CRH in the nTS (nucleus tractus solitarii) contributes to cardiorespiratory responses to hypoxia

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INTRODUCTION

Peripheral chemoreceptors located in the carotid body and aortic body monitor blood oxygen levels and send this information via afferent projections to the nucleus tractus solitarii (nTS) in the brainstem. The nTS then modulates and integrates this information before relaying it to other brain regions important in cardiorespiratory control. The major chemoreflex pathway that produces increases in respiration and mean arterial pressure in response to hypoxia involves activation of neurons in the nTS that project to the rostral ventrolateral medulla (RVLM). However, the paraventricular nucleus (PVN) of the hypothalamus is also activated by hypoxia (Berquin et. al, 2000) and cardiorespiratory responses to chemoreflex activation are blunted when the PVN is inhibited or lesioned—evidence that suggests the PVN is necessary for full expression of the chemoreflex (Reddy et. al, 2005, Olivan et. al, 2000).

The PVN receives input from multiple brainstem regions, including the nTS and caudal ventrolateral medulla (CVLM), and has projections to the RVLM, nTS and intermediolateral cell column (IML). Interestingly, PVN neurons that project to the RVLM or IML –which would be expected to contribute to cardiorespiratory responses— are not activated by hypoxia (Coldren et. al, 2013). However, nTS projecting PVN neurons are activated by hypoxia and nearly 90% of these neurons express corticotropin-releasing hormone (CRH) (Figure 1B; Ruyle et. al, 2015). Based on these results, we hypothesized that CRH neurons located within the PVN modulate cardiorespiratory responses to chemoreflex activation via a projection to the nTS.

The purpose of this study was to further characterize the pathway between the PVN and nTS and elucidate the importance of CRH in the nTS in increasing ventilation during chemoreflex activation. Immunohistochemistry was used to verify CRH terminals and CRH receptors in the nTS. Labeling with Synaptophysin (synaptic marker) and MAP2 (dendritic marker) was used to further examine the location of these receptors. Tyrosine hydroxylase (TH; catecholaminergic marker) was investigated as prior studies have shown that nTS catecholaminergic cells are activated by hypoxia and are required for full expression of the chemoreflex (King et. al, 2015). In order to evaluate ventilatory responses before and after lesion of nTS cells with CRH receptors, plethysmography experiments were conducted on rats injected with a CRH saporin into the nTS.

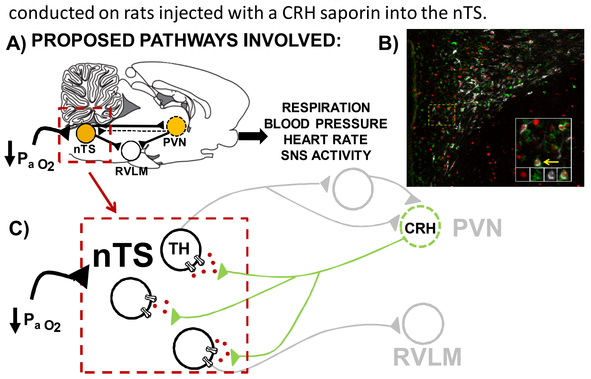

Figure 1: Experimental Model. A) Hypoxia activates a reciprocal nTS-PVN pathway to increase ventilation and arterial pressure **B)** CRH neurons located within the PVN that project to the nTS are activated by hypoxia. Photomicrograph of the PVN shows **Fos (red)**, **CRH (green)**, and nTS-projecting (white) cells. **C)** CRH projections from the PVN may influence nTS neurons to modulate cardiorespiratory responses

HYPOTHESIS

CRH released from PVN neurons acts at CRH receptors located in the nTS to modulate cardiorespiratory responses to chemoreflex activation.

If true, we also predict the following (Figure 1C):

- CRH is located at terminals in the nTS
- CRH receptors (CRHRs) are present in the nTS
- Lesion of nTS cells expressing CRHRs using a CRH-specific saporin will lead to a decreased respiratory response to acute hypoxia

RESULTS

CRH puncta consistent with terminals are present in the nTS and surround TH cells

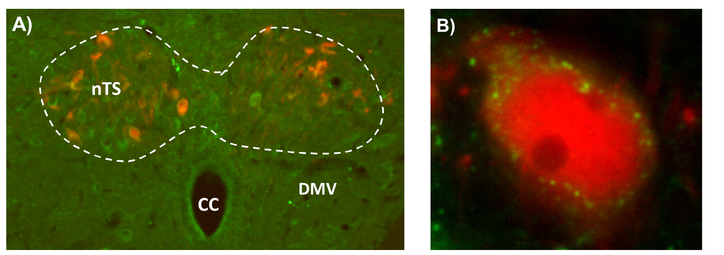

Figure 2. CRH is present in the nTS. A) 10X **CRH (green)**, **TH (red) B)** 60X merged image CRH, TH
Legend: Central canal (CC), dorsal motor nucleus of the vagus (DMV)

CRHR2 is located rostral-caudally throughout the nTS

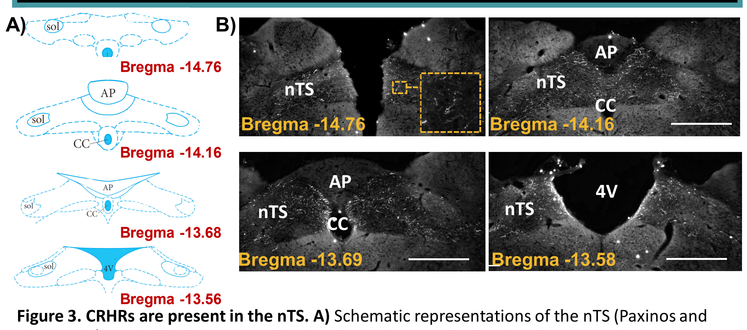

Figure 3. CRHRs are present in the nTS. A) Schematic representations of the nTS (Paxinos and Watson, 6th edition). **B)** Corresponding 4X photomicrographs showing fluorescent images of CRHR2 in the nTS. Scale bar: 1mm. Legend: Area Postrema (AP), 4th ventricle (4V), Solitary tract (Sol)

CRHRs do not co-label with dendritic or synaptic markers

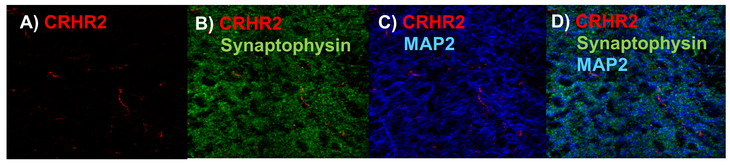

Figure 4. Determining the location of CRHR2 in the nTS. 60X photomicrographs of **A) CRHR2 (red) B)** CRHR2 and Synaptophysin (**green**; synaptic). **C)** CRHR2 and MAP2 (**blue**; dendritic). **D)** Merged image with CRHR2, Synaptophysin, and MAP2.

CRH saporin alters respiratory function under acute hypoxic conditions

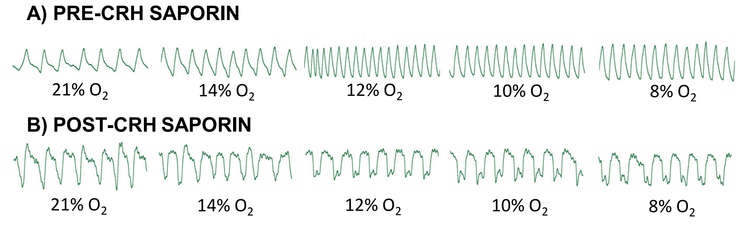

Figure 5. Plethysmography raw data (10s traces). Ventilatory responses to normoxia and hypoxia **A)** before and **B)** after administration of CRH saporin

Effects of lesion of nTS neurons with CRH receptors on ventilatory responses to hypoxia

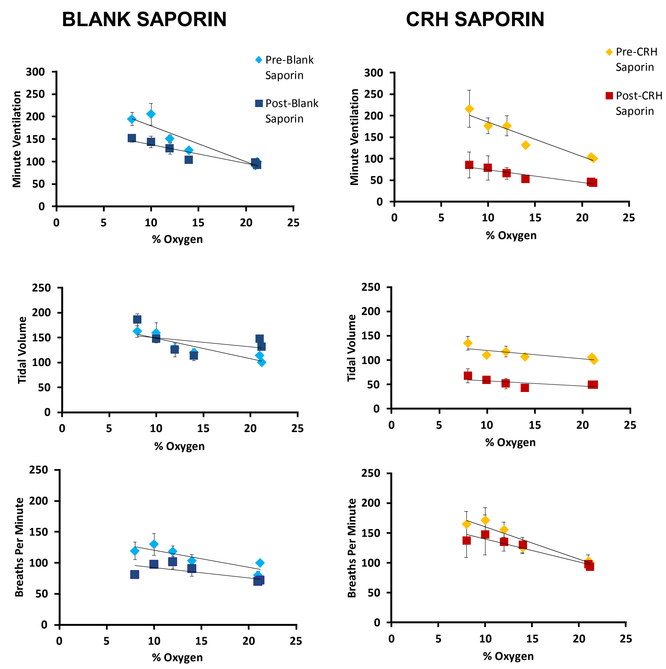

Figure 6. Effects of lesion in the nTS on ventilatory responses to peripheral chemoreceptor stimulation. Injection of CRH saporin (n=3) into the nTS reduced tidal volume and minute ventilation responses to hypoxia to a greater extent than Blank saporin (n=4).

Lesion of nTS cells expressing CRHRs using a CRH-specific saporin may lead to a decreased respiratory response under acute hypoxic conditions.

Effects of lesion of nTS neurons with CRH receptors on ventilatory responses to hypercapnia

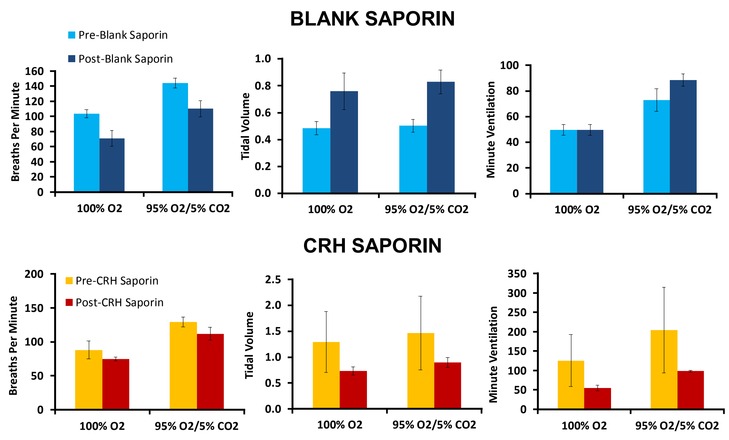

Figure 7. Effects of CRH saporin in the nTS on ventilatory responses to central chemoreceptor stimulation. Group data (n=7) showing depressed respiratory rate, tidal volume and minute ventilation in CRH saporin animals.

Data suggest that CRH and CRHRs in the nTS play a role in mediating the response to central chemoreceptor stimulation.

METHODS

PVN Microinjection: Male Sprague Dawley rats (240-310g) were anesthetized and placed in a stereotaxic apparatus. The brainstem at the level of the nTS was exposed and 4 (30-60nL) microinjections of Blank saporin (controls) or CRH saporin were injected bilaterally into the nTS. Rats were allowed 14 days to recover.

Immunohistochemistry (IHC): Standard IHC procedures were performed on 30µm hindbrain sections from naïve rats.

1° Antibodies	Rabbit anti-CRFR2 (1:5000)	Mouse anti-MAP2 (1:500)	Guinea pig anti-Synaptophysin (1:500)	**1° Antibodies**	Mouse anti-TH (1:1000)	Guinea pig anti-CRF (1:1000)
2° Antibodies	Cy2 donkey anti-rabbit IgG (1:200)	Cy5 donkey anti-mouse IgG (1:200)	Cy3 donkey anti-guinea pig IgG (1:200)	**2° Antibodies**	Cy2 Donkey anti-mouse IgG (1:200)	Cy3 donkey anti-guinea pig IgG (1:200)

Chemoreflex challenges: Prior to microinjection with CRH saporin or Blank saporin (controls), respiratory responses were assessed via plethysmography while the rats were conscious and unrestrained. Rats were conditioned to whole body plethysmography chambers (Data Sciences International) for two hour periods under room air over three days. On the day of the experiment, rats were exposed to normoxic (21% O_2), a range of hypoxic (14, 12, 10, 8% O_2), and hypercapnic (95% O_2/5% CO_2) conditions. A pressure transducer connected to a PowerLab (ADInstruments) data acquisition system was used to measure changes in chamber pressure and calculate respiratory rate (f), tidal volume (V_T) and minute ventilation (V_E=V_T x f). Oxygen saturation was measured using a pulse oximeter (MouseOx, StarrLife Sciences Corp.). Plethysmography studies were repeated after a two week recovery period following microinjection.

Analysis: An epifluorescent Olympus BX51 microscope was used to visualize rostral-caudal sections of the nTS. ImageJ software was used to compile images with positive labeling for CRF, CRFR2, TH, MAP2, and Synaptophysin. Plethysmography data were compiled and analyzed using PowerLab software and Microsoft Excel.

SUMMARY AND CONCLUSIONS

- CRH puncta consistent with terminals are located in the nTS and surround TH cells
- CRHR2 receptors are present in the nTS
- CRHR2 receptors can be found in close apposition to terminals and dendrites
- Rats exhibited decreased respiratory responses when cells with CRH receptors were lesioned with a CRH saporin in both hypoxic and hypercapnic conditions

Anatomic and functional data support a role for CRH and CRH receptors in the nTS in mediating cardiorespiratory responses to hypoxia

FUTURE DIRECTIONS

- Verify efficacy of CRH saporin injection in nTS
- Investigate if glial cells within the nTS contain CRH receptors
- Examine whether CRH receptors are expressed in RVLM projecting nTS neurons
- Optimize CRHR1 antibody for use in future studies

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