Orexin activates neurons in the paraventricular nucleus of the hypothalamus

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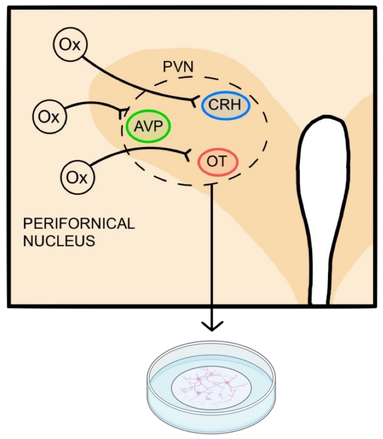
Introduction

- The paraventricular nucleus (PVN) of the hypothalamus is an integral site for the regulation of cardiorespiratory control and sympathetic nervous system activity in response to hypoxia.
- This area is comprised of oxytocin (OT), vasopressin (AVP), and corticotropin-releasing hormone (CRH) neurons that project the nucleus of the solitary tract or the posterior pituitary (Fig. 1).
- Exposure to chronic stressors, including low oxygen (hypoxia), leads to persistent PVN activation and increased sympathetic drive, hypertension, and cardiac arrythmias.

- Orexin (Ox) -producing neurons originating from the perifornical hypothalamus project to the PVN (Fig. 1).
- The Ox system has an important role in sleep-wakefulness cycles as well as other homeostatic processes.
- Ox within the PVN increases action potential discharge, elevating sympathetic tone, respiratory drive and blood pressure.
- Ox neurons are stimulated by hypoxia and facilitate activation of the PVN.
- Ox binds to the orexin 1 (Ox1R) or orexin 2 receptor (Ox2R) which are G protein-coupled receptors that raise intracellular calcium.
- The mechanism by which Ox activates PVN neurons, the intracellular signaling cascades involved, and the extent hypoxia modifies this response are not understood.

Figure 1. Diagram illustrating the projection of Ox neurons to the PVN. Ox neurons originate in the perifornical hypothalamus and project to the PVN which is comprised of OT, AVP, and CRH neurons. PVN neurons were collected and plated on coverslips for Ca^{2+} imaging. Created with BioRender.



Hypothesis: Orexin increases PVN activity primarily through activation of the Orexin 1 receptor

Methods

Animals

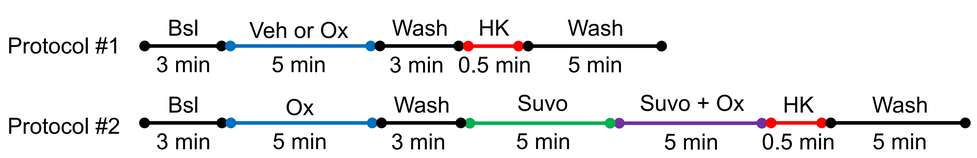
- PVN tissue was collected from male Sprague-Dawley rats (age 3-4 weeks).
- Neurons were dissociated from tissue, plated on poly-D-lysine-treated 15mm coverslips, and labeled with 1μM fura-2 AM to measure intracellular calcium using fluorescence intensity as an index of neuronal activity.

Calcium Imaging

- The coverslip was placed in an imaging dish and neurons were imaged using a 20x water immersion lens.
- While exposed to one of the following protocols, neurons were excited at 340nm and 380nm every 5 seconds and emission intensity at 510nm monitored.

Cells were exposed to one or more of the following pharmaceuticals or vehicle controls:

- 55mM Potassium Chloride (High K, HK), used to depolarize cells and confirm neuronal phenotype
- 10nM and 100nM Orexin-A (Ox)
- 10μM Suvorexant (Suvo), Ox1R and Ox2R receptor blocker



Analysis and Statistics

- Calculations of the raw 340nm/380nm ratio were performed in ImageJ.
- Ratio peaks were calculated using Python and Microsoft Excel.
- Neurons were eliminated from the data set if a) they did not have a clear elevation in Ca^{2+} elicited by HK depolarization or b) looked unhealthy under the bright field.
- Statistical analysis performed with GraphPad Prism using 1-way (RM) ANOVA *, $p < 0.05$ was considered significant.

Immunohistochemistry

- Rats were perfused with 4% paraformaldehyde.
- Tissue slices from the PVN were immunostained for OT, AVP, and CRH neurons.
- Dissociated neurons were fixed in 4% PFA and immunostained for neuronal markers (pan-neuronal antibody) to confirm neuronal identity.

Results

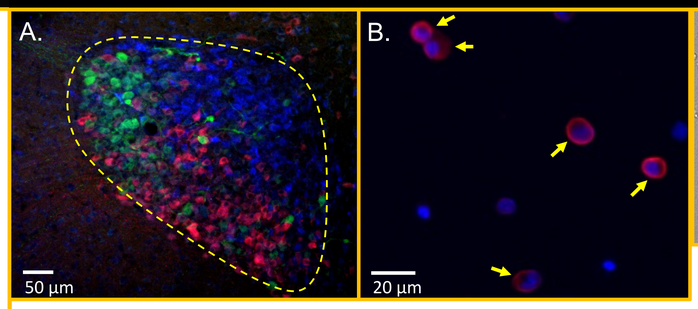


Figure 2. Identification of the PVN. (A) Immunohistochemistry of PVN slices stained for OT (red), AVP (green) and CRH (blue) neurons. (B) Dissociated PVN cells. Immunostaining of cells with pan-neuronal markers (red) confirms neuronal phenotype. DAPI staining (blue) labels nuclei. Note the majority of cells were immunoreactive for neuronal markers.

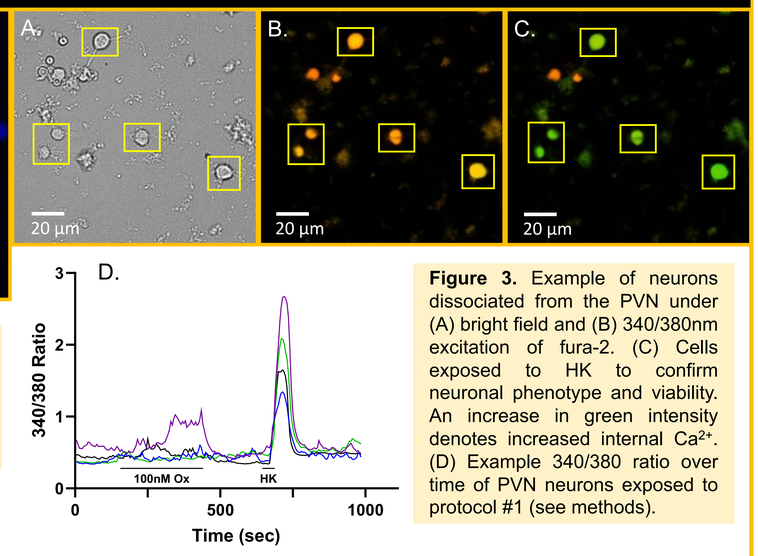
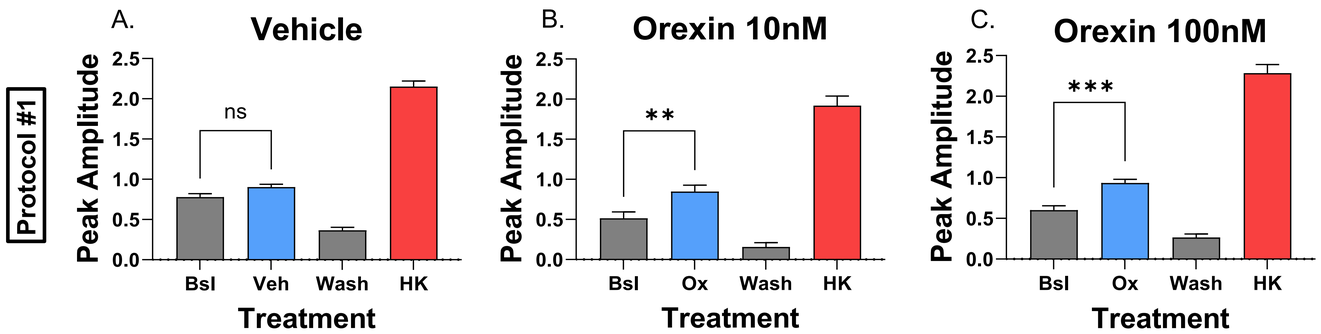


Figure 3. Example of neurons dissociated from the PVN under (A) bright field and (B) 340/380nm excitation of fura-2. (C) Cells exposed to HK to confirm neuronal phenotype and viability. An increase in green intensity denotes increased internal Ca^{2+}. (D) Example 340/380 ratio over time of PVN neurons exposed to protocol #1 (see methods).

Orexin receptor activation significantly increases intracellular calcium in PVN neurons



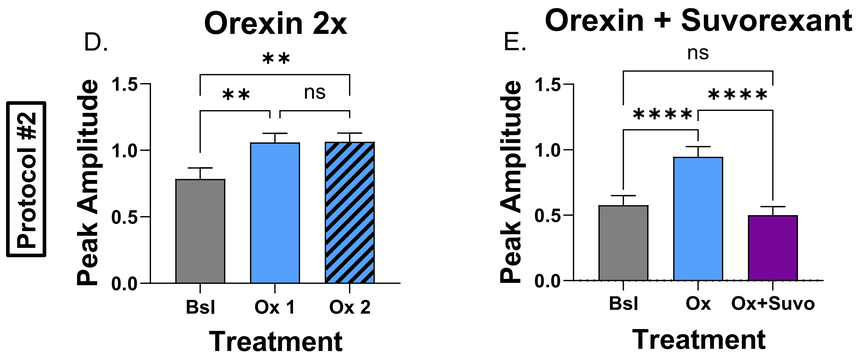
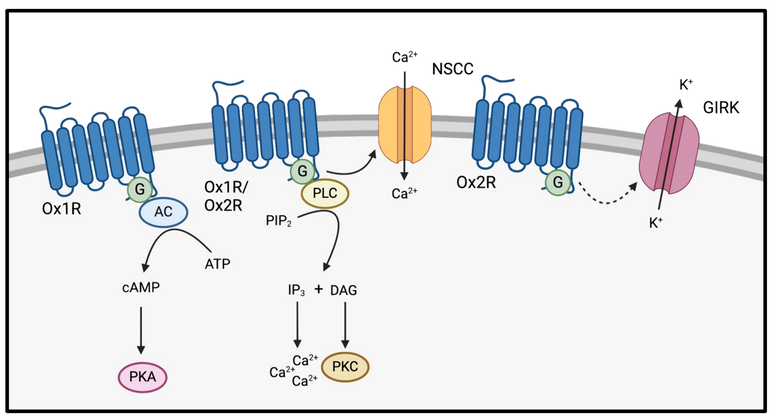


Figure 4. Peak Ca^{2+} during baseline (Bsl), vehicle (Veh), Ox and the OxR blocker Suvo. Exposure of PVN neurons to 10nM (B) and 100nM Ox (C) significantly increased the amplitude of Ca^{2+} relative to its initial Bsl. Veh (A) did not increase Ca^{2+}. HK increased Ca^{2+} in all groups. Repeated exposure to 100nM Ox (D) repeatably and significantly increased Ca^{2+}. The peak amplitude was not different between treatments. Conversely, prior exposure to Suvo (E) attenuated the Ox-mediated elevation of Ca^{2+}. 1-way (RM) ANOVA *, $p < 0.05$ was considered significant.

Conclusions and Future Directions

- Ox at 10 and 100nM increased intracellular calcium in PVN neurons.
- PVN neurons do not have a dose-dependent response to Ox.
- Suvo significantly decreases PVN response to Ox, indicating that Ox acts via OxRs.
- Current experiments are identifying the PVN phenotypes (OT, AVP, CRH) using immunocytochemistry, the specific receptor activated, and the intracellular pathways responsible (Fig. 5). Future experiments will examine the extent chronic hypoxia enhances Ox signaling in the PVN.

Figure 5. Pathways to be investigated of Ox1R and Ox2R signaling cascades. The OxRs are G protein-coupled receptors that are coupled with G_q or G_s subtypes. Stimulation of the G_q subtype activates the PLC-DAG-PKC or PLC-IP_3-Ca^{2+} pathways as well as a non-selective cation channel (NSCC). Stimulation of the Ox1R-mediated G_s subtype activates the AC-cAMP-PKA pathway. The Ox2R may also activate the G protein-coupled inwardly rectifying K^+ channel (GIRK). Adenylyl cyclase, AC; cyclic adenosine monophosphate; DAG, diacylglycerol; IP_3, inositol triphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C. Created with BioRender.



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