet. The change in MAP in response to air jet stress. * Denotes statistical difference.

The change in HR at 3, 6, 9, 12, and 15 minutes following air jet stress were 41.6±13.9, 37.1±9.86, 36.2±9.83, 39.7±12.7, and 37.5±12.5 bpm for ACSF microinjected rats, -34.9±10.1, -35.2±8.17, -30.1±9.63, -26.6±14.6, and -24.8±16.5 bpm for DPAT microinjected rats, and -20.1±15.5, -19.0±14.1, -20.3±11.4, -17.3±10.7, and -31.6±10.1 mmHg for muscimol microinjected rats, respectively (Figure 12). ANOVA with post hoc analysis revealed that these differences were statistically different at 3 ($p=0.002$), 6($p=0.001$), 9 ($p=0.001$), 12($p=0.005$), and 15 ($p=0.002$) minute averages.

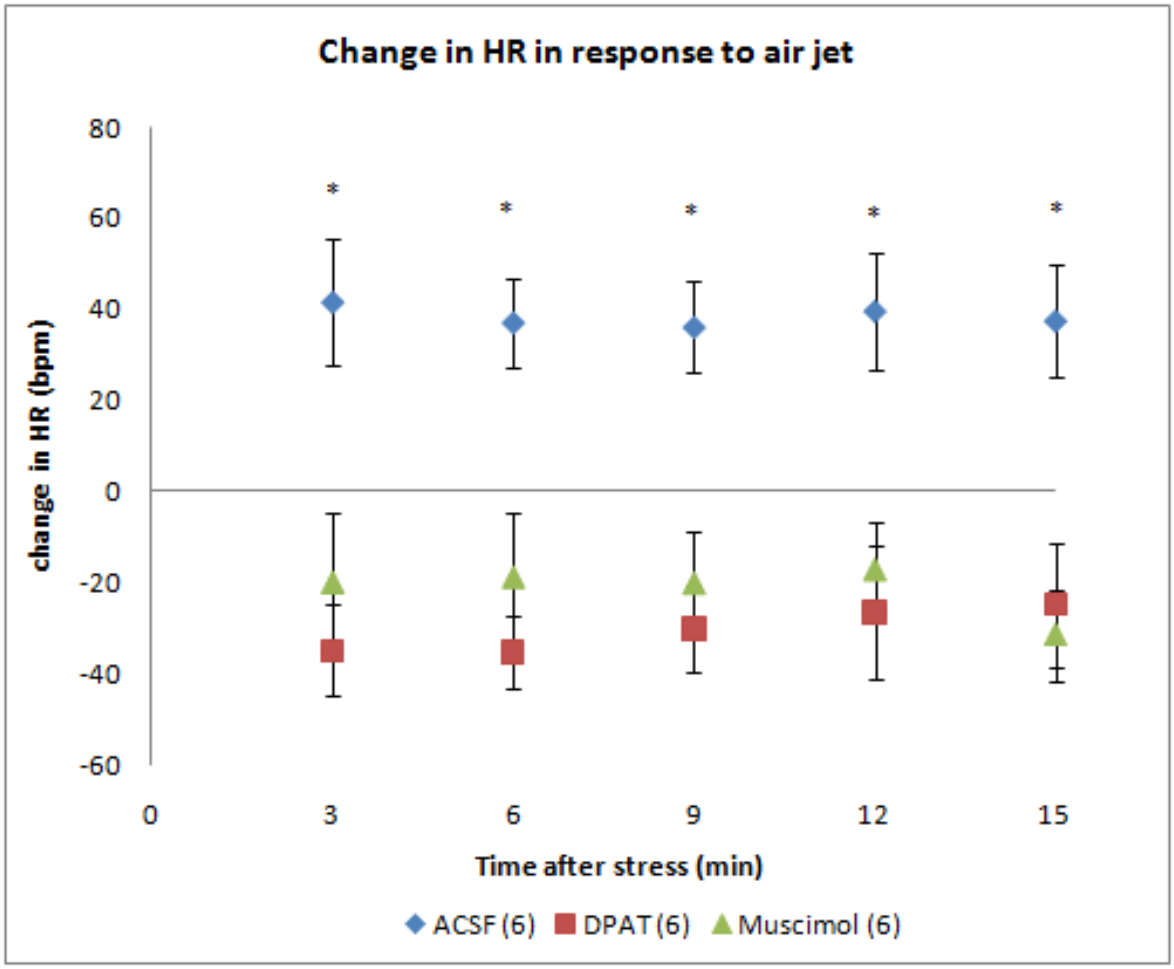

Figure 12. Change in HR in response to air jet. The change in HR in response to air jet stress. * Denotes statistical difference.

Restraint Stress

The restraint stress was used to elicit the rat to fully activate its "fight or flight" response. The changes in MAP at 3, 6, 9, 12, and 15 minutes following the restraint stress were 15.7±5.17, 18.7±6.73, 18.7±7.7, 19.1±6.31, and 20.3±8.31 mmHg for ACSF microinjected rats, 6.01±1.83, 0.11±1.73, -0.20±2.35, 1.01±3.78, and -0.25±3.67 mmHg for DPAT microinjected rats, and -3.08±2.82, -8.67±3.28, -11.2±3.58, -10.6±4.41, and -12.7±4.22 mmHg for muscimol microinjected rats, respectively (Figure 13). ANOVA with post hoc analysis revealed that these differences were statistically different at 3 ($p=0.001$), 6 ($p=0.002$), 9 ($p=0.002$), 12 ($p=0.003$), and 15 ($p=0.010$) minute averages. At 3 ($p=0.164$), 12 ($p=0.052$) and 15 ($p=0.059$) minutes, the difference between ACSF and DPAT microinjected groups were not statistically significant.

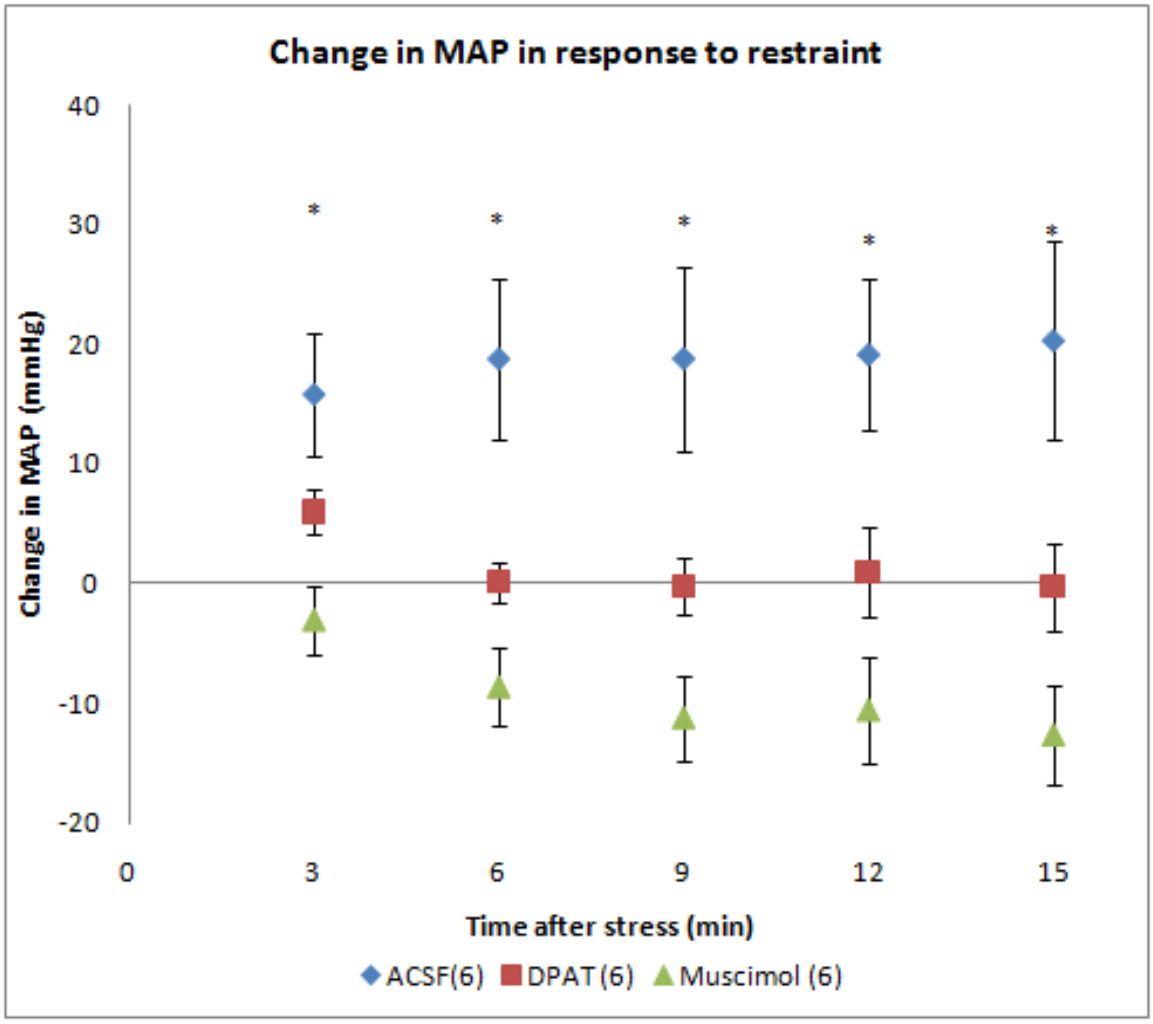

Figure 13. Change in MAP in response to restraint. The change in MAP in response to restraint. * Denotes statistical difference.

The change in HR at 3, 6, 9, 12, and 15 minutes following restraint were 69.1±20.5, 69.7±23.1, 78.7±18.5, 85.0±16.9, and 75.4±15.9 bpm for ACSF microinjected rats, 1.77±16.8, -11.6±7.63, -1.68±12.2, 5.32±16.1, and -1.57±16.7 bpm for DPAT microinjected rats, and -32.6±16.2, -36.7±15.4, -47.4±15.2, -50.4±15.4, and -49.7±15.7 mmHg for muscimol microinjected rats, respectively (Figure 14). ANOVA analysis revealed that these differences were statistically different at 3 ($p=0.004$), 6 ($p=0.004$), 9 ($p<0.001$), 12 ($p<0.001$), and 15 ($p<0.001$) minute averages.

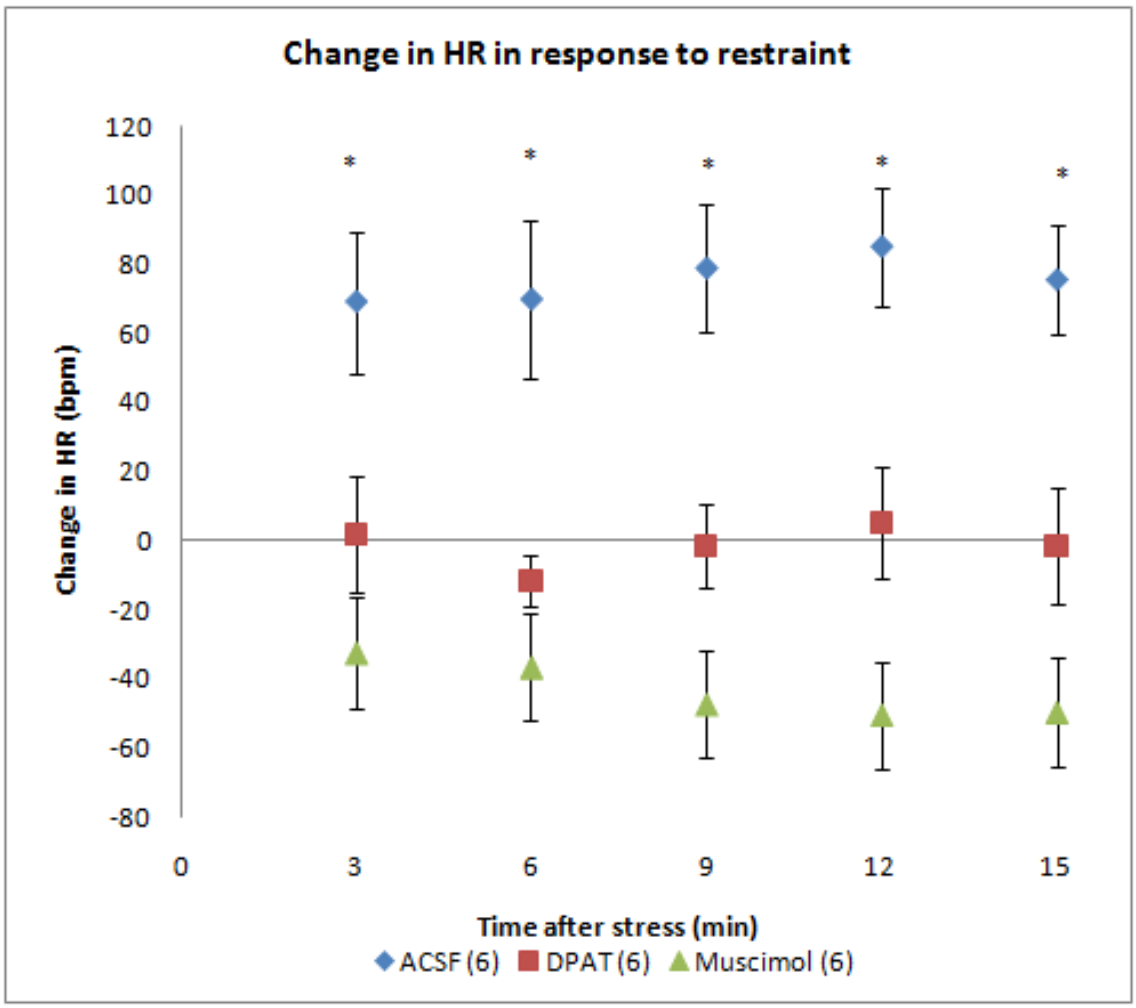

Figure 14. Change in HR in response to restraint. The change in HR in response to restraint. * Denotes statistical difference.

Discussion

During a stress response, there is usually a large increase in HR and moderate change in MAP. This study found that these responses were abolished by microinjections of muscimol and DPAT into the NRP. The lack of elevation in HR and MAP following microinjections of muscimol suggest that the NRP is responsible for normal elevations in HR and MAP. The abolition of the cardiovascular responses to stress following microinjections of DPAT suggests that these responses were largely mediated by the serotonin system. Additionally, this study was conducted in conscious rats. Previous research in this area has involved the use of anesthetized animals, which have confounded the data concerning the role of the NRP in regulation of the cardiovascular system (Coleman & Dampney 1995; McCall *et al.* 1987; Hof & Fozard 1989; Fozard *et al.* 1987). Therefore, this study has more direct application to how the cardiovascular system responds to stress in a normal animal and how alterations in this system may contribute to the etiology of SIDS.

Basal Cardiovascular Parameters

The pre stress MAP and HR of rats in the different microinjection treatments for each stress were not statistically different from each other. However, there was a wide range of basal MAP and HR values within each group. For example, the MAP of rats that were restrained had ranges of 96.8-138, 92.9-143, and 98.6-121 mmHg for the ACSF, DPAT and muscimol microinjected treatments, respectively. The variation in these variables are similar to the results of Charkoudian *et al.* (2010), whose study examined the intraspecies variation of cardiovascular parameters of rats and humans. Baseline HR and MAP parameters were similar to those in previous studies (Vianna,

Allen, & Carrive 2008; van den Buuse & Wegener 2005). The qualitative similarities between the cardiovascular systems of the rat and human reinforce the validity of the application of animal models to the study of human physiology.

Inhibition of Brainstem Neurotransmission

Muscimol inhibits neuronal cells by activating $GABA_A$ receptors that increases the permeability of chloride channels. Administration of muscimol likely activates inhibitory post synaptic receptors which decreases neuronal transmission. The inhibitory activity of the serotonergic system by activation of the $5HT_{1A}$ receptor stems from its role as an autoreceptor and/or as a post synaptic inhibitory receptor. Activation of this receptor has been found to inhibit the activity of adenylyl cyclase (Markstein *et al* 1986; Fayolle *et al*. 1988). The reduced intracellular concentration of cyclic adenosine monophosphate (cAMP) stemming from this inhibition leads to a decrease in the activity of protein kinase A (PKA), which is activated by cAMP. This leads to decreased stimulation of post synaptic cell (source) or inhibition of pre synaptic voltage-sensitive calcium channels, which are necessary for the exocytosis of synaptic vesicles (Dolphin 2003). The end result of $5HT_{1A}$ receptor activation is the inhibition of post synaptic neuronal firing and/or inhibition of exocytosis of synaptic vesicles containing serotonin.

Cardiovascular Responses to Stress

ACSF was used as a control in this study compared to studies that have used saline. Cerebrospinal fluid is a complex solution of ions, nutrients, and proteins, all of which act as solutes and may be necessary for signaling events. The use of saline may not have sufficient solutes and may cause cells in microinjected areas to swell (McNay & Sherwin 2003).

In all instances, rats that were microinjected with ACSF and stressed had increases in MAP and HR. However, the responses following the air jet stress was markedly lower than responses following the handling and restraint stress. This difference may reflect either the different nature of this stress compared to the other stressors or it may be due to the rats' perception of the stress. In both the handling and restraint stresses, the rats were physically touched and the cage top was opened, which may have set off different pathways in the brain upstream to the MR. During the air jet stress, the cage top was not opened and the rat was not handled. While the rat may have been stressed due to its uncertainty of the situation, it may have perceived the stress as environmental and not from a potential predator. The fear of predation, which can be tested using the open field test, has been shown to trigger cardiovascular responses (van den Buuse & Wegener). The differences in the magnitude of these responses could also be attributed to differences in the magnitude at which the neural centers that mediate the cardiovascular responses to stress were activated.

The increase in HR and MAP are the cardiovascular components in a multifactorial response to stress. The changes in these factors following microinjections of ACSF and stress indicated that neurons within the NRPs were intact and functional. The increases in MAP and HR are adaptive responses that allow for increased blood supply (and nutrients) to areas of the body (such as muscles) that would be used during stress. This may translate to an increase in an animal's ability to escape from a predator or ability to fend its territory from competitors.

Changes in MAP and HR following stress in animals microinjected with DPAT and muscimol were statistically different from those microinjected with ACSF (with a

few exceptions). The rats either had no change or a decrease in their cardiovascular parameters after stress following microinjections with DPAT or muscimol. Activation of $GABA_A$ receptors inhibited the activity of the entire NRP. This likely inhibited SNS premotor neurons in the NRP, and led to an inhibition of the SNS response to stress (Coleman & Dampney 1995). DPAT inhibited the cardiovascular responses in a similar fashion to muscimol. The neurons of the NRP are usually only active in response to stress (Nalivaiko, Ootska & Blessings 2005; Zaretsky *et al.* 2003). Activation of the $5HT_{1A}$ receptors by DPAT inhibited the release of serotonin by these neurons. The end result of microinjections of DPAT and muscimol were the same- SNS premotor neurons in the NRP were not activated.

The responses following microinjections of muscimol and DPAT were not significantly different from each other. This suggests that these responses are entirely mediated by the serotonin system in the NRP or that the $5HT_{1A}$ receptor can modulate other neurotransmitter systems in this region of the brain. Muscimol generated IPSPs on post-synaptic cells to inhibit action potentials generated by other signaling pathways (such as serotonin). DPAT likely inhibited the release of serotonin from pre-synaptic cells by activating autoreceptors, preventing the initiation of these action potentials. Alternatively, DPAT may have activated inhibitory post synaptic receptors that modulated the stress response. The $5HT_{1A}$ receptor is expressed pre- and post-synaptically. When expressed as a pre-synaptic autoreceptor, activation of the $5HT_{1A}$ receptor inhibits the cell from releasing 5HT into the synaptic cleft. $5HT_{1A}$ receptor is also found post-synaptically, mostly on the cell body and dendrites (Figure 15; Barnes & Sharp 1999, Darnall *et al.* 2005). Its role on post-synaptic neurons has not been

extensively studied and is not well understood. Given its role as a metabotrophic receptor, it cannot initiate an action potentials or IPSPs. Perhaps the $5HT_{1A}$ receptor has a modulatory effect when expressed post-synaptically. Other excitatory neurotransmitters, namely glutamate and dopamine, in the MR have been identified as being partially responsible in mediating the cardiovascular responses to stress (Alvarenga, Pires & Futuro Neto 2005; Edwards & Paton 2000; Kitahama *et al.* 2000; Vianna, Allen, & Carrive 2008). If microinjections of DPAT only affected the 5HT system, one would expect to see the effects of these other neurotransmitter systems, such as glutamate or dopamine that should have been activated during the stress response. Since the responses were abolished, it could be hypothesized that post-synaptic activation of the $5HT_{1A}$ receptor may have affected the activity of those non-serotonergic neurotransmitter systems. Although this has not been confirmed in the NRP, the $5HT_{1A}$ receptor has been found to be a modulator of other neurotransmitters (Figure 16; Khateb *et al.* 1993).

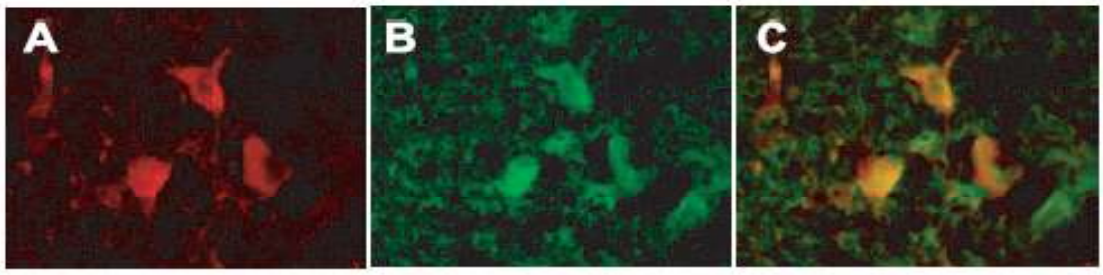

Figure 15. Distribution of the $5HT_{1A}$ receptor and tryptophan hydroxylase. Cells from the Nucleus Paragigantocellularis Lateralis were stained for the expression of the 5HT1A receptor and tryptophan hydroxylase. (A) Cells stained red were positive for tryptophan hydroxylase. (B) Cells stained green were positive for the 5HT1A receptor. (C) An overlay of images A and B. Orange cells expressed both the 5HT1A receptor and tryptophan hydroxylase. Adapted from Darnall *et al* (2005).

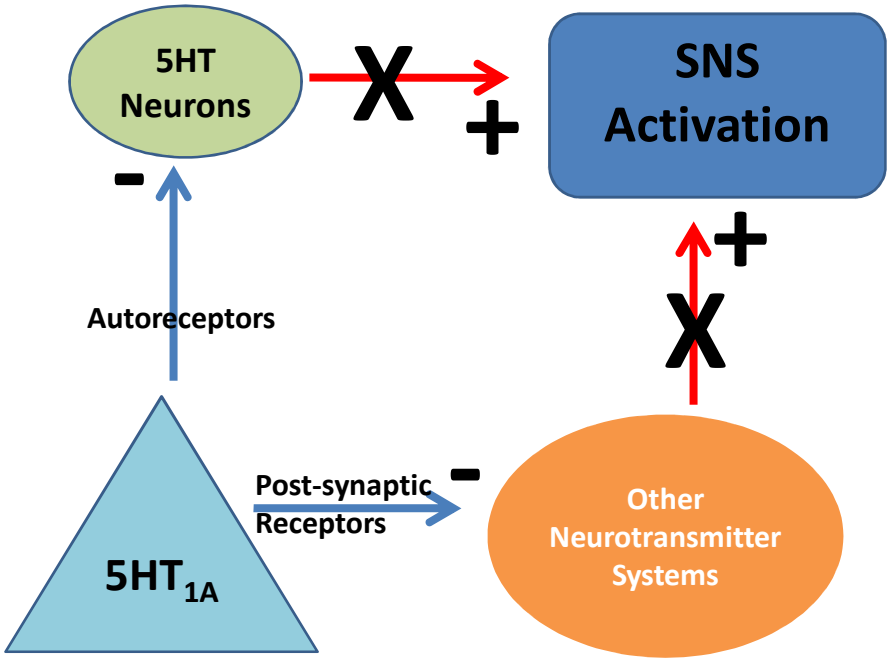

Figure 16. Possible mechanisms in which the $5HT_{1A}$ receptors in the NRP inhibits the SNS. A proposed role for 5HT1A in mediation of the SNS. As an autoreceptor, the receptor inhibits the activity of serotonergic neurons. As a post-synaptic receptor, it acts as an inhibitory modulator of other neurotransmitter systems. Activation of the serotonergic and other neurotransmitter systems in the NRP activates the SNS, which goes on to activate stress responses. This response is inhibited by the 5HT1A receptor.

While the abolishment of the normal cardiovascular response to stress following muscimol or DPAT injection is consistent with previous studies (Nalivaiko *et al*. 2005; Vianna, Allen, & Carrive 2008), there were noted differences. These previous studies have generally found that microinjections DPAT, muscimol, and other inhibitory pharmacologic agents into the MR attenuated, but did not abolish, the cardiovascular responses to stress. Generally, a response was still observed following microinjections and subsequent stress. A difference in this study compared to others is that the rats were microinjected in their home cage without being picked up. In previous studies, these animals were picked up and handled during microinjection. Taken together, this suggests not only that these stress responses were mediated by the MR, but also that the entirety of the response may be mediated by the 5HT system.

An important issue arises when comparing the results of this study to previous studies. Even though the animals were stressed in these previous studies, why were the cardiovascular responses not abolished as observed in this study (Nalivaiko *et al*. 2005; Vianna, Allen, & Carrive 2008)? Why was only an attenuation of the response observed? A suitable explanation may be that as these animals were picked up and handled for microinjection, the fight or flight response may have been fully engaged. The result of which may have been the release of hormones, including epinephrine, into the vasculature. In turn, the attenuated response may be due to an abolishment of the neural aspect of the response and the hormonal effects may have already been established. Since the rats were not handled during microinjections in this study, the fight or flight response may have been abolished altogether. The MR is a relay center for the descending stress signals from higher regions of the brain before they are relayed to the

body (Cao & Morrison 2003; Samuels, Zaretsky, & Dimicco 2002; Zaretsky *et al.* 2003). If instead of not only mediating the cardiovascular responses to stress, the MR also mediated the release of stress hormones into the vasculature, the differences between this study and the attenuated responses in previous studies could be accounted for. However, this explanation is only a proposed hypothesis and would need to be verified experimentally.

Clinical Implications

This study further expanded the understanding of the physiology of the MR and its role in regulating the cardiovascular responses to stress. If the MR is the central relay station of the brain before these signals are sent to the body, it can aid in the explanation of the etiology of SIDS. In SIDS, there are severe abnormalities in the 5HT system of the MR including the NRP. These abnormalities can lead a loss of the ability for stress signals from the brain to reach the body. Ultimately, this leads to an inability for the body to respond to stress that the brain may have detected, and subsequently to sudden death. While this is a possible mechanism for sudden death, it is a downstream effect of the biological cause of SIDS (Figure 16).

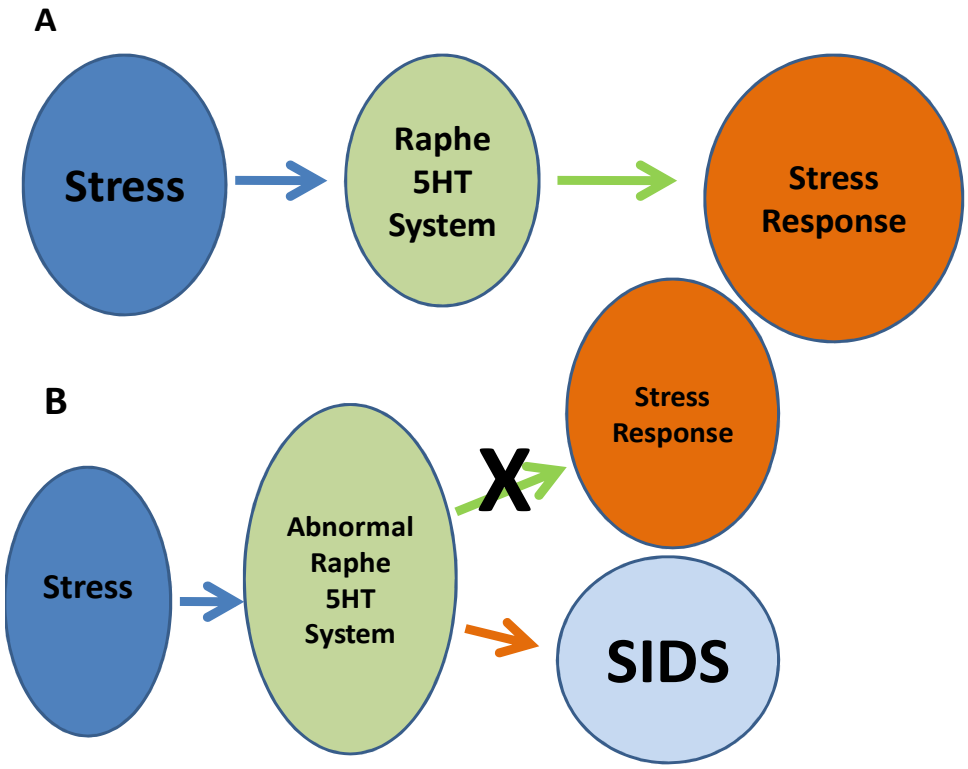

Figure 17. The role of the serotonin system in the MR on stress responses and its relationship to SIDS. (A) Under normal circumstances, stress leads to an activation of the serotonergic system of the MR, which leads to a stress response. (B) When the serotonergic system of the MR is abnormal, the response to stress is not activated. In infants, this leads to SIDS.

Patients who have hypertension may have an increase in the body's set point for regulation of MAP (Guyenet 2006; Osborn 2005). The MR is a cardiovascular control center and may potentially be partially responsible for the increase in set-point. Therapeutics that can selectively inhibit the 5HT system of the MR may be efficient in lowering the patient's MAP, however, the fidelity in the cardiovascular response to stress may be dangerously muted.

Conclusions

Microinjections of muscimol and DPAT into the NRP abolished cardiovascular stress responses that were seen in rats that were microinjected with ACSF. While this study provided insights into the mediation of cardiovascular responses to stress by the MR, it also posed new questions its mechanisms of action. It is possible that instead of being responsible solely for the cardiovascular response to stress, the MR may be responsible for the mediating many more components of the stress response, including the hormonal components. The loss of this response may be partially responsible for SIDS.

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