Acute hypoxia (AH) activates nNOS and astrocyte associated nNOS containing cells in the paraventricular nucleus of the hypothalamus (PVN)

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Introduction

Patients with chronic obstructive sleep apnea suffer from hypertension (Bradley 2009), which can last into waking hours (Narkiewicz and Somers 2003). Hypoxia experienced during apneic episodes excites chemoreceptor afferent nerves and causes increased activation of efferent sympathetic nerves and hypertension (Dempsey et al 2010). The paraventricular nucleus (PVN) of the hypothalamus integrates cardiorespiratory signals including those from the arterial chemoreflex (King et al 2012) and is activated during acute hypoxia (Berquin et al 2000). However, previous studies show that during acute hypoxia, hypotension is experienced rather than hypertension (King et al 2012). Consistent with this observation, pre-sympathetic, spinally projecting cells within the PVN do not exhibit increased activation during acute hypoxia. Rather, increased activation (as indicated by Fos-IR) was observed in cells containing neuronal nitric oxide synthase (nNOS); these cells were neither pre-sympathetic spinally projecting nor vasopressinergic (Coldren et al 2013). It is possible the activated nNOS containing cells are producing nitric oxide, which has a known inhibitory action on pre-sympathetic (Rossi et al 2010) and magnocellular neurons (Stern and Zhang 2005) within the PVN. Astrocytes and CRH-releasing neurons are known to contain nNOS and could be activated under hypoxic conditions. Moreover, along with the presynaptic terminal and postsynaptic neuron, astrocytes are an important component of the tripartite synapse and play an important role in modulation of synaptic transmission.

Hypothesis

Therefore, we hypothesize that activated nNOS containing cells (as indicated by Fos-IR) and activated pre-sympathetic spinally projecting cells will show increased association with astrocytes (as indicated by GFAP) under acute hypoxic compared to normoxic conditions.

Methods

Prior retrograde labeling of pre-sympathetic neurons in the PVN: Microinjection of 0.5% CtB-Alexa 555 (CtB, ~180 nl) into the spinal cord IML (T1-T3)

Animals and treatments
- Male Sprague-Dawely rats exposed to either acute hypoxia (AH, 10% oxygen for 3 hours) or normoxia (N, 21% oxygen)
- Euthanization and perfusion of rat with 4% PFA
- Whole brain tissue collection and cryoprotection in 30% sucrose for approximately 4 days
- Sectioning of forebrain via cryostat at 35 µm

Immunohistochemistry (IHC)
- 9 rats: acutely hypoxic (n=5) and normoxic (n=4)
- IHC was performed on free-floating sections (1 in 6 series)
- Primary antibodies- 24 hr incubation (overnight)
  - Mouse anti-nNOS: 1:2000 (Santa Cruz)
  - Rabbit anti-cFOS: 1:3000 (Calbiochem)
  - Guinea pig anti-GFAP: 1:500 (SYSY)
- Secondary antibodies- 2 hr incubation
  - Donkey anti-mouse Dylight 405 (AMCA)
  - Donkey anti-rabbit Alexa 488 (Cy2)
  - Donkey anti-guinea pig Alexa 647 (Cy5)

Analysis
- Z stack (10 x 2 µm) images were obtained for two rostral-caudal levels of the PVN containing the majority of spinally projecting cells using Olympus BX51 and Neurolucida software
- Immunopositive cells for the three fluorescent targets (nNOS, cFOS, GFAP) and the spinal tracer were counted ipsilateral to the retrograde tracer

Central Chemoreflex Pathways

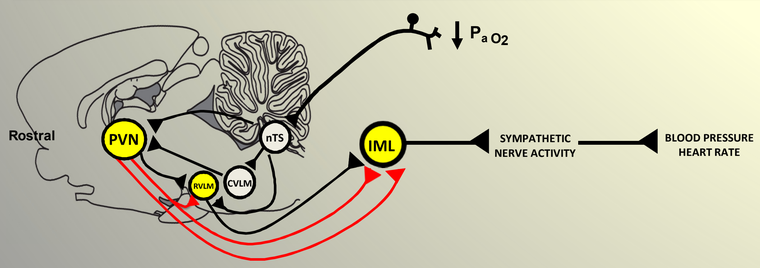


Figure 1. ARTERIAL CHEMOREFLEX PATHWAYS. Chemoreceptor afferents project to the nucleus tractus solitarii (nTS). The nTS projects to the PVN directly or to the caudal ventrolateral medulla (CVLM) which projects to the PVN. Pre-sympathetic spinally projecting efferent neurons project to the intermediolateral cell column (IML) directly or also to the rostral ventrolateral medulla which projects to the IML.

Modulation in the PVN

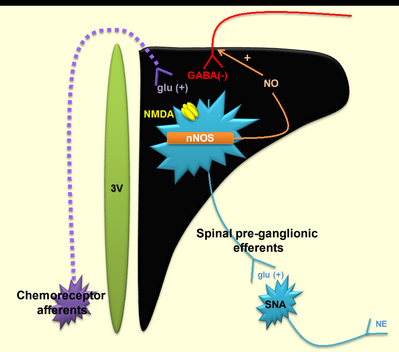
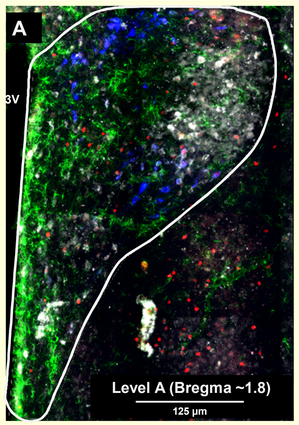
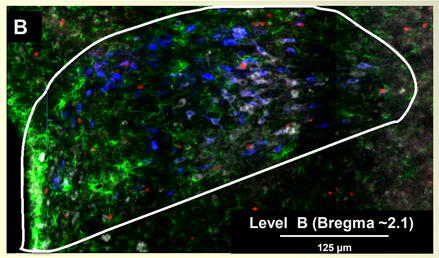


Figure 2. NITRIC OXIDE (NO) MODULATION OF PRE-SYMPATHETIC NEURONS IN THE PVN. Upon excitation by glutamate, nNOS within nNOS containing cells is activated and potentially releases NO, which has a stimulatory effect on release of the inhibitory neurotransmitter GABA at the nerve terminal within the PVN.

Immunohistochemistry





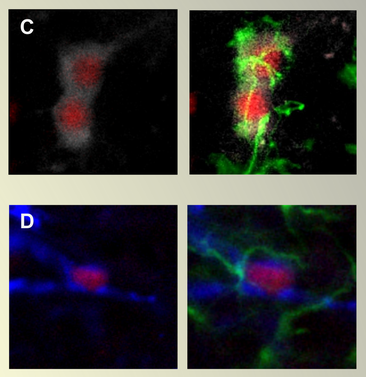


Figure 3. PVN SECTIONS FROM AH RATS (Fos-IR→ Red, nNOS-IR→white, astrocyte-IR→ green, spinally projecting tracer→ blue)

(A) Level A (Bregma ~1.8)
(B) Level B (Bregma ~2.1)
(C) Left: co-labeled Fos-IR and nNOS-IR cell. Right: co-labeled cell associated with an astrocyte
(D) Left: co-labeled Fos-IR and spinally projecting cell. Right: co-labeled cell associated with an astrocyte

Results

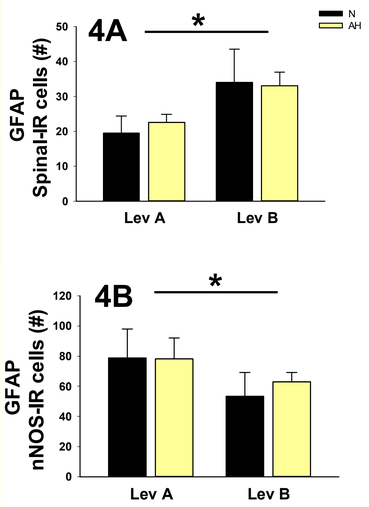


Figure 4. ASTROCYTE ASSOCIATED CELLS

(4A) Astrocyte associated spinally projecting neurons were located primarily in level B
(4B) Astrocyte associated nNOS-IR cells were located primarily in Level A

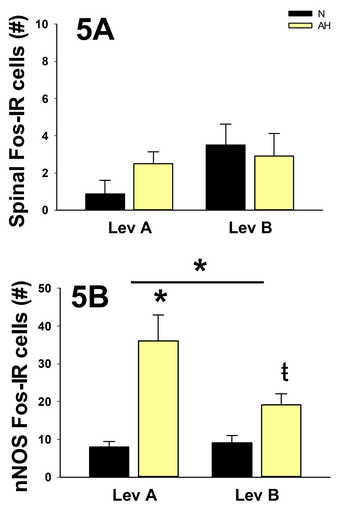


Figure 5. AH INCREASED NUMBER OF ACTIVATED nNOS CELLS

(5A) The number of activated spinally projecting cells was small and tended (P=0.08) to be greater in level B. AH was without effect.
(5B) AH activated nNOS cells: level A>level B

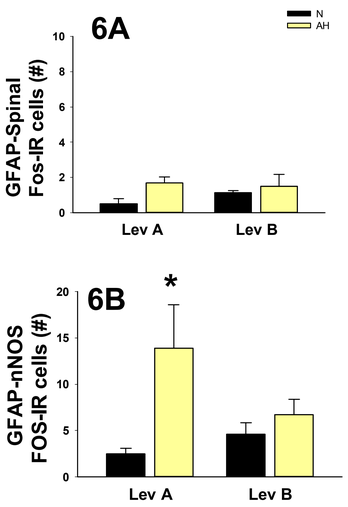


Figure 6. ACTIVATED nNOS CELLS WERE ASSOCIATED WITH ASTROCYTES

(6A) Few GFAP-spinally projecting cells were activated by AH
(6B) AH activated astrocyte associated nNOS cells in level A of the PVN

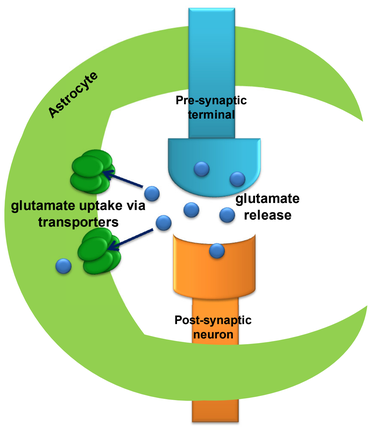


Figure 7: TRIPARTITE SYNAPSE. Astrocytes modulate synaptic transmission. Shown here, glutamate transporters on astrocytes remove glutamate from the synapse and limit excitatory effects. In addition (not shown), astrocytes are a source of nitric oxide which increases inhibitory transmission in the PVN.

Table 1. EFFECTS OF ACUTE HYPOXIA AND NORMOXIA ON CELLS WITHIN THE PVN. Mean cell counts and SEM for all cells. Two-way repeated measures ANOVA was performed (factors equal treatment and level). T-test were performed on each level and the total count for AH and N. * indicates a significant difference (P</= 0.05) from the two-way repeated measures ANOVA. ŧ indicates a significant difference found in the t-test. Total count data were analyzed by a t-test only.

PHENOTYPE		#FOS	#nNOS	# Spinal	#Spinal FOS	#nNOS FOS	#Spinal nNOS	#GFAP Spinal	#GFAP nNOS	#GFAP-Spinal-FOS	#GFAP-nNOS-FOS	%Spinal FOS	%nNOS FOS	%Spinal GFAP	% nNOS GFAP	%GFAP-Spinal-FOS	%GFAP-nNOS-Fos
Treatment		*AH>N	--	--	--	*AH>N	--	--	--	--	*AH>N	--	*AH>N	--	--	--	AH>N (P=0.11)
Level		N: 3>2 (P=0.10) *AH: 2>3	*2>3	*3>2	3>2 (P=0.08)	*AH: 2>3	*3>2	*3>2	*2>3	--	--	*N: 3>2 *AH: 2>3	*AH: 2>3	--	--	*AH: 2>3	--
Level A (Bregma ~1.8)	Norm	20±4	201.38±23	51±5	1±1	8±1	16±2	20±5	79±20	1±0	3±1	1±1	4±1	41±13	38±7	3±2	4±1
	AH	*121±25	223±22	54±14	3±1	*36±7	21±6	23±2	78±14	ŧ2±0	*14±5	ŧ5±1	*17±4	50±11	37±7	8±2	ŧ17±5
Level B Bregma (~2.1)	Norm	29±3	143±20	94±14	4±1	9±2	26±3	34±10	53±16	1±0	5±1	4±1	6±1	35±5	37±8	4±1	10±3
	AH	*48±4	173±12	93±7	3±1	ŧ19±3	34±5	33±4	63±6	2±0	8±2	3±1	11±2 (P=0.09)	37±5	38±6	4±2	11±2
Total	Norm	50±5	344±42	145±10	4±2	17±3	42±5	54±14	132±34	2±0	7±1	3±1	5±1	36±7	38±7	4±1	6±2
	AH	ŧ170±26	396±31	147±19	5±2	ŧ55±9	55±10	56±2	141±19	3±1	21±6 (P=0.08)	4±1	ŧ14±3	41±6	37±7	6±2	14±3 (P=0.07)

Summary and Conclusion

As seen in previous studies, a larger proportion of nNOS containing cells was seen in level A and a larger proportion of spinally projecting cells was seen in Level B. Rats exposed to acute hypoxia show an overall increased cellular activation (Fos-IR) and activated 14% of nNOS containing cells in the PVN (primarily level A).

Current data indicate that acute hypoxia activated 17% nNOS containing cells associated with astrocytes in level A. Although a substantial number of spinally projecting cells were associated with astrocytes, very few of these cells were activated by AH (6%).

The majority (62%) of cells activated (Fos-IR) by acute hypoxia are phenotypically unknown.

Nitric oxide is a highly diffusible inhibitor of sympathetic outflow from the PVN, and increased activation of nNOS could serve as an early compensatory response to prevent hypertension found in apneic patients. Considering the role of astrocytes in modulating synaptic transmission, it is possible that astrocytes closely associated with activated cells contribute to nNOS production in the PVN during acute hypoxic insult.