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ACUTE EXERCISE-INDUCED ACTIVATION OF PHOX2B-EXPRESSING NEURONS OF THE RETROTRAPEZOID NUCLEUS IN RATS MAY INVOLVE THE HYPOTHALAMUS

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Abstract—The rat retrotrapezoid nucleus (RTN) contains neurons that have a well-defined phenotype characterized by the presence of vesicular glutamate transporter 2 (VGLUT2) mRNA and a paired-like homeobox 2b (Phox2b)-immunoreactive (ir) nucleus and the absence of tyrosine hydroxylase (TH). These neurons are important to chemoreception. In the present study, we tested the hypothesis that the chemically-coded RTN neurons (ccRTN) (Phox2b^{+}/TH^{-}) are activated during an acute episode of running exercise. Since most RTN neurons are excited by the activation of perifornical and lateral hypothalamus (PeF/LH), a region that regulates breathing during exercise, we also tested the hypothesis that PeF/LH projections to RTN neurons contribute to their activation during acute exercise. In adult male Wistar rats that underwent an acute episode of treadmill exercise, there was a significant increase in c-Fos immunoreactive (c-Fos-ir) in PeF/LH neurons and RTN neurons that were Phox2b^{+}TH^{-} ($p < 0.05$) compared to rats that did not exercise. Also the retrograde tracer Fluoro-Gold that was injected into RTN was detected in c-Fos-ir PeF/LH ($p < 0.05$). In summary, the ccRTN neurons (Phox2b^{+}TH^{-}) are excited by running exercise. Thus, ccRTN neurons may contribute to both the chemical drive to breath and the feed-forward control of breathing associated with exercise.

Key words: central autonomic pathways, breathing, exercise, c-Fos immunoreactivity, Phox2b, medulla oblongata.

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Abbreviations: ANOVA, analysis of variance; ccRTN, chemically-coded retrotrapezoid nucleus neurons; c-Fos-ir, c-Fos immunoreactive; CPG, central pattern generator; DMH, dorsal medial hypothalamus; FG, Fluoro-Gold; ir, immunoreactive; Pa_{CO_2}, partial pressure of arterial carbon dioxide; Pa_{O_2}, partial pressure of arterial oxygen; PAG, periaqueductal gray; PE, polyethylene; PeF/LH, perifornical and lateral hypothalamus; PFA, paraformaldehyde; Phox2b, paired-like homeobox 2b; PVH, paraventricular nucleus of the hypothalamus; RTN, retrotrapezoid nucleus; RVLM, rostral ventrolateral medulla; TH, tyrosine hydroxylase; VGLUT2, vesicular glutamate transporter 2; VLM, ventrolateral medulla; VMH, ventromedial hypothalamus.

INTRODUCTION

The retrotrapezoid nucleus (RTN) within the ventrolateral medulla (VLM) is an important center of chemoreception and is characterized by neurons that express high levels of the transcriptor factor paired-like homeobox 2b but lack tyrosine hydroxylase (Phox2b^{+}TH^{-}). These CO_2-activated excitatory neurons innervate the entire ventral respiratory column (Stornetta et al., 2006). Recently, we demonstrated that ventrolateral medullary neurons are activated after acute exercise in rodents (Barna et al., 2012). Although we presumed that these neurons were RTN chemosensitive neurons, we did not identify their exact phenotype.

In the present study, we refer to the chemosensitive neurons as the chemically-coded RTN neurons (ccRTN neurons) to distinguish them from other types of neurons in this area (Lazarenko et al., 2009; Marina et al., 2010). ccRTN neurons are activated by hypercapnia in anesthetized rats and are uniformly activated by acidification in slices; and the selective activation of ccRTN neurons *in vivo* stimulates breathing (Abbott et al., 2009; Lazarenko et al., 2009; Marina et al., 2010). Their acute inhibition or chronic destruction eliminates or markedly attenuates breathing and the ability of CO_2 to elicit breathing in anesthetized or unrestrained awake rats (Takakura et al., 2006, 2008, 2013; Marina et al., 2010). A very elegant genetic study revealed the importance of ccRTN neurons in respiratory chemoreception (Ramanantsoa et al., 2011). The authors created a mouse model that expresses the Phox2b Congenital Central Hypoventilation Syndrome mutation in neurons of rhombomere 3 and 5 lineage (*Phox2b*^{27alacki}; *Egr2*$^{cre/+}$). This offspring lacked all Phox2b neurons but unexpectedly survived to adulthood, despite a complete loss of respiratory responses to CO_2. Their survival was attributed to a respiratory compensation via peripheral chemoreceptors (Ramanantsoa et al., 2011). Collectively, this evidence suggests that the ccRTN neurons provide a substantial fraction of the excitatory drive to the central pattern generator (CPG) at rest and are required for the homeostatic regulation of breathing by CO_2.

The ccRTN neurons presumably contribute to the increased respiratory activity associated with various behaviors and during hypothalamic stimulation, including central command, emotions, sleep and thermoregulation

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(Hilton and Redfern, 1986; Waldrop et al., 1988; Dimicco et al., 2002; Zhang et al., 2006; Tanaka and McAllen, 2008). Given that hypothalamic stimulation activates the ccRTN neurons vigorously (Fortuna et al., 2009) and that selective stimulation of the same neurons activates breathing (Abbott et al., 2009), the RTN is likely a relay for the central command of respiration during exercise. This view is also consistent with the notion that RTN drives active expiration (Janczewski and Feldman, 2006; Abdala et al., 2009; Takakura et al., 2013).

The present experiments were designed to test the hypothesis that acute exercise activates ccRTN neurons and this activation is mediated, at least in part, by exercise-induced activation of hypothalamic inputs to the RTN.

RESULTS

Activation of ccRTN Phox2b-expressing neurons after acute exercise

The first set of experiments was designed to test if ccRTN neurons are activated by acute treadmill exercise in rats. We used the proto-oncogene product c-Fos as a measure of cell activation. ccRTN neurons can be identified histologically as Phox2b$^+$/TH$^-$ cells. All Phox2b$^+$ cells in this region contain vesicular glutamate transporter 2 (VGLUT2) mRNA (Stornetta et al., 2006), and the Phox2b$^+$/TH$^-$ cells are readily distinguished from ChAT$^+$ neurons in the facial motor nucleus and serotonergic neurons in the raphe nucleus (Takakura et al., 2008; Takakura and Moreira, 2013).

We compared c-Fos expression in two groups of rats: acute exercise and no-exercise. Exercise-activated ccRTN neurons were identified by the presence of c-Fos immunoreactive (c-Fos-ir) in their nuclei. Fig. 1 illustrates the ccRTN in a control rat and in a rat that performed acute exercise. In the control rat, Phox2b$^+$/TH$^-$ neurons (ccRTN neurons; white arrow) lacked expression of c-Fos-ir; whereas in the exercise rat, a large proportion of these cells had immunoreactivity for c-Fos (Fig. 1A–F). The neurons that were Phox2b$^+$/TH$^+$ (blue arrow in Fig. 1) are presumably C1 neurons.

The number of Phox2b$^+$TH$^-$ neurons that are also c-Fos-positive were identified and counted in a one-in-six series of transverse sections (one section every 240 μm). Counts were made on both sides of the brain and throughout the portion of the VLM depicted in gray in Fig. 2A–E. As a control population, we also counted the number of Phox2b$^-$/TH$^-$ c-Fos-ir neurons located in the nearby parapyramidal region (Fig. 2A–E). These presumably serotonergic cells were Phox2b$^-$/TH$^-$ (Takakura and Moreira, 2013). Acute exercise increased the number of c-Fos$^+$ neurons within the ccRTN neurons (Fig. 2F, G). We counted the number of cells identified at all levels of the region of interest and multiplied this number by six. We then applied the 0.81 Abercrombie correction factor as previously determined on identically prepared histological material (Takakura et al., 2008; Barna et al., 2012). From this, we identified that the number of c-Fos$^+$ ccRTN neurons in no-exercise and acute exercise groups as 15 ± 2 and 384 ± 14, respectively (one-way analysis of variance (ANOVA); $p = 0.016$; Fig. 2G). The number of ccRTN neurons that were Phox2b-ir was significantly greater than the number of neurons that were activated during acute exercise (Fos$^+$Phox2b$^+$). Specifically, ccRTN neurons that were Phox2b$^-$/TH$^-$ c-Fos-ir neurons represented only 18% of the total cell count (2112 ± 71 vs. 384 ± 14, $p = 0.0265$; data not shown). However, the number of ccRTN (Phox2b$^+$TH$^-$) neurons that are activated in acute exercise is 77% compared to total c-Fos-ir RTN neurons (384 ± 14 vs. 497 ± 28, $p = 0.0398$; Fig. 2H).

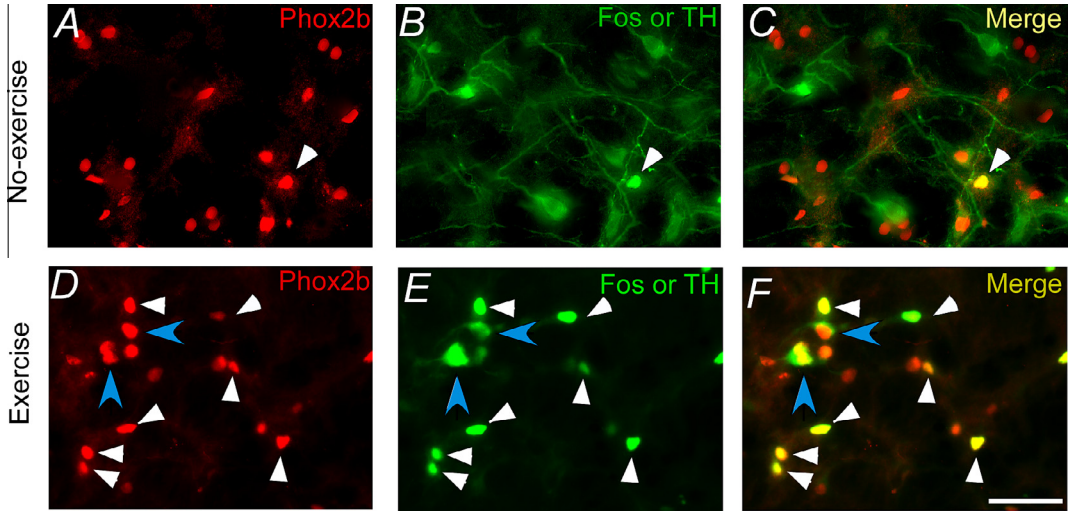

Fig. 1. Acute exercise triggers c-Fos expression by the ccRTN. c-Fos expression (green nuclei – Alexa 488) in the RTN region under control conditions (no-exercise) (A–C) and after acute exercise (D–F). ccRTN neurons were identified by the presence of a Phox2b-ir nucleus (red nuclei – Cy3) and the absence of TH-ir (green cytoplasm – Alexa 488). In the no-exercise case, the nuclei of most of the Phox2b$^+$/TH$^-$ lacked c-Fos-ir and appear red. In the acute exercise group, the majority of the ccRTN had a c-Fos-ir nucleus and therefore appears yellow. The neurons that were positive for both Phox2b and TH are probably C1 neurons. White arrows represent Fos$^+$/Phox2b$^+$/TH$^-$ neurons while blue arrows indicate Phox2b$^+$/TH$^+$. Scale bar = 40 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)