Hypoxia Increases TRPM3 Function Via Influx of Extracellular Calcium in Vagal Sensory Neurons

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ABSTRACT

Hypoxic (Hx) exposure elevates blood pressure, heart rate and respiration via reflex afferent and brain activation. Transient receptor potential (TRP) channels contribute to the afferent-evoked Hx response. The TRPV1 subfamily is a critical component and upregulated in the chronic intermittent hypoxia (CIH) model of sleep apnea. In addition to TRPV1, TRPM3 contributes to afferent and glutamate signaling, yet its contribution to Hx-afferent signaling is unknown. We hypothesize that TRPM3 increases neuronal activity in Hx. TRPM3 expression and function was tested in rat afferent neurons dissociated from the nodose-petrosal ganglia (NPG). Activity was monitored via Fura-2 Ca^{2+} imaging. Protein expression was examined via immunocytochemistry. Neurons were examined following one of four conditions: 1) normoxia, in which neurons were examined the same day of dissociation, 2) 24-hr normoxia or 3) 24-hr Hx in which cells were incubated for 24hrs in 21% or 1% O_2, or 4) immediately after in vivo CIH in rats exposed to episodic 6% O_2 (45 sec), 10 episodes/hr, 8 hrs/day, 10 days. In normoxia, TRPM3 activation with Pregnenolone (Preg, 30 and 100 μM) or CIM0216 (1 and 5 μM) increased intracellular Ca^{2+} concentration; both were blocked by the TRPM3 antagonist Ononetin (20 μM), confirming drug specificity. Elevated Ca^{2+} by Preg was eliminated by bathing cells in 0 mM Ca^{2+}, suggesting Ca^{2+} elevation occurs via opening of membrane-bound TRPM3. Following 24-hr Hx, Preg elevation of Ca^{2+} was greater than in 24-hr normoxic controls. The proportion of Preg responders was similar in 24-hr norm and Hx. After CIH, the number of Preg responders and Ca^{2+} elevation were similar to normoxia. Immunocytochemistry demonstrated TRPM3 membrane expression in NPG neurons. Altogether, these results suggest a Hx and pattern-specific increase in TRPM3 function, driven by the influx of extracellular calcium.

INTRODUCTION

Maintenance of O_2 supply to the central nervous system is critical for homeostasis. In response to environmental or physiological stressors, the cardiorespiratory system adapts both acutely and chronically. The immediate physiological response to low arterial O_2, or hypoxia, is a robust increase in respiration, blood pressure (BP), and sympathetic nerve activity. Peripheral baroreceptors detect beat-to-beat changes in BP, while chemoreceptors detect the reduced partial pressure of O_2. These afferent sensory fibers have their somas in the peripheral nodose-petrosal ganglia (NPG), where their central projections terminate and are integrated in the nucleus tractus solitarii (nTS, **Figure 1**).

Transient Receptor Potential channels (TRPs) are expressed by both the sensory neurons and nTS. TRP channels are cell membrane channels that conduct calcium (Ca^{2+}, **Fig 1**), a cation used in cell signaling and crucial for neurotransmitter release. TRPV1 is expressed in the NPG and jugular ganglia (JG), is activated by capsaicin, and senses physiological temperature. Several other TRP channels are located in these ganglia, including TRPA1, TRPM8 (activated by cold and menthol), and TRPM3, where they are often co-expressed in single neurons. For instance, activation of TRPA1 and TRPV1 additively enhance intracellular Ca^{2+} upon their stimulation. Additionally, TRPM3 operates in conjunction with TRPV1 in vagal afferent activity and central glutamate signaling.

Specific subfamilies of TRP receptors in the vagal circuit contribute to the reflex responses after exposure to Hx. For instance, TRPA1 in the vagus plays a role in responding to both low and high O_2 levels. We and others have shown that TRPV1 is increased in expression and function to enhance afferent activity in acute Hx and acute and chronic intermittent Hx. However, the contribution of TRPM3 in afferent signaling following Hx is unknown.

Given the role of TRPM3 and TRPV1 in glutamatergic signaling in the vagal afferent pathway, we ***hypothesize*** that TRPM3 will exhibit increased activity after hypoxia (**Fig 1**).

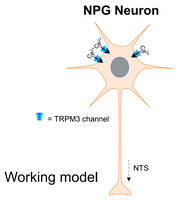

Fig 1. Working model

METHODS

NPG Dissociation and Culture. Male Sprague-Dawley rats (3-6 weeks) were anesthetized with Isoflurane and decapitated. The NPG were extracted immediately after, placed into ice-cold DMEM, and cut up with microdissection scissors. Tissue chunks were placed into Collagenase and Dispase solution and left in a 37-degree shaking water bath for 3x15 mins, with mild trituration in between the intervals. After this treatment, a thorough trituration took place, and the cells were plated onto PDL/Laminin coverslips and allowed to sit in the incubator for 1.5 hours before flooding.

Exposure Protocols. Cells were examined following one of four exposures.

- *Normoxia.* Neurons were examined the same day as dissociation.
- *24-Hour Normoxia.* Chips were incubated in 21% O_2, 5% CO_2.
- *24-Hour Hypoxia.* Chips were incubated in a hypoxic chamber for 24hrs in 1% O_2, 5% CO_2.
- *CIH.* In vivo rats were exposed to episodic 6% O_2 (45 sec), 10 episodes/hr, 8 hrs/day, for 10 days, before processing.

Fura-2 Ca^{2+} Imaging. After the waiting periods (for 24-hr norm and 24-hr Hx) or after directly after flooding (for normoxia and CIH), the cells were loaded with Fura-2 dye and incubated for 30 mins. Following 1x10 min wash in recording solution, the cells were placed on an Olympus IX71 microscope where wash continued for another 20 min. Fura-2 was excited at 340 and 380 nm; emission was 520 nm and acquired using μManager software.

Drugs used. (initially dissolved in DMSO and subsequently in recording solutions):

- TRPM3 agonist: Pregnenolone sulfate sodium salt, Tocris
- TRPM3 agonist: CIM 0216, Tocris
- TRPM3 antagonist: Ononetin, Tocris

Immunocytochemistry. After dissociation and culturing, 2% paraformaldehyde in 0.01 M phosphate buffered saline (PBS) was applied to fix the cells. After 15 minutes, cells were rinsed 3x in PBS and stored at 4ºC until use. After 3x10 min PBS wash, cells were permeabilized in 0.01% saponin in PBS (10 mins), then blocked in 1% bovine serum albumin (BSA) and 10% normal donkey serum (NDS) in PBS for 30 mins. Overnight incubation in Rabbit anti-TRPM3 antibody (1:500, 4ºC, Novus biologicals) was followed by 3x10 min PBS rinse and secondary donkey anti-rabbit Cy2 (1:500, 90 min, Jackson Labs). After a final PBS rinse (3x10 min) coverslips were covered with ProLong Gold Antifade Reagent with DAPI and placed on gelatin-coated slides before imaging. Cells were imaged with the Neurolucida 360 program on a fluorescent microscope.

Analysis and Statistics. For Ca^{2+} imaging, peaks and areas of the responses were determined using OriginPro and Excel software, and statistics were examined using Graphpad Prism. Peak data are shown as change from initial baseline (ΔF). Significance was set at $p<0.05$. Data are shown as mean ± SEM.

RESULTS

Activation of TRPM3 increase intracellular calcium in NPG neurons

Increase in Ca^{2+} in response to agonist Pregnenolone

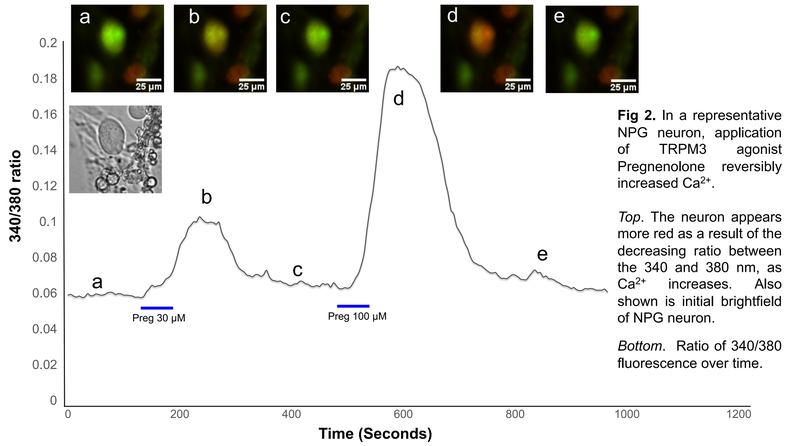

Fig 2. In a representative NPG neuron, application of TRPM3 agonist Pregnenolone reversibly increased Ca^{2+}.

Top. The neuron appears more red as a result of the decreasing ratio between the 340 and 380 nm, as Ca^{2+} increases. Also shown is initial brightfield of NPG neuron.

Bottom. Ratio of 340/380 fluorescence over time.

TRPM3 antagonist blocks Preg and CIM agonist–induced Ca^{2+} increase

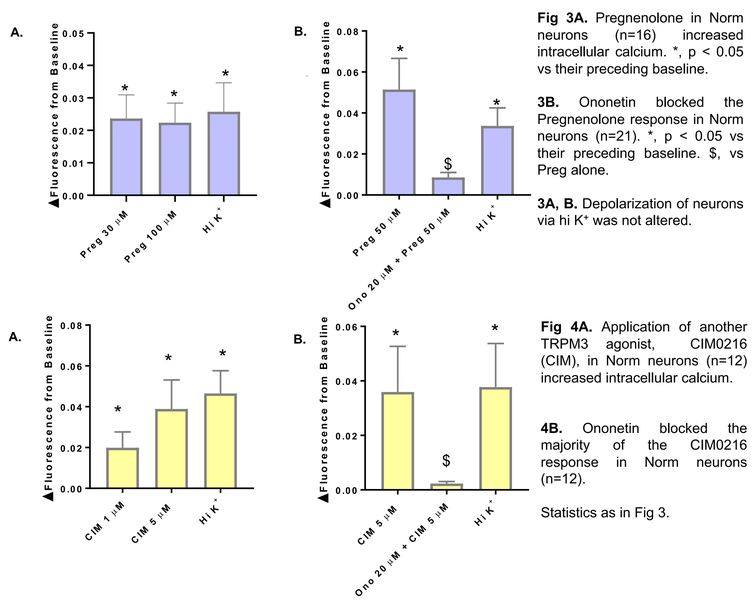

Fig 3A. Pregnenolone in Norm neurons (n=16) increased intracellular calcium. *, $p < 0.05$ vs their preceding baseline.

3B. Ononetin blocked the Pregnenolone response in Norm neurons (n=21). *, $p < 0.05$ vs their preceding baseline. $, vs Preg alone.

3A, B. Depolarization of neurons via hi K^+ was not altered.

Fig 4A. Application of another TRPM3 agonist, CIM0216 (CIM), in Norm neurons (n=12) increased intracellular calcium.

4B. Ononetin blocked the majority of the CIM0216 response in Norm neurons (n=12).

Statistics as in Fig 3.

TRPM3-induced Ca^{2+} influx is dependent on extracellular calcium

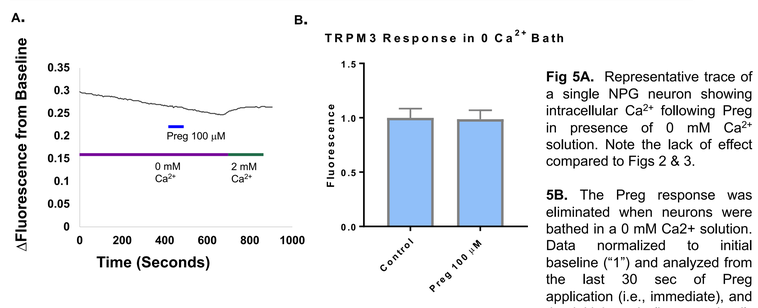

Fig 5A. Representative trace of a single NPG neuron showing intracellular Ca^{2+} following Preg in presence of 0 mM Ca^{2+} solution. Note the lack of effect compared to Figs 2 & 3.

5B. The Preg response was eliminated when neurons were bathed in a 0 mM Ca2+ solution. Data normalized to initial baseline ("1") and analyzed from the last 30 sec of Preg application (i.e., immediate), and the initial wash (i.e, sustained). Note lack of change (n=39).

Hypoxia increases TRPM3 Activity in NPG Neurons

Ca^{2+} Responses to TRPM3 activation are greater following 24-Hour Hypoxia

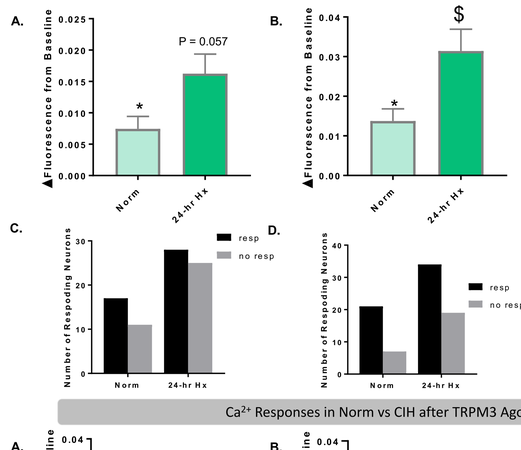

Fig 6 A. Application of 30 μM Preg increased intracellular Ca^{2+} to a greater extent in 24-hr Hx neurons (n=53) compared to 24-hr Norm (n=28). **B.** The same effect was seen using 100 μM Preg. A, B. *, $p < 0.05$ vs baseline, $, $p < 0.05$, Norm vs Hx.

C, D. The increase in Ca^{2+} occurred despite the similar number of cells that respond to Preg in Norm and Hx (C, 30 μM; D, 100 μM).

Ca^{2+} Responses in Norm vs CIH after TRPM3 Agonist Preg

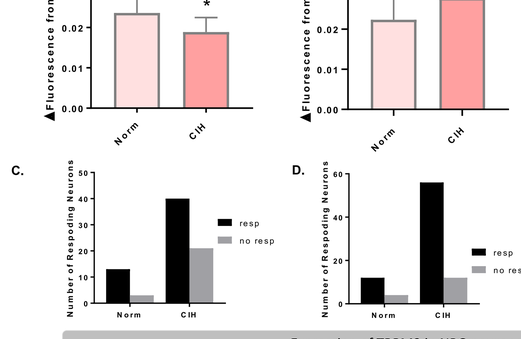

Fig 7 A. Application of 30 μM Preg increased intracellular Ca^{2+} similarly in Norm neurons (n=16) compared to CIH (n=68). **B.** The same effect was seen using 100 μM Preg. A, B. *, $p < 0.05$ vs baseline.

C, D. Proportion of responders to non responders was not significantly different between CIH and normoxic neurons (C, 30 μM; D, 100 μM).

Expression of TRPM3 in NPG neurons

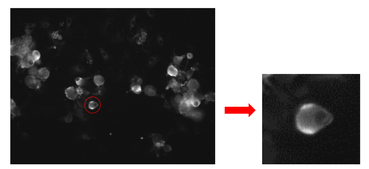

Fig 8. Representative expression of TRPM3 in NPG neurons. Fluorescence of 1:500 Rabbit anti TRPM3 in 24-hr Hx NPG neurons.

SUMMARY and CONCLUSIONS

- TRPM3 activation increases intracellular Ca^{2+} due to the influx of extracellular Ca^{2+}
- 24-hour hypoxic neurons respond to TRPM3 agonists to a greater extent than normoxic neurons
- No difference between TRPM3 activity in CIH vs normoxic NPG neurons
- TRPM3 is expressed in NPG neurons, consistent with their functional expression

FUTURE DIRECTIONS

- PCR of dissociated norm, 24-hypoxic, and CIH NPG neurons
- Increase n's of immunocytochemistry; confirm subcellular expression
- Examine physiological contribution by recording of action potential discharge in vagal nerve while simultaneously examining somal calcium and conduction velocity

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