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Paraventricular nucleus neurons target nucleus tractus solitarii via CRH receptors and oxytocin in hypoxia

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INTRODUCTION

The arterial chemoreflex increases ventilation, sympathetic nervous system activity, and arterial blood pressure to maintain oxygen delivery in hypoxic conditions. Chemoreceptors in the aortic and carotid bodies sense arterial oxygen, and chemoafferents convey this information to the nucleus tractus solitarii (nTS) in the brainstem. The nTS integrates this input and sends signals to nuclei in the brainstem and forebrain that are involved in ventilation and sympathetic nervous system regulation. The output from the nTS to the ventrolateral medulla (VLM) is considered to be the primary reflex pathway mediating autonomic and ventilatory responses to hypoxia. Another pathway contributing to the response to hypoxia is a projection from the nTS to the hypothalamic paraventricular nucleus (PVN). The PVN is an integrative nucleus important in control of respiration, cardiovascular function, and sympathetic nerve activity. Hypoxia activates (indicated by Fos immunoreactivity, IR) nTS projections to the PVN, and the response to hypoxia is blunted when the PVN is inhibited or lesioned. Together, these data suggest the PVN is important in control of sympathetic, pressor, and respiratory responses to hypoxia. However, the efferent pathway from the PVN that mediates cardiorespiratory responses to hypoxia is not fully understood. The PVN sends projections to the VLM and spinal cord nuclei involved in control of respiration and sympathetic nervous system function; these projections, however, do not express Fos following hypoxia, indicating that they are not activated by the chemoreflex. Projections from the PVN to the nTS do express Fos after exposure to hypoxia, suggesting this pathway may contribute to chemoreflex responses. The majority of nTS-projecting PVN neurons express either oxytocin or CRH; in hypoxia, most (85-90%) activated nTS-projecting PVN neurons express CRH. Thus, the PVN may mediate responses to hypoxia via CRH acting on receptors in the nTS. We have shown that the predominant CRH receptor in the nTS, CRFR2, colocalizes extensively with oxytocin, and also with synaptophysin, suggesting CRH modulates oxytocin release in the nTS presynaptically.

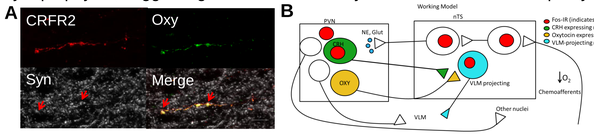


Figure 1: **A:** Photomicrographs of coronal section of nTS displaying CRFR2-IR (red), Oxytocin-IR (green) and synaptophysin-IR (grey). Arrows denote colocalization of CRFR2, oxytocin, and synaptophysin. **B: Working Model:** Information from carotid and aortic bodies is integrated in the nTS, which sends projections to the PVN. The PVN sends projections to the VLM, pre-Bötzinger complex, intermediolateral cell column, phrenic motor nucleus, and nTS; however, only nTS-projecting PVN neurons are activated by hypoxic conditions. The majority of these activated PVN neurons express CRH. We have previously shown that CRH receptors (CRFR2) and oxytocin colocalize extensively in the nTS, along with synaptophysin, suggesting CRH acts presynaptically on oxytocin terminals to influence chemoreflex response.

HYPOTHESIS

- CRFR2- and oxytocin-expressing puncta make close appositions onto nTS neurons that are activated in Hx, in particular VLM-projecting neurons.

METHODS

Animals: Male Sprague Dawley rats (250-350g) were used; n=6 hypoxic (10% oxygen), n=5 normoxic (21% oxygen).

Retrograde labeling from the VLM: Anesthetized rats were placed in a stereotaxic apparatus, and the brainstem was exposed. A small burr hole was made in the skull. Coordinates for the VLM were as follows: 0.7 mm rostral to calamus scriptorius (CS), 1.5-1.7 mm lateral to midline, and 3.8 mm ventral to the dura. Retrograde tracer Cholera toxin B (CtB; 1%, conjugated to Alexafluors 488 or 555) was injected bilaterally (30 nl). Rats were allowed 7-10 days to recover and to allow retrograde transport of CtB to the nTS.

Hypoxia Exposure : Following recovery from microinjection surgery, conscious rats were acclimated for 2 hours to a hypoxic chamber (Biospherix) on 3 days at room air prior to the final experiment. On the day of the experiment, conscious rats were placed in the same hypoxic chamber and exposed to either normoxic (21% O_2, n=5) or hypoxic (10% O_2, n = 6) conditions for 2 hrs.

Tissue preparation: Following hypoxic exposure, rats were deeply anesthetized (5% Isoflurane) and transcardially perfused with heparinized Dulbecco's Modified Eagle Medium (DMEM, Sigma) followed by 200-400 ml 4% paraformaldehyde (PFA). Hindbrain sections were cut at 30 microns using a vibrating Microtome (VT 1000s; Leica, Germany).

Immunohistochemistry (IHC): IHC was performed on free-floating hindbrain sections. Protocols for IHC were as follows: (**1**) IHC targeting activation marker Fos-IR (Goat anti-Fos, 1:500, sc52g), CRFR2-IR (Rabbit anti-CRFR2 1:5000, abcam ab150510), and neuronal marker HUC/D-IR (Mouse anti-HU C/D 1:1000, ThermoFisher A-21271); and (**2**) IHC targeting Fos-IR (Rabbit anti-Fos 1:3000, sc-52) and Oxy-IR (Mouse anti-oxytocin, 1:2000, Millipore 2709170). Fluorescent secondary antibodies were used to visualize primary antibodies . The intrinsic fluorescence of the retrograde tracer CtB was used to visualize VLM-projecting cells.

Quantitative Analysis: An epifluorescent Olympus BX51 microscope was used with spinning disc confocal to visualize sections containing caudal nTS approximately 360 µm caudal to the CS. 60X image stacks were taken at 0.5µm between images. Stacks were converted to z- and 3-D y-axis projections (Z Project and 3D Project ImageJ plug-ins) to visualize interactions between oxytocin and VLM-projecting neurons in 3-D. Oxytocin may function as a volume transmitter over distances less than 3µm; we classified oxytocin-VLM projecting neuron interactions under 3µm as near interactions, interactions under 1µm as close appositions, and interactions with no visible space as contacts. Interactions were counted for both inactive and active (Fos-IR) VLM-projecting neurons using the Cell Counter plug-in through ImageJ software.

RESULTS

INJECTION SITE VERIFICATION

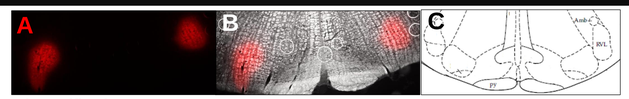


Figure 2: VLM bilateral injection site verification
A: Epifluorescent photomicrograph of VLM. **B**: Bright field photomicrograph of same section, with epifluorescent photomicrograph overlaid. **C**: Diagram (Paxinos and Watson, 6th edition) illustrating VLM anatomic location demonstrated in photomicrographs. VLM, rostral ventrolateral medulla; Amb, nucleus ambiguus; py, pyramidal tract.

PVN Projections Contact Activated nTS Neurons

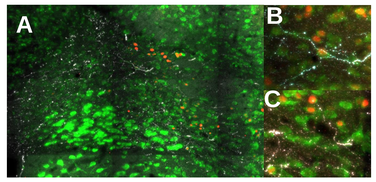
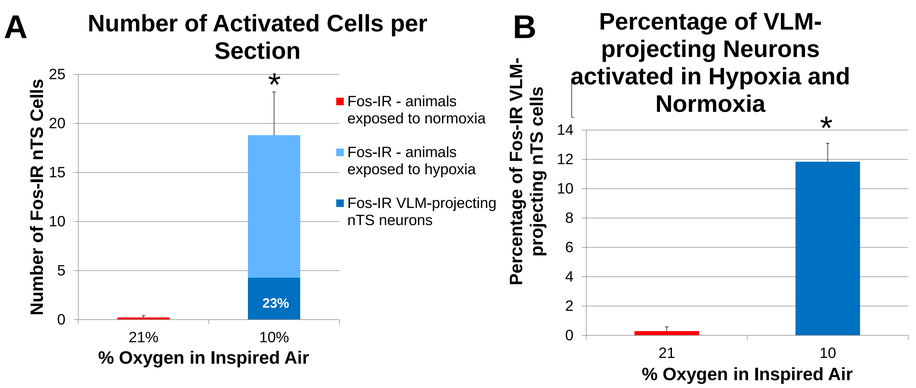


Figure 3: CRFR2-IR fibers closely appose Fos-IR nTS neurons. **A**: Merged photomicrograph of an nTS section from a rat exposed to hypoxia (10% O_2). CRFR2-IR (white) fibers and puncta made close appositions to activated (Fos-IR, red) nTS neurons (HUC/D-IR, green). **B:** High magnification photomicrograph of Fos-IR nTS neuron surrounded by CRFR2-IR fibers. **C:** High magnification photomicrograph of CRFR2 appositions on the soma of activated nTS neuron.

VLM-projecting nTS Neurons are Activated by Hypoxia



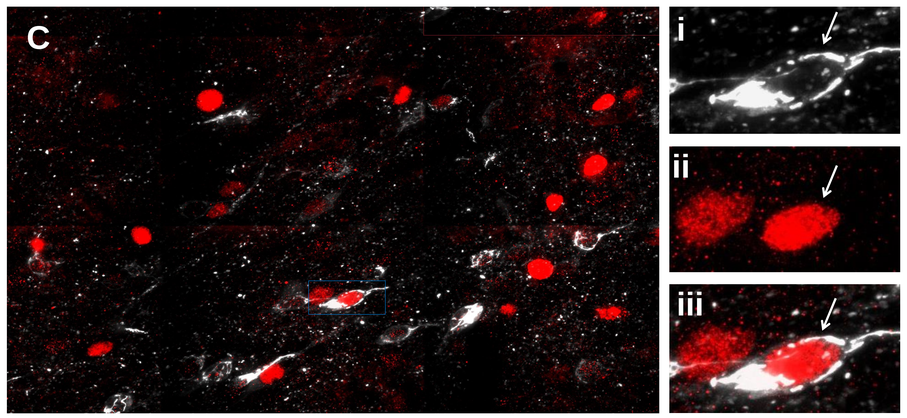
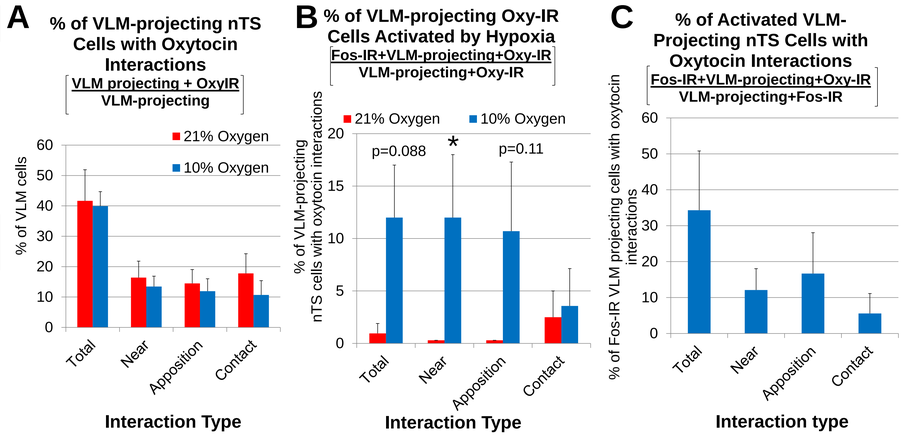


Figure 4: VLM projecting neurons expressed Fos-IR in response to hypoxia, indicating acute activation and suggesting involvement in chemoreflex responses. **A:** Fos-IR in hypoxic and normoxic animals. More nTS cells were activated following hypoxia than normoxia; of these hypoxia-activated cells, 23% project to the VLM. **B:** Consistent with previous studies, greater than 11% of VLM projecting neurons are activated in hypoxia, more than in normoxia. **C:** Separate and merged photomicrographs of the nTS showing Fos-IR (red) and CtB fluorescence (white), demonstrating activated VLM-projecting nTS cells. **Ci-iii:** Higher magnification of Fos-IR VLM-projecting nTS neuron. *, p<0.05

Oxytocin Interacts with VLM Projecting Neurons



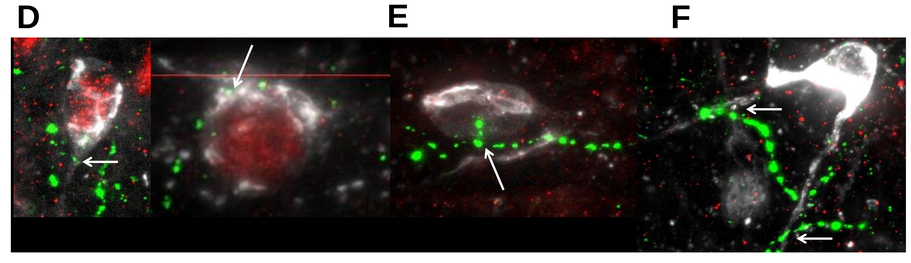


Figure 5: VLM projecting neurons interact with oxytocin. Interactions were characterized as near (≤3µm), apposition (≤1µm), or contact (no visible space). **A:** VLM projecting nTS neurons exhibit interactions with oxytocin. No significant difference was found between hypoxia and normoxia. **B:** Following hypoxia, more VLM-projecting nTS neurons that interact with oxytocin were activated compared to normoxia. **C:** Some VLM-projecting nTS neurons activated following hypoxia interact with oxytocin. Normoxic data excluded due to lack of Fos-IR. **D-F:** Merged photomicrographs of the nTS showing Fos-IR (red), VLM-projecting cells (white), and oxytocin fibers and puncta (green). **D:** Examples of activated VLM-projecting nTS cells with oxytocin interactions. **E:** Non-activated VLM-projecting cell with oxytocin interaction. **F:** VLM-projecting nTS cell exhibiting multiple interactions. *, p<0.05

SUMMARY

- PVN projections to the nTS contact nTS neurons that exhibit Fos-IR following hypoxia
- VLM-projecting nTS neurons exhibit Fos-IR following hypoxic exposure
- Oxytocin interacts with VLM-projecting nTS neurons
- VLM-projecting nTS neurons with oxytocin interactions exhibit Fos-IR after hypoxic exposure
- Fos-IR VLM-projecting neurons make primarily near and appositional oxytocin interactions

CONCLUSION

- nTS projections to the VLM are activated after hypoxia, suggesting involvement in response to hypoxia
- nTS-projecting PVN neurons may influence activation of nTS neurons in chemoreflex responses
- Oxytocin may influence activation of VLM-projecting nTS neurons in chemoreflex responses

FUTURE DIRECTIONS

- Further studies on the interactions between oxytocin and VLM-projecting nTS neurons
- Studies to evaluate other phenotypes of nTS neurons that have been shown to be activated after exposure to hypoxic conditions
  - PVN-projecting neurons
  - Catecholaminergic neurons