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Research Paper

Raphe gene expression changes implicate immune-related functions in ventilatory plasticity following carotid body denervation in rats

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ABSTRACT

The regulation of blood gases in mammals requires precise feedback mechanisms including chemoreceptor feedback from the carotid bodies. Carotid body denervation (CBD) leads to immediate hypoventilation (increased $PaCO_2$) in adult rats, but over a period of days and weeks ventilation normalizes due in part to central (brain) mechanisms. Here, we tested the hypothesis that functional ventilatory recovery following CBD correlated with significant shifts in medullary raphe gene expression of molecules/pathways associated with known or novel forms of neuroplasticity. Tissue punches were obtained from snap frozen brainstems collected from rats 1–2 days or 14–15 days post-sham or post-bilateral CBD surgery (verified by physiologic measurements), and subjected to mRNA sequencing to identify, quantify, and statistically compare gene expression level differences among these groups of rats. We found the greatest number of gene expression changes acutely after CBD (154 genes), with fewer changes in the weeks after CBD (69–80 genes) and the fewest changes in expression among the time control groups (39 genes). Little or no changes were observed for multiple genes associated with serotonin- or glutamate receptor-dependent forms of neuroplasticity. However, an unbiased assessment of gene expression changes using a bioinformatics pathway analysis highlighted multiple changes in gene expression in signaling pathways associated with immune function. These included several growth factors and cytokines associated with peripheral and innate immune systems. Thus, these medullary raphe gene expression data support a role for immune-related signaling pathways in the functional restoration of blood gas control after CBD, but little or no role for serotonin- or glutamate receptor-mediated plasticity.

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1. Introduction

Continuous gas exchange through ventilation is critical to maintain homeostatic levels of oxygen, CO_2 and pH to sustain mammalian life. Disease processes that compromise the ventilatory system at the level of the lung, circulation, and/or neural control centers in the brainstem can ultimately lead to ventilatory insufficiency. However, the ventilatory control system appears to have an innate capacity for compensation, as evidenced by partial or complete recovery from experimental and/or therapeutic neural lesions. This partial or complete restoration of ventilatory function is a form of respiratory neuroplasticity, for which there are several examples and potential mechanisms (Forster, 2003; Kinkead et al., 2001; Mitchell et al., 2001).

Carotid body denervation (CBD) has been performed in multiple mammalian species including humans (Dahan et al., 2007; Dahan et al., 2008), and in most cases this leads to hypoventilation and elimination of hypoxic ventilatory responses (Miller et al., 2013; Mouradian et al., 2012; Bisgard et al., 1976). However, the degree and duration of eupneic hypoventilation (increase in $PaCO_2$) was variable among species, lasting for only a few weeks (Mouradian et al., 2012) up to 20 or more years (Honda, 1992). Bilateral CBD in adult rats also elicits a significant and immediate hypoventilation, but in contrast to many other species the CBD-induced hypoventilation was rapidly and completely resolved within 2 weeks. Bilateral CBD in adult rats also elicits a significant and immediate hypoventilation, but in contrast to many other species the CBD-induced hypoventilation was rapidly and completely resolved within 2 weeks (Mouradian et al., 2012). In contrast, hypoventilation after CBD in ponies persisted for up to 2 years, at which time there was 30–40% recovery of their hypoxic ventilatory responses which was not due to reinnervation or a return of function of the denervated carotid chemoreceptors (Bisgard et al., 1980). Subsequent aortic arch denervation eliminated the recrudescent hypoxic response, but failed to alter eupneic $PaCO_2$. Thus, increased activity of aortic body chemoreceptors and not a reinnervation of the carotid bodies accounted for plasticity in the hypoxic ventilatory responses, but did not account for the plasticity in eupneic breathing which may be occurring within the CNS.

Abbreviations: CBD, carotid body denervation; SD, Sprague Dawley; V_E, minute ventilation; V_T, tidal volume; 5-HT, serotonin.

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Medullary serotonergic (5-HT) raphe neurons are embedded in the neural network generating ventilatory pattern and rhythm and provide a major source of excitatory neuromodulation to pre-motor and motor nuclei throughout the CNS (Hodges and Richerson, 2010). 5-HT receptor activation alone is sufficient to induce and is involved in the maintenance of central respiratory plasticity (Hodges and Richerson, 2008), and contributes to the recovery of eupneic breathing after peripheral nerve injury (Mitchell et al., 2000) (Kinkead et al., 1998). 5-HT can initiate the long-lasting increase in respiratory motor output that follows acute intermittent hypoxia (i.e. phrenic long-term facilitation, pLTF) (Fuller et al., 2001), and the upregulation of 5-HT innervation has been shown after recovery of respiratory function after cervical dorsal rhizotomy (CDR) (Kinkead et al., 1998) and thoracic dorsal rhizotomy (TDR) (Mitchell et al., 2000). Furthermore, the 5-HT receptors appear to mediate the establishment of oxygen chemosensitivity in the aorta following carotid body denervation (CBD) in developing piglets (Serra et al., 2002a). These and other data highlight an important role of the 5-HT system in facilitating specific forms of respiratory neuroplasticity and/or recovery following perturbations.

In addition, activity-dependent neuroplasticity is a major mechanism governing neuroplasticity in higher brain regions. However, it may also contribute to recovery of function within the brainstem under conditions of altered respiratory activity with chronic hypoxia (Kline et al., 2007) or following injury. Activity-dependent neuroplasticity, or homeostatic plasticity, is thought to be a cell-autonomous process that restores the activity of a neuron or neural network from a reduced state to normal through upregulation of specific glutamatergic (AMPA and NMDA) receptor subunits. This process may theoretically occur in brainstem nuclei following experimental peripheral deafferentation like that after CDR, TDR, or CBD. Indeed, we have recently demonstrated that CBD in adult goats leads to severe hypoventilation 3–5 days post-CBD, which modestly but significantly recovered within 30 days post-CBD (Miller et al., 2014). Expression of glutamatergic receptor subunits GluA2 and GluN1 was decreased in several brainstem nuclei acutely after CBD, but then were restored to pre-CBD levels by 30 days post-CBD. This suggests that despite an initial decrease, the compensatory increase in AMPA and NMDA receptor levels may contribute to the functional restoration of eupneic breathing after peripheral nerve sectioning.

Given that the rat exhibits a relatively robust ventilatory recovery/plasticity after CBD, it is an ideal model for studies aiming to elucidate brainstem-specific mechanisms of neuroplasticity following peripheral nerve injury/sectioning. Herein we utilized mRNA Sequencing (Puissant et al., 2015) (RNASeq) to measure gene expression changes in the 5-HT neuron-rich medullary raphe nuclei at two time points after CBD in rats to gain insights into mechanisms driving the restoration of breathing following CBD. Here we tested the hypothesis that the restoration of eupneic breathing acutely or chronically after CBD depends on 5-HT-specific and/or glutamate receptor-dependent changes in medullary raphe gene expression. Concomitantly, we sought to determine if there are additional genes and/or signaling pathways that may contribute to the restoration of ventilation after CBD in adult rats.

2. Methods

Adult (7–8 weeks) male Sprague Dawley rats ((Harlan) SD; n = 39) were used in this study. All rats were housed in the Biomedical Research Center, allowed access to low salt chow (Dyets 0.4% NaCl) and water ad libitum, and maintained on a 12:12 h light/dark cycle. All experimental protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee prior to commencing the study.

2.1. Experimental design

All rats were chronically instrumented with indwelling femoral artery and vein catheters as previously described (Mouradian et al., 2012). Three or more days later, ventilation was measured and arterial blood was sampled while breathing room air (RA; $F_IO_2 = 0.21$, bal. N_2) for 20 min or hypoxic gas ($F_IO_2 = 0.12$, bal. N_2) in a 10 L flow-through, whole body plethysmograph. Arterial blood was sampled (0.4 mL) during the last 3 min of RA or hypoxia. In additional studies, ventilatory responses to venous injections of NaCN (3 mg mL^{-1}) or saline (0.9% NaCl) were also measured in RA as previously described (Mouradian et al., 2012). Rats then underwent sham or CBD surgery and breathing studies were repeated at regular intervals thereafter (1–2, 4–5, 9–10, and 14–15 days post-surgery). Hypoxic and NaCN ventilatory responses and corresponding blood gases were measured pre- and 1 or 2 days post-surgery. RA ventilation and blood gases were measured at each of the indicated intervals. A total of four groups of rats were used. The first group of rats was denervated and euthanized 1–2 days post-CBD ("CBD Acute") and a second group of rats was denervated and euthanized 14–15 days post-CBD ("CBD Chronic"). Each denervation group had a corresponding time control, sham-denervated group of rats ("Sham Acute" and "Sham Chronic"). This study design permitted subsequent gene and biological function comparisons associated with peak hypoventilation (CBD Acute vs Sham Acute), the recovery process (CBD Acute vs CBD Chronic), and significant recovery of ventilation (CBD Chronic vs Sham Chronic) while controlling for time and surgery (Sham Acute vs Sham Chronic; Fig. 1).

2.2. Surgical protocols

All catheterization, sham, and CBD surgeries were carried out using aseptic technique as previously described (Mouradian et al., 2012). Rats received intraoperative injections of Carprofen (Rimadyl; 5 mg kg^{-1} i.p.) for analgesia and enrofloxacin (Baytril; 1 mg $(100\ g)^{-1}$) to prevent infection. Additional Carprofen was given BID for 48 h, and Baytril (1 mg/100 mL) supplied in the drinking water continuously after surgery.

2.3. Tissue collection and mRNA sequencing

Upon completion of physiological protocols, the rats were deeply anesthetized (20% isoflurane in propylene glycol) and upon respiratory arrest the brain extracted, embedded with embedding media and flash frozen and stored (−80 °C) until being placed into a chilled stainless steel matrix for ~1 mm serial sectioning (Puissant et al., 2015). Frozen tissue punches (~0.85 mm in diameter) were collected from the ventral midline from 2 serial sections beginning with the one containing Obex as previously described (Puissant et al., 2015), and stored in Eppendorf tubes (−80 °C) until RNA extraction.

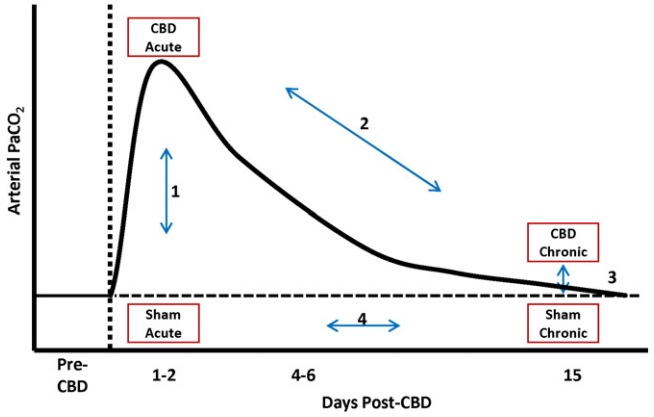

Fig. 1. Study design showing the four raphe transcriptome comparisons employed to identify gene expression changes and biological functions associated with peak hypoventilation (#1, Acute CBD vs Sham Acute), the recovery process (#2, CBD Acute vs CBD Chronic), and significant recovery of ventilation (#3, CBD Chronic vs Sham Chronic) while controlling for time and surgery (#4, Sham Acute vs Sham Chronic).