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Research report

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

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ABSTRACT

Dysfunction in serotonin (5HT) neurotransmission in the brainstem of infants may disrupt protective responses to stress and increase the risk for Sudden Infant Death Syndrome (SIDS). The raphé pallidus (NRP) and other brainstem nuclei are rich in 5HT and are thought to mediate stress responses, including increases in blood pressure (BP) and heart rate (HR). Determining how 5HT neurotransmission in the brainstem mediates responses to stress will help to explain how dysfunction in neurotransmission could increase the risk of SIDS. It was hypothesized that alterations in neurotransmission in the NRP, specifically activation of the $5HT_{1A}$ receptor subtype, would block cardiovascular responses to various types of exogenous stress. Using aseptic techniques, male Sprague-Dawley rats were instrumented with radiotelemetry probes which enabled non-invasive measurement of BP and HR. An indwelling microinjection cannula was also stereotaxically implanted into the NRP for injection of drugs that altered local 5HT neurotransmission. Following a one week recovery period, rats were microinjected with either muscimol ($GABA_A$ receptor agonist), 8-OH-DPAT (agonist to the inhibitory $5HT_{1A}$ receptor), or a vehicle control (artificial cerebral spinal fluid; ACSF) immediately prior to exposure to one of three stressors: handling, air jet, or restraint. Physical handling and restraint of the animal were designed to elicit a mild and a maximal stress response respectively; while an air jet directed at the rat's face was used to provoke a psychological stress that did not require physical contact. All three stressors elicited similar and significant elevations in HR and BP following ACSF that persisted for at least 15 min with BP and HR elevated by ~14.0 mmHg and ~56.3 bpm respectively. The similarity in the stress responses suggest even mild handling of a rat elicits a maximal sympathoexcitatory response. The stress response was abolished following 8-OH-DPAT or muscimol microinjection suggesting the cardiovascular responses to stress are mediated by the NRP and likely involve the $5HT_{1A}$ receptor. Impairment in $5HT_{1A}$ receptor function in the NRP likely impairs the normal cardioprotective responses to stress and may contribute to the etiology of SIDS.

1. Introduction

Sudden Infant Death Syndrome (SIDS) is a leading cause of infant mortality and is thought to result, in part, from an infant's inability to perceive and/or respond to stress. There is increasing evidence that developmental abnormalities in serotonin (5-hydroxytryptamine or 5HT) neurotransmission in select brainstem nuclei contribute to the etiology of SIDS [31,36]. Specifically, there are an increased number of 5HT neurons and a decreased binding to the $5HT_{1A}$ receptor subtype in select brainstem areas such as the raphé nuclei. These neurons innervate motor neurons in the intermediolateral horn of the spinal cord and likely coordinate the sympathetic cardiovascular reflex response to stress, including the elevation in heart rate (HR) and blood pressure (BP) [26]. In response to stress, efferent signals from higher brain centers, such as the hypothalamus, converge in the raphé nuclei before being sent to the body [3,8,14,15,34]. In this way the raphé act as a relay station for effector responses to stress stimuli [18]. Therefore, a developmental abnormality in 5HT neurotransmission in the raphé nuclei of an infant could significantly hinder the protective stress response and increase the risk for SIDS.

Numerous studies suggest that the $5HT_{1A}$ receptor subtype in the medullary raphé (MR) mediates protective responses to stress [2,11,24,27,28,32,38]. Systemic administration of 8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT), a selective $5HT_{1A}$ receptor agonist, resulted in bradycardia and a decrease in BP [7,10] suggesting the $5HT_{1A}$ receptor has an inhibitory effect on sympathetic outflow [3,4]. Similar sympatholytic effects were evident when 8-OH-DPAT was administered via microinjection directly to select nuclei of the MR [20,25]. Localized activation of the $5HT_{1A}$ receptor in the MR blocked the shivering response to cold stress in sleeping piglets [2] and the increase in HR and brown adipose tissue

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activation in response to skin cooling in anesthetized rats [24,27]. The $5HT_{1A}$ receptor also mediates the cardiovascular responses to other stressors such as conditioned fear [38], fever induction [28], and psychological stress [37]. Transgenic $5HT_{1A}$ knockout models of mice found cardiovascular responses to be exacerbated when exposed to stressful stimuli [11,32]. These data suggest that the inhibitory $5HT_{1A}$ receptor in the MR reduces the activity of neurons that help mediate the cardiovascular responses to various forms of stress [19,28].

Inconsistencies in these studies and the use of anesthetized preparations have unfortunately confounded the results. Activation of the $5HT_{1A}$ receptor in select MR nuclei, such as the raphé pallidus (NRP), resulted in bradycardia and decreased BP in anesthetized animals [5,9,13,21] while studies in conscious animals found that these responses were either much weaker or absent [7,16]. Other studies that have selectively targeted the NRP used only the initial changes (s) in HR and BP to determine the maximum changes in the cardiovascular response to stress [28]. The more sustained responses (min) to stress that may be essential for the protective effect were not considered. Thus, the role of the $5HT_{1A}$ receptors in the MR, specifically the NRP, in mediation of protective cardiovascular responses to stress remains unclear.

In the present study, the role of the NRP in mediating cardiovascular responses to several types of stress (handling, air jet, and restraint) in conscious rats was explored. Animals were physically handled or restrained to evoke a mild or a maximal stress response respectively. In addition, rats were exposed to an air jet directed at their face to provoke a moderate psychological stress that did not involve physically handling the animal. We hypothesized that pharmacologic inhibition of the NRP, and selective activation of the inhibitory $5HT_{1A}$ receptor, would block the sympathoexcitation associated with the cardiovascular response to various forms of environmental stress in conscious rats. Muscimol ($GABA_A$ receptor agonist) or 8-OH-DPAT ($5HT_{1A}$ receptor agonist) was microinjected into the NRP prior to stress while HR and BP were measured. If 5HT neurotransmission in the NRP is an important mediator of the cardiovascular response to environmental stress, then alteration in the function of the $5HT_{1A}$ receptor will alter the protective increase in BP and HR following exogenous stress exposure. If 5HT neuron development and/or neurotransmission in the NRP are impaired in victims of SIDS as suggested [31], then the protective responses to environmental stress may be inadequate, and may contribute to its etiology.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (≥9 weeks old; 250–375 g; $N = 84$) were included in this study. All surgeries were performed on rats anesthetized with 75 mg/kg ketamine and 15 mg/kg xylazine (intra-abdominal injection) and were conducted using aseptic techniques. Following surgical instrumentation, all animals were given 1 mg/kg indomethacin on rat treats each day for two days for pain management and a one week recovery period before experimentation. All methods were performed with approval from James Madison University's animal care and use committee.

2.2. Biotelemetry and stereotaxic surgery

A stainless steel cannula (20 ga; 26 mm long) targeting the midline NRP was stereotaxically implanted (2.3 mm caudal to lambda and 9.2 mm ventral to the skull surface at the midline) into the rat's skull. This allowed microinjection of drugs into the NRP that altered neurotransmission. The cannula was secured with at least 3 screws anchored into the rat's skull and reinforced with dental cement.

Immediately following stereotaxic surgery, a biotelemetric probe (PhysioTel PA-C40 small animal transmitter; DataSciences International; St. Paul, MN) used to measure BP and HR was implanted in the abdominal cavity of the rats. A 2.5 cm midline abdominal incision along the lina alba was made to gain access to the abdominal cavity. The descending aorta was then isolated to insert the attached cannula between the celiac and renal branches, according to the manufacture's protocol, and held in place using tissue adhesive (LiquidVet Rapid; MedRep Express; Prescott, AZ) and 1 cm^2 piece of sterilized filter paper. The probe was then secured to the abdominal muscles using non-absorbable silk sutures. The abdominal muscle layer was closed with non-absorbable silk sutures and the skin was closed using surgical staples.

2.3. Microinjection protocol

Prior to stress induction, an injection cannula (25 ga, 28 mm) that extended past the indwelling cannula 2.0 mm was used to deliver drugs to the NRP. The injection cannula was attached to a Hamilton syringe (7000 series) via a 20 cm piece of PE-50 tubing, which allowed 300 nl of volume to be injected at a rate of 100 nl/min, as well as allowing free movement of the rats. After microinjection, a 100 nL air bolus was administered and the injection cannula was left in the brain for 1 min to allow drug diffusion and to prevent reflux through the indwelling cannula. Artificial cerebrospinal fluid (ACSF; 128 mM NaCl, 3.0 mM KCl, 1.3 mM $CaCl_2$, 1.0 Mg Cl_2, 21.0 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, 1.0 mM D-glucose, pH = 7.4 titrated by bubbling in CO_2) [22] was used as a vehicle control. 30 mM of muscimol, the inhibitory $GABA_A$ receptor agonist, or 8-OH-DPAT, the inhibitory $5HT_{1A}$ receptor subtype agonist, were also injected in randomized trials. The 30 mM dose was chosen because it is the highest concentration of 8-OH-DPAT that will dissolve into ACSF.

2.4. Data collection

The intra-abdominal radiotelemetry probes allowed for continuous data collection without disturbing the animal. The rat's cage was placed above telemetry receiver plates and HR and BP data were collected every 30 s for at least 15 min prior to microinjection and at least 15 min after the end of a stress. Data prior to microinjection were averaged and used as a baseline measure in which to compare the post stress data. Data from the onset of microinjection until the end of the stress was not used for analysis due to the stress effect of the microinjection protocol on the animal.

2.5. Experiments

Prior to microinjections, all rats were allowed to acclimate to the lab environment in their home cage until BP (remained within ±10 mmHg) and HR (remained within ±25 beats per minute (bpm)) had stabilized for at least 15 min. No more than 3 stress experiments were conducted on each rat to avoid acclimation to microinjection procedure. Microinjection without stress was not assessed because the microinjection procedure alone typically activates the autonomic response to stress. Only one experiment was conducted per day, and experiments were conducted on consecutive days. The combination of drug injected and stress used to provoke a cardiovascular response was randomized however, the microinjection was always performed immediately prior to the onset of stress. The procedure for stress induction is described below.

2.5.1. Handling

The rats were held at approximately the midpoint of their tail while being lifted a few centimeters above the floor of their cage for 30 s. They were then released and the lid of the cage was replaced. Handling the animal for longer than 30 s usually caused the rats to try and escape. The goal of this experiment was to elicit a mild stress response so the handling of the animal was limited to 30 s.

2.5.2. Air jet

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 s. The nozzle was aimed at the rat's face while the rats freely moved around the cage during the course of the stress. The goal of this experiment was to expose the rat to an unfamiliar stimulus and elicit a moderate psychological stress response that did not require physically handling the animal.

2.5.3. Restraint

The rats were held around the thoracic region behind the front limbs in their cage for 60 s. While the rats were able to move their limbs and freely breathe, they were not able to move their bodies. The goal of this experiment was to elicit a maximal stress response as if the animal was captured by a predator.

2.5.4. Histological verification

After experiments were completed, the rats were euthanized and 300 nl of a 1% solution of potassium permanganate was microinjected into the brainstem of the rats (in the same manner as the drug injections). After transcardial perfusion with heparinized saline followed by 5% formalin, the brains were prepared for histological analysis to confirm injection sites at the NRP. Targeting of the NRP was deemed successful if the potassium permanganate stain was within ±1.5 mm of the caudal end of the facial nucleus which is −2.3 mm from the interaural line, within 1.0 mm of the ventral surface, and within 0.75 mm of the midline as indicated by comparison to a rat brain stereotaxic atlas [33]. The mean coordinates for injection were -1.9 ± 0.1 mm caudal to interaural line, 0.7 ± 0.1 mm from midline, and 0.5 ± 0.1 mm from the ventral surface. An example of the injection site is shown in Fig. 1. Twenty three of the 32 rats (71%) that survived the surgical instrumentation