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Role of TrkB during the postnatal development of the rat carotid body

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Abstract

Brain-derived neurotrophic factor (BDNF) supports innervation of the carotid body by neurons projecting from the petrosal ganglion. Although carotid body glomus cells also express TrkB, BDNF's high affinity receptor, the role of BDNF in carotid body growth and O₂ sensitivity has not been studied. Neonatal rats were treated with the TrkB antagonist K252a (100 µg kg⁻¹, i.p., b.i.d.) or vehicle on postnatal days P0–P6 and studied on P7. Carotid body volume was decreased by 35% after chronic K252a ($P < 0.001$); a reduction in carotid body size was also elicited using the more selective TrkB antagonist ANA-12 (500 µg kg⁻¹, i.p., b.i.d.). In contrast, single-unit chemoafferent responses to 5% O₂, measured *in vitro*, were unaffected by chronic K252a administration. Normoxic and hypoxic ventilation, measured by head-body plethysmography, were also normal after chronic K252a administration, but acute K252a administration produced a slower, deeper breathing pattern during the transition into hypoxia. These data suggest that BDNF regulates postnatal carotid body growth but does not influence the development of glomus cell O₂ sensitivity.

Keywords

Brain-derived neurotrophic factor; BDNF; Control of breathing; Hypoxic ventilatory response; K252a; ANA-12

1. Introduction

Neurotrophic factors are a heterogeneous group of proteins that support the growth, maturation, and maintenance of neurons. Two groups of neurotrophic factors linked to respiratory control are the nerve growth factor (NGF) family, or neurotrophins (which include brain-derived neurotrophic factor (BDNF)), and the glial cell line-derived neurotrophic factor (GDNF) family of ligands (which includes GDNF) (Huang and

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Reichardt, 2001; Ogier et al., 2013). Neonatal mice lacking functional *bdnf* or *gdnf* genes exhibit slow, erratic breathing with a marked increase in the frequency of apneas (Erickson et al., 1996, 2001). BDNF-deficient mice also exhibit impaired ventilatory responses to hyperoxia despite normal hypercapnic ventilatory responses, suggesting that BDNF deficiency selectively impairs peripheral O₂ sensitivity (Erickson et al., 1996). In contrast, mice lacking the GDNF receptor RET exhibit depressed hypercapnic ventilatory responses (Burton et al., 1997). These studies in intact animals, along with studies in reduced preparations (e.g., Balkowiec and Katz, 1998; Huang et al., 2005), demonstrate that BDNF and GDNF play important and often complementary roles in the normal development of the respiratory network.

Neurotrophic factors and their receptors are expressed at all levels of the respiratory control system, including peripheral chemoreceptors, primary chemoafferent neurons, brainstem regions responsible for sensory integration and rhythmogenesis, respiratory motoneurons, and the neuromuscular junction (Katz, 2005; Mantilla and Sieck, 2008; Porzionato et al., 2008; Ogier et al., 2013). Within the central nervous system, BDNF acutely modulates the electrical and synaptic properties of neurons within the pre-Bötzinger complex, nucleus tractus solitarius (nTS), and the nucleus Kolliker-Fuse (reviewed in Ogier et al., 2013). For example, exogenous BDNF application alters respiratory burst frequency of pre-Bötzinger neurons recorded from mouse brainstem slices in an age-dependent manner; BDNF increases burst frequency in embryonic preparations (Bouvier et al., 2008) and slows it in newborns (Thoby-Brisson et al., 2003). BDNF also promotes the survival of pontine noradrenergic neurons (Guo et al., 2005). Within the peripheral nervous system, BDNF plays a critical role in the development of the chemoafferent pathway between the carotid body and the brainstem. The carotid body, which serves as the principal site of O₂ sensing for the respiratory control system, is innervated by dopaminergic afferent neurons projecting from the petrosal ganglion via the carotid sinus nerve. Newborn mice lacking either BDNF or its high affinity receptor TrkB exhibit a dramatic reduction in the total number of dopaminergic neurons in the nodose-petrosal ganglion complex (Erickson et al., 1996, 2001); BDNF promotes survival of chemoafferent neurons by inhibiting proapoptotic genes (Hellard et al., 2004). The carotid body is an important source of BDNF and other neurotrophic factors for the developing afferent neurons (Hertzberg et al., 1994; Brady et al., 1999). Surgical removal of the carotid body causes chemoafferent neuron degeneration *in vivo*, but these cells can be rescued by replacing the carotid body with implants containing BDNF (Hertzberg et al., 1994).

Neurotrophic factors are primarily expressed by the O₂-sensitive glomus (type I) cells within the carotid body (Izal-Azcárate et al., 2008; Porzionato et al., 2008; Atanasova and Lazarov, 2013, 2014). Although most research on carotid body BDNF expression has focused on the role of carotid body-derived BDNF in sustaining chemoafferent neurons (Katz, 2005; Chavez-Valdez et al., 2012; Ogier et al., 2013), carotid body glomus cells express TrkB as well (Wang and Bisgard, 2005; Atanasova and Lazarov, 2013). This raises the possibility that BDNF released by carotid body glomus cells acts in an autocrine or paracrine manner to promote glomus cell proliferation, survival, and/or functional maturation. For example, rats exposed to chronic postnatal hyperoxia exhibit reduced carotid body BDNF expression

(Dmitrieff et al., 2011; Chavez-Valdez et al., 2012). These rats also exhibit smaller carotid bodies due to decreased cell division and increased apoptosis (Erickson et al., 1998; Wang and Bisgard, 2005; Dmitrieff et al., 2012; Bavis et al., 2013), consistent with a link between insufficient BDNF signaling and carotid body hypoplasia. Autocrine or paracrine activation of TrkB receptors could also influence postnatal maturation of O₂ transduction mechanisms or synaptic transmission with afferent neurons. To begin testing these hypotheses, we chronically administered TrkB receptor antagonists to neonatal rats for the first postnatal week and assessed carotid body size and *in vitro* carotid body responses to hypoxia. Ventilation was also measured to determine whether observed changes in carotid body morphology and/or systemic inhibition of TrkB altered postnatal development of hypoxic responses at the whole animal level.

2. Methods

2.1. Experimental animals

Late-gestation pregnant Sprague-Dawley rats were obtained from a commercial supplier (Charles River Laboratories) and housed under standard conditions (12:12 light cycle with lights on 08:00 – 20:00, food and water *ad libitum*). All experimental protocols were approved by the Bates College Institutional Animal Care and Use Committee.

Two TrkB receptor antagonist drugs were used in this study, the non-selective TrkB receptor inhibitor K252a (Sigma-Aldrich) and the more selective inhibitor ANA-12 (Tocris). K252a was prepared at a working concentration of 10 $\mu\text{g ml}^{-1}$ in 25% dimethyl sulfoxide (DMSO; Sigma-Aldrich). ANA-12 was prepared to a working concentration of 50 $\mu\text{g ml}^{-1}$ in 5% DMSO and 19% Cremophor EL (CrEL; Calbiochem). DMSO and CrEL solutions were prepared in 0.9% saline (Vedco). Drugs were administered by intraperitoneal injection of 0.01 ml per gram of body mass, resulting in final dosages of 100 $\mu\text{g kg}^{-1}$ (K252a) or 500 $\mu\text{g kg}^{-1}$ (ANA-12) for each injection. For comparison, littermates were injected with equivalent volumes of the appropriate vehicle (DMSO or DMSO+CrEL).

For chronic studies, each pup was injected twice daily at approximately 12-h intervals (typically 08:00 and 20:00) from the day of birth (P0) through the sixth postnatal day (P6); pups received only one injection on P0 if they were born after 12:00. Carotid body morphology (K252a and ANA-12) or physiological measurements (K252a only) were then completed the following day (i.e., P7). To study the acute effects of K252a on breathing, an additional group of rats received a single injection of K252a approximately 30 min prior to ventilation measurements. Rat pups were derived from a total of 9 litters (7 K252a, 2 ANA-12) for the chronic studies and from 4 litters for the acute K252a study, with approximately half of the individuals in each litter receiving the drug and the remainder receiving the vehicle. Both male and female pups were studied with approximately equal representation in each treatment group.

2.2 Carotid body volume

Carotid bifurcations were harvested *en bloc* after euthanasia with CO₂ and decapitation; decapitation was performed well caudal to the carotid bifurcations to avoid damaging the carotid bodies. Carotid bifurcations were fixed for 1 hour in 4% paraformaldehyde (PFA) in

0.1 M phosphate buffered saline (PBS; pH 7.4) followed by cryoprotection in 30% sucrose in PBS (24 h at 4 °C). Bifurcations were then embedded in Tissue-Tek O.C.T. compound (Sakura Finetek), flash frozen on dry ice, and stored at –80 °C. Samples were sectioned at 10 µm with a cryostatic microtome and mounted onto slides (Fisherbrand Superfrost Plus). Slides were then stained with hematoxylin and eosin (H & E) and imaged with a Nikon Eclipse 80i microscope attached to a 2 megapixel camera and NIS Elements Software. Carotid body area within each section was calculated using Image J software (version 1.43u; National Institutes of Health); investigators were blinded to the treatment group during image analysis. Carotid body volume was calculated from the area of the carotid body in each section, section thickness, and total number of sections containing the carotid body.

2.3. Single-unit carotid chemoafferent activity

Immediately following ventilation measurements (see Section 2.4), subsets of rats were deeply anesthetized with CO₂ and decapitated. The carotid bifurcation and petrosal-nodose ganglion complex were removed *en bloc* and placed in an ice-cold, oxygenated (95% O₂, 5% CO₂) saline solution containing (in mM): 125 NaCl, 5 KCl, 2 CaCl₂, 1 Na₂HPO₄, 1 MgSO₄, 24 NaHCO₃, and 5 dextrose. Tissue was removed from the carotid body preparation using a solution of 0.1% collagenase (collagenase P; Roche Diagnostics) and 0.02% protease (type XIV; Sigma-Aldrich) in oxygenated saline with gentle agitation for 30 min at 37 °C. The preparation was then further dissected to isolate the carotid body, carotid sinus nerve (CSN), glossopharyngeal nerve, and petrosal ganglion. This preparation was transferred to a perfusion chamber (RC-22C; Warner Instruments) on the stage of an inverted microscope (Eclipse TE-300; Nikon). The chamber was perfused with saline solution bubbled with 21% O₂ (5% CO₂, balance N₂) that was delivered at a rate of ~3 mL min⁻¹ through stainless steel tubing (Upchurch Scientific). Fluid passing through the perfusion chamber was warmed to approximately 37 °C using an in-line heater.

A suction electrode was used to record single-unit nerve activity from soma in the petrosal ganglion. An extracellular amplifier (EX-1; Dagan Instruments) was employed to amplify pipette potential (2,000–5,000 ×), which was passband-filtered (0.1–2 kHz), digitized (10 kHz sample rate), and recorded to a computer (Powerlab 8/30 and Chart 6 software; AD Instruments). For identification of individual chemoafferent cells under baseline conditions (21% O₂, 5% CO₂, balance N₂), the carotid body was stimulated (~200 µA × 0.05 ms pulse duration) at 0.5–1 Hz (Isostim A320; World Precision Instruments) using a glass electrode (filled with 1 M NaCl). If a stimulus elicited a single orthodromic action potential, the stimulator was turned off and baseline nerve activity was recorded for 2 min. The response to acute hypoxia (5% O₂, 5% CO₂, balance N₂) was then recorded for 2 min. The preparation was returned to baseline conditions (21% O₂, 5% CO₂, balance N₂) for at least 10 min before searching for a second chemoreceptor unit and repeating the protocol; no more than two recording were made for each carotid body-petrosal ganglion complex. Single-unit chemoafferent activity was discriminated off-line (Spike Histogram Module v.1; ADInstruments). The number of spikes was counted for the final 1 min of baseline and in 1-s bins throughout the duration of acute hypoxia. Peak firing activity in hypoxia was determined from a 3-s moving average of spike frequency.

2.4. Ventilation measurements

Ventilation was measured in neonatal rats using head-body plethysmography as described elsewhere (Bavis et al., 2014). After weighing, the rat was sealed into the plethysmograph chamber with a flexible collar separating the head and body compartments; petroleum jelly was applied around the animal's neck to ensure an airtight seal. Air mixtures were passed through the head compartment at a flow rate of 1.5 L min^{-1} . A pneumotach (MLT1L; ADInstruments) connected to a differential pressure transducer (ML141, ADInstruments) monitored airflow as gas was displaced from the body compartment during breathing; the system was calibrated by injecting 0.5 mL of air into the body compartment. Output from the pressure transducer was recorded at a sampling rate of 1000 Hz, passed through a high pass filter (0.1 Hz), and integrated to obtain respiratory volumes (PowerLab 8SP and LabChart 7 software with the spirometry extension; ADInstruments). Air temperature within the body compartment was measured continuously (IT-18 thermocouple; Physitemp Instruments) and was regulated at $32\text{--}34^\circ\text{C}$ (i.e., within the thermoneutral zone for neonatal rats; Malik and Fewell, 2003).

Following a 10-min adjustment period (21% O_2 , balance N_2), baseline ventilation was recorded for approximately 5 min. Gas concentrations were then switched to 12% O_2 (balance N_2) for 10 min to assess the HVR. Baseline ventilation was determined from 40–60 s of the record, excluding sighs and movement artifacts. Hypoxic ventilation was determined from 10–15 s of the record after the 1st, 5th, and 10th minutes of hypoxia.

2.5. Statistical analysis

Body mass, carotid body volume, and peak carotid chemoafferent activity were compared between treatment groups (i.e., drug vs. vehicle) using unpaired t-tests (Prism 6.04; GraphPad Software). Baseline chemoafferent neuron discharge rates were not normally distributed and the treatment groups had unequal variance, so treatment groups were compared using a Mann-Whitney U test (Prism 6.04). Ventilation was compared among groups by two-way repeated measures ANOVA (factor 1: drug; factor 2: time) followed by Tukey's multiple comparison tests (SigmaPlot 12.5; Systat Software). Statistical significance determined at $P < 0.05$. Values are presented as mean $\pm$ SEM unless otherwise indicated.

3. Results

3.1. Body mass after chronic K252a or ANA-12 administration

Body mass at P7 was similar between rats chronically administered K252a ($13.5 \pm 0.2 \text{ g}$, $n=31$) and rats that only received the vehicle ($13.7 \pm 0.2 \text{ g}$, $n=28$) ($P=0.42$). Likewise, body mass was unaffected by ANA-12 treatment ($15.3 \pm 0.6 \text{ g}$ vs. $15.6 \pm 0.6 \text{ g}$ in the vehicle group; both $n=8$) ($P=0.70$). However, rats from both chronic drug studies (regardless of whether they received drug or vehicle) tended to weigh less than the age-matched rats used in the acute K252a study ($16.9 \pm 0.4 \text{ g}$, $n=30$); this could reflect the stress of repeated handling / injection or an adverse effect of DMSO in the vehicle.

3.2. Carotid body volume after chronic K252a or ANA-12 administration

Chronic K252a treatment reduced carotid body volume by 35% ($P<0.001$; Fig. 1A). Similar results were obtained using the more selective TrkB antagonist ANA-12 which reduced carotid body volume by 18% ($P=0.03$; Fig. 1B).

3.3. Single-unit chemoafferent activity after chronic K252a administration

Single-unit chemoafferent activity was not altered by chronic K252a treatment (Fig. 2). Baseline discharge rates were variable, but median activity was not different between treatment groups (0.4 vs. 0.2 imp s^{-1} in the K252a and vehicle groups, respectively) ($P=0.24$; Fig. 2A). Peak activity during 5% O_2 was also similar between the K252a and vehicle treatment groups ($P=0.22$; Fig. 2B). Comparable results were obtained when peak hypoxic responses were calculated as the change in discharge rate during 5% O_2 (activity = 14.6 ± 2.2 vs. 11.8 ± 1.3 imp s^{-1} in the K252a and vehicle groups, respectively; $P=0.26$).

3.4. Ventilation after acute or chronic K252a administration

A subset of rats was not injected with K252a until P7, at which time individuals received a single dose of K252a approximately 30 min before recording baseline ventilation. Acute K252a administration did not alter minute ventilation during normoxia or hypoxia (main effect for drug, $P=0.35$; drug $\times$ time, $P=0.95$) (Fig. 3C). Both groups of rats increased ventilation during acute hypoxia (main effect for time, $P<0.001$), and this HVR was biphasic with ventilation being slightly less during the 10th minute of hypoxia compared to the 1st minute independent of the drug treatment ($P=0.03$). Despite the similarity of the overall HVR, the pattern of breathing was affected by K252a. Acute K252a exposure blunted the frequency response to 12% O_2 (drug $\times$ time, $P=0.004$), but this was only apparent during the early phase of the HVR (i.e., $P=0.001$ between K252a and vehicle groups during the 1st minute of hypoxia) (Fig. 3A). Conversely, the tidal volume response tended to be greater in K252a rats although this did not reach statistical significance (drug $\times$ time, $P=0.30$) (Fig. 3B).

For rats that received twice-daily injection of K252a from P0 through P6, ventilation appeared normal on P7 (i.e., >12 hours after the final injection). Chronic K252a did not alter minute ventilation during normoxia or hypoxia (main effect for drug, $P=0.35$; drug $\times$ time, $P=0.24$) (Fig. 4C). As in the acute K252a experiments, the HVR was moderately biphasic (main effect for time, $P<0.001$; 10th min vs. 1st min, $P=0.003$). Neither the frequency responses nor tidal volume responses to hypoxia were altered by chronic K252a administration (drug and drug $\times$ time, all $P>0.05$) (Figs. 4A and 4B).

4. Discussion

4.1. Effects on TrkB receptor inhibition on carotid body development

Chronic inhibition of TrkB receptors with K252a or ANA-12 during the postnatal period reduced carotid body size. This result is consistent with the hypothesis that endogenous BDNF normally acts through autocrine and paracrine pathways to promote cell proliferation and/or cell survival during postnatal growth. Indeed, BDNF and other neurotrophic factors have these roles in other cell populations (reviewed in Huang and Reichardt, 2001). BDNF

is the most likely TrkB ligand given its abundant expression in the developing carotid body (Brady et al., 1999; Wang and Bisgard, 2005; Izal-Azcárate et al., 2008; Porzionato et al., 2008; Dmitrieff et al., 2011; Chavez-Valdez et al., 2012; Atanasova and Lazarov, 2013, 2014), but we cannot exclude a role for neurotrophin-4/5 (NT4) which also binds to TrkB. However, there currently is no evidence that NT4 is expressed in the carotid body (Porzionato et al., 2008).

Although K252a is widely used to antagonize TrkB, this drug inhibits a variety of protein kinases so some of the observed effects on carotid body growth may reflect inhibition of other Trk receptor subtypes. Both TrkA (which binds nerve growth factor (NGF)) and TrkC (which binds neurotrophin-3 (NT3)) receptor subtypes have been detected by immunohistochemistry in the carotid body, particularly in glomus cells (Atanasova and Lazarov, 2013, 2014). Although NGF has been implicated in perinatal carotid body growth *in vivo* (Aloe and Levi-Montalcini, 1980), others have questioned this result since subsequent experiments showed no effect of NGF on glomus cell survival *in vitro* (Nurse and Vollmer, 1997; Porzionato et al., 2008). Importantly, ANA-12 is a highly selective TrkB antagonist (Cazorla et al., 2011) and this drug had qualitatively similar effects on carotid body volume. Quantitative differences between K252a and ANA-12 (i.e., -35% vs. -18% change in carotid body volume) could reflect differences in the effective concentration or half-life of the drugs; dosages were selected from the literature rather than being optimized for the current study. Indeed, preliminary experiments using a lower dosage of K252a (50 $\mu\text{g kg}^{-1}$, administered i.p. once daily) tended to produce a smaller change in carotid body size than the dosage ultimately used in the present study (S.C. Fallon and R.W. Bavis, unpublished data). Likewise, larger or more frequent doses of ANA-12 may have produced greater deficits in carotid body size. In any event, the data presented here strongly support a role for TrkB activation (and by extension BDNF) in normal carotid body growth.

It was not possible to assess the independent contributions of different cell types with the techniques used in the present study (i.e., H & E staining), so it is not clear whether reductions in carotid body size reflected the loss of O₂-sensitive glomus cells, sustentacular (type II) cells, or both. Glomus cells comprise approximately 30% of the volume of the neonatal carotid body (Erickson et al., 1998), so it does not seem possible that changes in glomus cell numbers alone can explain the observed reduction in carotid body size after K252a administration. TrkB is highly expressed in glomus cells and chemoafferent nerve fibers (Wang & Bisgard, 2005), but modest TrkB expression has been reported recently for a subset of sustentacular cells in adult rat carotid bodies (Atanasova and Lazarov, 2013, 2014). The glia-like sustentacular cells comprise approximately 20% of the cells in the carotid body (Kumar and Prabhakar, 2012) and may also serve as precursors for glomus cells (Pardal et al., 2007). Therefore, TrkB activation may regulate carotid body growth through multiple cell types. Whether these receptors regulate cell division, suppress apoptosis, or both in the carotid body warrants further study. Studies using cell type-specific labeling paired with markers for cell division and apoptosis should be able to resolve these questions.

In contrast to its effects on carotid body size, chronic inhibition of TrkB (and other Trk receptor subtypes) with K252a had no measurable effect on the carotid chemoreceptor

responses to hypoxia. This suggests that BDNF signaling through TrkB does not contribute to the development of glomus cell O₂ sensitivity (e.g., development of O₂ transduction mechanisms) or synaptic transmission. It is likely that chronic TrkB inhibition reduced the total number of chemoafferent neurons (Hertzberg et al., 1994; Erickson et al., 1996, 2001), and thus the whole-nerve CSN response to hypoxia, but this cannot be verified with the single-unit recording technique employed in the current study.

4.2. Effects on TrkB inhibition on breathing

Despite the fact that chronic K252a reduced carotid body size (and presumably caused chemoafferent neuron degeneration), normoxic and hypoxic ventilation were not affected by this treatment. This is somewhat surprising given the importance of carotid bodies to the acute HVR and the dramatic respiratory dysfunction observed in BDNF null mice (Erickson et al., 1996). There are several potential explanations for these findings. First, there may be sufficient redundancy in the respiratory control system to buffer the loss of some proportion of the carotid chemoreceptor cells. Although bilateral carotid body denervation nearly abolishes the HVR, normal ventilatory responses can be mounted after unilateral carotid body excision (i.e., 50% loss of carotid body tissue) (Busch et al., 1983; Cragg and Khrisanapant, 1994); carotid body size was reduced by only 35% in the present study. For comparison, studies that have attributed attenuated HVR to hyperoxia-induced carotid body hypoplasia often report 60–75% reductions in carotid body size (Bavis et al., 2013). Similarly, mice heterozygous for the BDNF mutation (*bdnf*^{+/-}) exhibit nearly normal breathing despite significant loss of chemoafferent neurons (i.e., 32% reduction in dopaminergic neurons from the nodose-petrosal ganglion complex; Erickson et al., 1996). Alternatively, plasticity in other components of the respiratory control system may have compensated for carotid body impairment over the course of the treatment (e.g., upregulation of aortic bodies, increased CNS gain) (Forster, 2003). Finally, *bdnf*^{-/-} mice completely lack BDNF during both prenatal and postnatal development whereas K252a was administered after birth. Although chemoafferent neurons continue to require trophic support during the postnatal period (Hertzberg et al., 1994), it is likely that prenatal BDNF insufficiency contributes to the more severe respiratory dysfunction Erickson and colleagues (1996) observed in the knockout mice.

BDNF can acutely modulate synaptic transmission (Huang and Reichardt, 2001; Carvalho et al., 2008), and this has been demonstrated in respiratory neurons as well using reduced preparations (Ogier et al., 2013; Gao et al., 2014). Acute administration of K252a did modulate the breathing pattern in intact, conscious neonatal rats; this acute effect probably was not evident in rats receiving chronic K252a since the last injection occurred more than 12 hours prior to ventilation measurements. Rats tended to breathe more slowly and deeply after TrkB inhibition, and this was most prominent during the transition into hypoxia. This result suggests that BDNF normally exerts a stimulatory effect on breathing at this age, but the location of the relevant TrkB receptors cannot be determined since K252a was administered systemically. Rhythm regulating neurons in the pre-Bötzinger complex are sensitive to BDNF, but it has been reported that BDNF's influence on respiratory frequency transitions from excitatory to inhibitory between the fetal period (E16.5) and the early postnatal period (P1–P4) in mice (Thoby-Brisson et al., 2003; Bouvier et al., 2008). Thus,

the respiratory effects of K252a administration observed in P7 neonatal rats in the present study (i.e., reduced breathing frequency) are opposite to those predicted based on neonatal mouse brainstem preparations (Thoby-Brisson et al., 2003). Whether these discordant results reflect differences in the species, the age, or the type of preparations (intact vs. reduced) used remains to be determined.

4.3. Conclusion

The localization of both BDNF and its high affinity receptor TrkB in carotid body cells generally, and within glomus cells in particular, suggested that BDNF could regulate carotid body growth and/or functional development through autocrine and paracrine signaling pathways. To our knowledge, the present study provides the first experimental evidence that TrkB activation supports carotid body growth *in vivo*. Although TrkB signaling does not appear to influence the development of O₂ sensitivity of individual carotid chemoreceptor cells, TrkB activation appears to modulate breathing pattern in neonatal rats during respiratory challenges. These results suggest that abnormal BDNF/TrkB signaling (whether of genetic or environmental origin) could compromise the development of the carotid body and carotid body-mediated reflexes, potentially leading to respiratory dysfunction. Indeed, reduced BDNF expression in the carotid chemoafferent pathway has been proposed to contribute to attenuated hypoxic responses in rats reared in moderate hyperoxia (Dmitrieff et al., 2011; Chavez-Valdez et al., 2012). Perinatal hyperoxia has also been linked to abnormal respiratory control in preterm infants (Katz-Salamon et al., 1994; Bates et al., 2014), but whether abnormal BDNF/TrkB receptor expression contributes to this respiratory dysfunction remains to be determined.

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References

- Aloe L, Levi-Montalcini R. Comparative studies on the effects elicited by pre- and postnatal injections of anti-NGF, guanethidine, and 6-hydroxydopamine in chromaffin and ganglion cells of the adrenal medulla and carotid body in infant rats. *Adv Biochem Psychopharmacol.* 1980; 25:221–226. [PubMed: 7446255]
- Atanasova DY, Lazarov NE. Immunohistochemical localization of some neurotrophic factors and their receptors in the rat carotid body. *Neurosci Med.* 2013; 4:284–289.
- Atanasova DY, Lazarov NE. Expression of neurotrophic factors and their receptors in the carotid body of spontaneously hypertensive rats. *Respir Physiol Neurobiol.* 2014; 202:6–15. [PubMed: 25034384]
- Balkowiec A, Katz DM. Brain-derived neurotrophic factor is required for normal development of the central respiratory rhythm in mice. *J Physiol.* 1998; 510(Pt 2):527–533. [PubMed: 9706001]
- Bates ML, Farrell ET, Eldridge MW. Abnormal ventilatory responses in adults born prematurely. *N Engl J Med.* 2014; 370(6):584–585. [PubMed: 24499235]
- Bavis RW, Fallon SC, Dmitrieff EF. Chronic hyperoxia and the development of the carotid body. *Respir Physiol Neurobiol.* 2013; 185(1):94–104. [PubMed: 22640932]
- Bavis RW, van Heerden ES, Brackett DG, Harmeling LH, Johnson SM, Blegen HJ, Logan S, Nguyen GN, Fallon SC. Postnatal development of eupneic ventilation and metabolism in rats chronically exposed to moderate hyperoxia. *Respir Physiol Neurobiol.* 2014; 198:1–12. [PubMed: 24703970]

- Bouvier J, Autran S, Dehorter N, Katz DM, Champagnat J, Fortin G, Thoby-Brisson M. Brain-derived neurotrophic factor enhances fetal respiratory rhythm frequency in the mouse preBötzing complex *in vitro*. *Eur J Neurosci*. 2008; 28(3):510–520. [PubMed: 18702723]
- Brady R, Zaidi SI, Mayer C, Katz DM. BDNF is a target-derived survival factor for arterial baroreceptor and chemoafferent primary sensory neurons. *J Neurosci*. 1999; 19(6):2131–2142. [PubMed: 10066266]
- Burton MD, Kawashima A, Brayer JA, Kazemi H, Shannon DC, Schuchardt A, Costantini F, Pachnis V, Kinane TB. RET proto-oncogene is important for the development of respiratory CO₂ sensitivity. *J Auton Nerv Syst*. 1997; 63(3):137–143. [PubMed: 9138245]
- Busch MA, Bisgard GE, Mesina JE, Forster HV. The effects of unilateral carotid body excision on ventilatory control in goats. *Respir Physiol*. 1983; 54(3):353–361. [PubMed: 6672918]
- Carvalho AL, Caldeira MV, Santos SD, Duarte CB. Role of the brain-derived neurotrophic factor at glutamatergic synapses. *Br J Pharmacol*. 2008; 153(Suppl 1):S310–S324. [PubMed: 18059328]
- Cazorla M, Prémont J, Mann A, Girard N, Kellendonk C, Rognan D. Identification of a low-molecular weight TrkB antagonist with anxiolytic and antidepressant activity in mice. *J Clin Invest*. 2011; 121(5):1846–1857. [PubMed: 21505263]
- Chavez-Valdez R, Mason A, Nunes AR, Northington FJ, Tankersley C, Ahlawat R, Johnson SM, Gauda EB. Effect of hyperoxic exposure during early development on neurotrophin expression in the carotid body and nucleus tractus solitarii. *J Appl Physiol*. 2012; 112(10):1762–1772. [PubMed: 22422797]
- Cragg PA, Khrisanapant W. Is the second carotid body redundant? *Adv Exp Med Biol*. 1994; 360:297–299. [PubMed: 7872104]
- Dmitrieff EF, Wilson JT, Dunmire KB, Bavis RW. Chronic hyperoxia alters the expression of neurotrophic factors in the carotid body of neonatal rats. *Respir Physiol Neurobiol*. 2011; 175(2):220–227. [PubMed: 21094282]
- Dmitrieff EF, Piro SE, Broge TA Jr, Dunmire KB, Bavis RW. Carotid body growth during chronic postnatal hyperoxia. *Respir Physiol Neurobiol*. 2012; 180(2–3):193–203. [PubMed: 22138179]
- Erickson JT, Conover JC, Borday V, Champagnat J, Barbacid M, Yancopoulos G, Katz DM. Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. *J Neurosci*. 1996; 16(17):5361–5371. [PubMed: 8757249]
- Erickson JT, Mayer C, Jawa A, Ling L, Olson EB Jr, Vidruk EH, Mitchell GS, Katz DM. Chemoafferent degeneration and carotid body hypoplasia following chronic hyperoxia in newborn rats. *J Physiol*. 1998; 509(2):519–526. [PubMed: 9575300]
- Erickson JT, Brosenitsch TA, Katz DM. Brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor are required simultaneously for survival of dopaminergic primary sensory neurons *in vivo*. *J Neurosci*. 2001; 21(2):581–589. [PubMed: 11160437]
- Forster HV. Plasticity in the control of breathing following sensory denervation. *J Appl Physiol*. 2003; 94(2):784–794. [PubMed: 12531915]
- Gao XP, Liu Q, Nair B, Wong-Riley MT. Reduced levels of brain-derived neurotrophic factor contribute to synaptic imbalance during the critical period of respiratory development in rats. *Eur J Neurosci*. 2014; 40(1):2183–2195. [PubMed: 24666389]
- Guo H, Hellard DT, Huang L, Katz DM. Development of pontine noradrenergic A5 neurons requires brain-derived neurotrophic factor. *Eur J Neurosci*. 2005; 21(7):2019–2023. [PubMed: 15869495]
- Hellard D, Brosenitsch T, Fritsch B, Katz DM. Cranial sensory neuron development in the absence of brain-derived neurotrophic factor in BDNF/Bax double null mice. *Dev Biol*. 2004; 275(1):34–43. [PubMed: 15464571]
- Hertzberg T, Fan G, Finley JC, Erickson JT, Katz DM. BDNF supports mammalian chemoafferent neurons *in vitro* and following peripheral target removal *in vivo*. *Dev Biol*. 1994; 166(2):801–811. [PubMed: 7813797]
- Huang EJ, Reichardt LF. Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci*. 2001; 24:677–736. [PubMed: 11520916]

- Huang L, Guo H, Hellard DT, Katz DM. Glial cell line-derived neurotrophic factor (GDNF) is required for differentiation of pontine noradrenergic neurons and patterning of central respiratory output. *Neuroscience*. 2005; 130(1):95–105. [PubMed: 15561428]
- Izal-Azcárate A, Belzunegui S, San Sebastián W, Garrido-Gil P, Vázquez-Claverie M, López B, Marcilla I, Luquin MA. Immunohistochemical characterization of the rat carotid body. *Respir Physiol Neurobiol*. 2008; 161(1):95–99. [PubMed: 18280799]
- Katz DM. Regulation of respiratory neuron development by neurotrophic and transcriptional signaling mechanisms. *Respir Physiol Neurobiol*. 2005; 149(1–3):99–109. [PubMed: 16203214]
- Katz-Salamon M, Lagercrantz H. Hypoxic ventilatory defense in very preterm infants: attenuation after long term oxygen treatment. *Arch Dis Child Fetal Neonatal Ed*. 1994; 70:F-90–95. [PubMed: 8154920]
- Kumar P, Prabhakar NR. Peripheral chemoreceptors: function and plasticity of the carotid body. *Compr Physiol*. 2012; 2(1):141–219. [PubMed: 23728973]
- Malik SS, Fewell JE. Thermoregulation in rats during early postnatal maturation: importance of nitric oxide. *Am J Physiol*. 2003; 285(6):R1366–R1372.
- Mantilla CB, Sieck GC. Trophic factor expression in phrenic motor neurons. *Respir Physiol Neurobiol*. 2008; 164(1–2):252–262. [PubMed: 18708170]
- Nurse CA, Vollmer C. Role of basic FGF and oxygen in control of proliferation, survival, and neuronal differentiation in carotid body chromaffin cells. *Dev Biol*. 1997; 184(2):197–206. [PubMed: 9133430]
- Ogier M, Kron M, Katz DM. Neurotrophic factors in development and regulation of respiratory control. *Compr Physiol*. 2013; 3:1125–1134. [PubMed: 23897682]
- Pardal R, Ortega-Sáenz P, Durán R, López-Barneo J. Glia-like stem cells sustain physiologic neurogenesis in the adult mammalian carotid body. *Cell*. 2007; 131(2):364–377. [PubMed: 17956736]
- Porzionato A, Macchi V, Parenti A, De Caro R. Trophic factors in the carotid body. *Int Rev Cell Mol Biol*. 2008; 269:1–58. [PubMed: 18779056]
- Thoby-Brisson M, Cauli B, Champagnat J, Fortin G, Katz DM. Expression of functional tyrosine kinase B receptors by rhythmically active respiratory neurons in the pre-Bötzinger complex of neonatal mice. *J Neurosci*. 2003; 23(20):7685–7689. [PubMed: 12930808]
- Wang ZY, Bisgard GE. Postnatal growth of the carotid body. *Respir Physiol Neurobiol*. 2005; 149(1–3):181–190. [PubMed: 15914098]

Highlights

- Neonatal rats were treated with the TrkB antagonists K252a or ANA-12.
- Chronic treatment with K252a or ANA-12 reduced carotid body size.
- Chronic K252a did not alter chemoafferent or ventilatory responses to hypoxia.
- Acute K252a altered breathing frequency, but not minute ventilation, in hypoxia.

Fig. 1. Carotid body volumes for P7 rats after chronic treatment with the TrkB antagonists (A) K252a (n= 10 K252a, 10 vehicle) or (B) ANA-12 (n= 8 ANA-12, 8 vehicle). Values are mean $\pm$ SEM. * $P < 0.05$ vs. vehicle-injected rats.

Fig. 2.

Single-unit carotid chemoafferent nerve activity during (A) 21% O₂ (baseline) and (B) 5% O₂ for P7 rats after chronic treatment with vehicle (n= 20 units) or K252a (n= 15 units). Baseline discharge rates were not normally distributed and are therefore represented by box plots in panel A; horizontal lines in the box represent the 1st, 2nd (median), and 3rd quartiles, and the whiskers extend from the minimum to the maximum values. In panel B, values are mean $\pm$ SEM. No significant differences were detected between groups.

Fig. 3.

Hypoxic ventilatory response for P7 rats after receiving an acute injection of vehicle ($n = 15$) or K252a ($n = 15$). (A) Respiratory frequency, (B) tidal volume, and (C) minute ventilation were measured in 21% O_2 (baseline, BL) and in 12% O_2 . All values are reported as mean $\pm$ SEM. Where there was a significant drug $\times$ time interaction (panel A), $*$ $P < 0.05$ vs. vehicle-injected rats at the same time point, † $P < 0.05$ vs. BL within the same drug treatment group, and ‡ $P < 0.05$ vs. 1st minute of hypoxia within the same drug treatment group. Where only the main effect of time was significant (panels B and C), ^a $P < 0.05$ vs. BL and ^b $P < 0.05$ vs. the 1st minute of hypoxia.

Fig. 4.

Hypoxic ventilatory response for P7 rats after chronic treatment with vehicle (n= 18) or K252a (n = 20). (A) Respiratory frequency, (B) tidal volume, and (C) minute ventilation were measured in 21% O₂ (baseline, BL) and in 12% O₂. All values are reported as mean ± SEM. Ventilation changed significantly over time: ^a $P < 0.05$ vs. BL and ^b $P < 0.05$ vs. the 1st minute of hypoxia.