Please cite this article in press as: Cerpa VJ et al. Medullary 5-HT neurons: Switch from tonic respiratory drive to chemoreception during postnatal development. Neuroscience (2016), http://dx.doi.org/10.1016/j.neuroscience.2016.09.002

Neuroscience xxx (2016) xxx–xxx

MEDULLARY 5-HT NEURONS: SWITCH FROM TONIC RESPIRATORY DRIVE TO CHEMORECEPTION DURING POSTNATAL DEVELOPMENT

VERONICA J. CERPA, [a,b] **YUANMING WU,** [a,b] **EDUARDO BRAVO,** [b]* **FRIDA A. TERAN,** [b] **RACHEL S. FLYNN** [a] **AND GEORGE B. RICHERSON** [a,b,c,d]

[a] *Department of Neurology, Yale University, New Haven, CT 06510, United States*

[b] *Department of Neurology, University of Iowa, Iowa City, IA 52242, United States*

[c] *Department of Molecular Physiology & Biophysics, University of Iowa, Iowa City, IA 52242, United States*

[d] *Veterans Affairs Medical Center, Iowa City, IA 52242, United States*

Abstract—Serotonin (5-HT) neurons contribute to respiratory chemoreception in adult mice, but it is unclear whether they play a similar role in neonatal mice. We studied breathing during development in *Lmx1b*$^{f/f/p}$ mice, which lack 5-HT neurons. From postnatal days 1–7 (P1–P7), ventilation of *Lmx1b*$^{f/f/p}$ mice breathing room air was 50% of WT mice ($p < 0.001$). By P12, baseline ventilation increased to a level equal to WT mice. In contrast, the hypercapnic ventilatory response (HCVR) of neonatal *Lmx1b*$^{f/f/p}$ and WT mice was equal to each other, but were both much less than adult WT mice. By P21 the HCVR of WT mice increased to near adult levels, but the HCVR of *Lmx1b*$^{f/f/p}$ mice had not changed, and was 42% less than WT mice. Primary cell cultures were prepared from the ventromedial medulla of neonatal mice, and patch-clamp recordings were made from neurons identified as serotonergic by expression of a reporter gene. In parallel with developmental changes of the HCVR *in vivo*, 5-HT neurons had little chemosensitivity to acidosis until 12 days *in vitro* (DIV), after which their response increased to reach a plateau around 25 DIV. Neonatal *Lmx1b*$^{f/f/p}$ mice displayed high mortality and decreased growth rate, and this worsened in hypoxia. Mortality was decreased in hyperoxia. These results indicate that maturation of 5-HT neurons contributes to development of respiratory CO_2/pH chemoreception during the first few weeks of life in mice *in vivo*. A defect in the 5-HT system in early postnatal life decreases survival due in part to hypoxia. © **2016 IBRO. Published by Elsevier Ltd. All rights reserved.**

*Corresponding author.
E-mail address: eduardo-bravo@uiowa.edu (E. Bravo).

Abbreviations: AHP, afterhyperpolarization; CI, chemosensitivity index; DIC, differential interference contrast; EYFP, enhanced yellow fluorescent protein; *f*R, respiratory frequency; HCVR, hypercapnic ventilatory response; RTN, retrotrapezoid nucleus; SIDS, sudden infant death syndrome; VE, ventilation; VO_2, oxygen consumption; VT, tidal volume.

http://dx.doi.org/10.1016/j.neuroscience.2016.09.002
0306-4522/

Key words: serotonin, chemoreception, *Lmx1b*, respiratory.

INTRODUCTION

5-HT neurons play a role in respiratory control (Al-Zubaidy et al., 1996; Feldman et al., 2003; Hodges et al., 2008; Niebert et al., 2011; Pena and Ramirez, 2002; Ptak et al., 2009) and chemosensory drive (Nattie et al., 2004; Richerson, 2004; Li and Nattie, 2008; Corcoran et al., 2009; Hodges and Richerson, 2010a; Ray et al., 2011; Brust et al., 2014; Teran et al., 2014). Their contribution to chemoreception has been characterized in adult mice, but not in neonatal mice. There are a variety of mouse models with dysfunction in their 5-HT system, such as Pet1$^{-/-}$ mice that have a 70% reduction of 5-HT neurons, *Lmx1b*$^{f/f/p}$ mice that lack >99% of 5-HT neurons, serotonin transporter (5-HTT) knockout mice that exhibit an absence of 5-HT reuptake and a decreased rate of synaptic 5-HT clearance, and others including dietary tryptophan depletion or tryptophan hydroxylase knockout mice (Holmes et al., 2003; Erickson et al., 2007; Li and Nattie, 2008; Alenina et al., 2009; Hodges et al., 2009; Cummings et al., 2010; Penatti et al., 2011). Some of these mice display respiratory abnormalities, high mortality, and growth retardation in the early postnatal period. *Lmx1b*$^{f/f/p}$ mice allow characterization of the effect of nearly complete absence of 5-HT neurons in the CNS. These mice have severely decreased baseline breathing in the neonatal period. This normalizes during postnatal development (Hodges et al., 2009), but these mice display a blunted response to hypercapnia as adults (Hodges et al., 2008).

Pet1$^{-/-}$ and *Lmx1b*$^{f/f/p}$ mice both have a low respiratory frequency (f_R) as neonates, but tidal volume (V_T) is lower only in *Lmx1b*$^{f/f/p}$ mice relative to their WT littermates (Hodges et al., 2009). There is a reduction in minute ventilation (V_E) in Pet1$^{-/-}$ null mice at P4 due to low f_R, but this is compensated by an increase in V_T at P14-15 (Cummings et al., 2010). Similarly, V_E from *Lmx1b*$^{f/f/p}$ mice normalizes around P12, coincident with the age at which apneas cease to occur (Hodges et al., 2009). The response to 5% CO_2 has previously been characterized in P4.5 Pet1$^{-/-}$ mice, and the change in f_R or V_T was not different compared to age-matched WT mice (Erickson et al., 2007). In addition, P5 mice that had previously been deficient in dietary tryptophan during gestation do not display an abnormal response to 7% CO_2 (Penatti et al., 2011). It is possible that the

chemoreceptive response early in development might not have much dependence on 5-HT neurons, and instead may be elicited primarily by chemosensitive neurons in other central nuclei, such as those in the retrotrapezoid nucleus (RTN) (Ramanantsoa et al., 2011; Kumar et al., 2015; Ruffault et al., 2015), locus coeruleus (Pineda and Aghajanian, 1997; Johnson et al., 2008) or nucleus tractus solitarius (Dean et al., 1990), or by peripheral chemoreceptors. That would be consistent with the findings that the chemosensitivity index (CI) of rat ventromedial medullary neurons increases with age in brain slices and in culture over the first few postnatal weeks (Wang and Richerson, 1999), and that the percentage of medullary 5-HT neurons that are chemosensitive, and the size of their response, is greater in brain slices prepared from mice aged P23-P26 than those aged P14-P18 (Brust et al., 2014).

It has previously been shown that neonatal *Lmx1b*$^{f/f/p}$ mice have a decrease in baseline ventilation compared to WT littermates (Hodges et al., 2009) and that adult *Lmx1b*$^{f/f/p}$ mice have a decrease in CO_2 chemoreception (Hodges et al., 2008), but the effect on chemoreception of genetic deletion of 5-HT neurons has never been examined in neonatal *Lmx1b*$^{f/f/p}$ mice. Here we compared the hypercapnic ventilatory response (HCVR) of *Lmx1b*$^{f/f/p}$ and WT mice from P1 to P21 to test the hypothesis that there is a switch in the role of 5-HT neurons from providing tonic respiratory drive in neonatal mice, to contributing to the robust HCVR of adult mice. We then used patch-clamp recordings from a large number of identified 5-HT neurons *in vitro* to test the hypothesis that there is a developmental increase in cellular chemosensitivity, and that the age at which this occurs coincides with the age at which there is an increase in the HCVR of WT mice *in vivo*. Finally, we tested the hypothesis that the absence of 5-HT neurons in neonatal *Lmx1b*$^{f/f/p}$ mice causes impaired growth rate and decreased survival, and this is due in part to hypoxia caused by the decrease in tonic respiratory drive at this age. Our results confirm that 5-HT neurons are important for baseline breathing in neonatal mice, and show for the first time that they do not play a significant role in central CO_2 chemoreception until they develop intrinsic chemosensitivity after 2–3 postnatal weeks. After that age, the increase in chemosensitivity of 5-HT neurons contributes to the large increase in the HCVR that normally occurs with maturation.

EXPERIMENTAL PROCEDURES

All experiments were done in accordance with the guidelines of the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health and were approved by the Yale University and University of Iowa Animal Care and Use Committees.

Animals

Generation of *Lmx1b*$^{f/f/p}$ mice, in which nearly all central nervous system 5-HT neurons fail to develop, has previously been described (Zhao et al., 2006). These mice are homozygous for floxed *Lmx1b*, and hemizygous for ePet-cre. Generation of ePet-EYFP mice, in which 5-HT neurons express the fluorescent reporter enhanced yellow fluorescent protein (EYFP), has also previously been described (Scott et al., 2005).

Genotypes were verified in all mice. Tail samples were obtained at P0-P1, digested and subjected to PCR with the following primers: Flox1: 5′ AGGCTCCATCCATTCTTCTC 3′. Flox2: 5′ CCACAATAAGCAAGAGGCAC 3′. Cre1: 5′ ATTTGCCTGCATTACCGGTCG 3′. Cre2: 5′ CAGCATTGCTGTCACTTGGTC 3′. YFP1: 5′ GAACTCCAGCAGGACCATGT 3′. YFP2: 5′ TAT ATC ATG GCC GAC AAG CA 3′. PCR products were analyzed via agarose gel electrophoresis. Breeding of *Lmx1b*$^{f/f/p}$ and ePet-EYFP mice was as previously reported (Scott et al., 2005; Hodges et al., 2008).

Whole-body plethysmography

Physiological studies were conducted to measure f_R, V_T, V_E and oxygen consumption (VO_2) in awake *Lmx1b*$^{f/f/p}$ mice (n = 15) and their WT littermates (n = 14) from five litters using stop-flow (P1) or flow-through (P4, P7, P12 and P21) whole-animal plethysmography under thermoneutral conditions (30 °C) as described in previous studies (Hodges and Richerson, 2008; Hodges et al., 2009; Cerpa et al., 2014). All experiments were performed in the daytime (light) period. All parameters were determined at each age studied, except VO_2, which was determined only for P4-P21 as it was not possible to obtain VO_2 for P1 mice due to the small chamber flow rate. The plethysmography chamber gas flow rate was measured using an electronic gas flowmeter (PV500CCMVADA-CP, Cole-Parmer) and PO_2 was measured with an O_2 sensor (S3-A; AEI Technologies). Baseline breathing was measured for more than 30 min in room air (21% O_2 – balance N_2), followed by 10 min in 7% CO_2/21% O_2 (balance N_2). Mice were returned to their home cage, and approximately 4 h later were placed in the plethysmograph and exposed for 30 min to room air, followed by 10 min in hypoxia (10% O_2 – balance N_2). Data were collected from the last 5 min of each gas exposure. Mice were observed continuously, and data were excluded from analysis during any behaviors that resulted in a breathing pattern other than eupnea (e.g. vocalization, licking, chewing, sniffing, walking, etc.), and were analyzed off-line using software custom-written in Matlab (Matlab, R2007b) from data collected only during episodes of quiet wakefulness (periods with eyes open when mice responded to a mild stimulus such as tapping the chamber). Rectal temperatures were measured in P1-P7 neonates (BAT-12, Physitemp Instruments) before and after experiments. Telemetric temperature probes (IPTT-300, BMDS) were implanted subcutaneously between the scapulae in mice at P8, and then used to measure body temperature during studies of P12 and P21 mice.

Quantification of changes in ventilation

V_T and V_E were calculated using the method of Drorbaugh and Fenn (Drorbaugh and Fenn, 1955). V_T was calibrated using a small animal ventilator (Minivent, Harvard Apparatus) by injecting 30–100 μl of air into the plethysmograph

Please cite this article in press as: Cerpa VJ et al. Medullary 5-HT neurons: Switch from tonic respiratory drive to chemoreception during postnatal development. Neuroscience (2016), http://dx.doi.org/10.1016/j.neuroscience.2016.09.002