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Anatomic changes in multiple brainstem nuclei after incremental, near-complete neurotoxic destruction of the pre-Bötzinger Complex in adult goats

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ABSTRACT

Abrupt, bilateral destruction of the pre-Bötzinger Complex (preBötC) leads to terminal apnea in unanesthetized goats and rats. In contrast, respiratory rhythm and pattern and arterial blood gases in goats during wakefulness and sleep are normal after incremental (over a month) destruction of >90% of the preBötC. Here, we tested the hypothesis that the difference in effects between abrupt and incremental destruction of the preBötC are a result of time-dependent plasticity, which manifests as anatomic changes at sites within the respiratory network. Accordingly, we report data from histological analyses comparing the brainstems of control goats, and goats that had undergone bilateral, incremental, ibotenic acid (IA)-induced preBötC lesioning. A major focus was on the parafacial respiratory group/retrotrapezoid nucleus (pFRG/RTN) and the pontine respiratory group (PRG), which are sites thought to contribute to respiratory rhythmogenesis. We also studied the facial (FN), rostral nucleus ambiguus (NA), medullary raphé (MRN), hypoglossal (HN), and the dorsal motor vagal (DMV) nuclei. Neuronal counts, count region area (mm^2), and neuronal densities were calculated using computer-assisted analyses and/or manual microscopy to compare control and preBötC-lesioned animals. We found that within the ventral and lateral medulla 2 mm rostral to the caudal pole of the FN (presumed pFRG/RTN), there were 25% and 65% more ($P < 0.001$) neurons, respectively, in preBötC-lesioned compared to control goats. Lesioned goats also showed 14% and 13% more ($P < 0.001$) neurons in the HN and medial parabrachialis nucleus, but 46%, 28%, 7%, and 17% fewer ($P < 0.001$) neurons in the FN, NA, DMV, and Kölliker-Fuse nuclei, respectively. In the remaining sites analyzed, there were no differences between groups. We conclude that anatomic changes at multiple sites within the respiratory network may contribute to the time-dependent plasticity in breathing following incremental and near-complete destruction of the preBötC.

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1. Introduction

The brainstem sites and mechanisms of respiratory rhythm and pattern generation remain controversial. Over the past century, three sites have been proposed to individually or collectively mediate this function. The three sites are (1) the pneumotaxic center or pontine respiratory group (PRG), which includes the Kölliker-Fuse nucleus (KFN), and the medial and lateral parabrachial nuclei (MPBN and LPBN: Lumsden, 1923; St John and Bledsoe, 1985), (2) the parafacial respiratory group/retrotrapezoid nucleus (pFRG/RTN: Onimaru and Homma, 2003), and (3) the pre-Bötzinger Complex (preBötC) (Smith et al., 1991). Recently, it was hypothesized that a "core circuit of components that constitute the neural machinery for respiratory rhythm and shaping inspiratory and expiratory motor patterns" includes the preBötC and surrounding medullary nuclei with necessary inputs from other medullary and pontine sites (Smith et al., 2009). Supporting this concept are findings in awake goats (Wenninger et al., 2004b) and rats (Tan et al., 2008) demonstrating that abrupt destruction of the preBötC causes cessation of phasic diaphragm activity and terminal apnea.

In contrast, when the presumed preBötC is destroyed incrementally over a month by progressively increasing the volume injections of a neurotoxin through microtubules chronically implanted into the preBötC, breathing rhythm and pattern, and arterial blood gases fully recover after acute perturbations in goats during wakefulness and sleep (Krause et al., 2009b). These findings suggest that incremental preBötC lesioning allows for a time-dependent plasticity within the respiratory network, consistent with an "emerging view that the brainstem respiratory network has rhythmogenic capabilities at multiple hierarchical levels, which allows flexible, state-dependent expression of different rhythmo-

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genic mechanisms under different physiological and metabolic conditions and enables a wide repertoire of respiratory behaviors" (Smith et al., 2009). This concept predicts that the time-dependent plasticity after preBötC lesions would occur at another brainstem site with putative rhythmogenic capabilities, which includes the PRG and/or the pFRG/RTN. Accordingly, we report herein data addressing the hypothesis that with incremental destruction of the preBötC, anatomic changes occur in the PRG and/or the pFRG/RTN. In addition, to determine whether anatomic changes would be restricted to sites thought to contribute to rhythmogenesis, we also determined whether anatomic changes occurred at other sites including the facial (FN), rostral nucleus ambiguus (NA), medullary raphé (MRN), hypoglossal (HN), and the dorsal motor vagal (DMV) nuclei.

2. Methods

Data reported herein were obtained from 13 adult goats, 7 of which were euthanized upon arrival at the laboratory (referred to as controls hereafter). The remaining 6 goats underwent two surgical procedures for instrumentation required to determine the effect of incremental destruction of the preBötC on respiratory rhythm and pattern (Wenninger et al., 2004a,b; Krause et al., 2009b). We have reported previously for these goats, the physiological data obtained under awake and sleeping states and the post mortem histological data documenting destruction of the presumed preBötC and surrounding region (Krause et al., 2009b). Prior to initiation of all studies, the protocols and procedures were reviewed and approved by the Medical College of Wisconsin Institutional committee for care and use of research animals.

All goats were euthanized (intravenous B-euthansia), before being perfused with physiological buffer solution (PBS) and fixed with 4% paraformaldehyde in phosphate buffer. The brainstems were excised and placed in 4% paraformaldehyde in PBS for 24 h, and then in 20% and 30% sucrose solutions, sequentially, before freezing. The brainstem of 1 goat was frozen sectioned (25 μm) in the sagittal plane and stained with cresyl violet (Nissl) to delineate the rostrocaudal orientation of respiratory nuclei in adult goats (Fig. 1A). The brainstems of the other goats were serially frozen sectioned (25 μm) transversely from obex to the superior colliculi (Fig. 1B–E), adhered to slides and dried. Serial sections were divided into four series, such that every fourth section (every 100 μm) was contained within a respective series, and a specific stain applied to each series. One series was stained with cresyl violet (Nissl) to profile the total number of neurons in each of the analyzed regions. A second series spanning the PRG region was immunostained with a primary antibody targeting muscarinic type-2 (M2) receptors (mouse anti-M2 (1:200); Millipore), and a biotinylated anti-mouse secondary (1:100), avidin-horseradish peroxidase complex (avidin-linked HRP) and diaminobenzidine (DAB; Vector ABC Elite, Vector Laboratories). These sections were used to identify the LPBN, MPBN, and KFN (Fig. 1B; (Bonis et al., 2010)). For all goats, a third series of sections from obex to +2 mm rostral to the caudal edge of FN were immunoprocessed for neurokinin-1 (NK1) receptors by complexing an anti-NK1 receptor primary antibody (1:5000; Sigma), an anti-mouse secondary, avidin-linked HRP and DAB. In 4 preBötC-lesioned goats, some sections from the fourth series were stained with hematoxylin and eosin from obex to 2 mm rostral to the caudal pole of FN to identify dead or dying neurons (Wenninger et al., 2001; Hodges et al., 2004). Finally, in 2 control and 2 experimental goats, some sections from the fourth tissue series were immunostained with antibodies targeting NeuN (1:1000; Millipore) from −2 mm to +2 mm from the caudal aspect of the facial nucleus (FN) to verify that the Nissl-stained cells were neurons.

2.1. Computer-assisted analyses

Initially we focused on the presumed pFRG/RTN region and the PRG. Thus, Nissl-stained tissue sections in the rostral medulla and rostral pons were imaged every 100 μm at 4× magnification utilizing a Nikon Eclipse E400 microscope (equipped with a SPOT Insight color camera and software) after Kohler alignment, flat field subtraction and white-balance correction. Approximately 20 image files per slide (.TIFF) were then photomerged in Adobe Photoshop, calibrated, and analyzed after importation into MetaMorph Offline v. 7.1.3.0 (Molecular Devices Corp., Sunnyvale, CA). The areas analyzed within the medullary image sequences spanned 2 mm caudal to, and 2 mm rostral to the caudal-most aspect of the FN. The FN was identified by the prominence of large (30–40 μm) motor neurons ∼1–2 mm from the ventral surface and ∼4–5 mm lateral from the midline as previously described in the goat (Dean et al., 1999). Within each photomerged image, the FN was outlined manually (Trace Region tool), the neurons were counted (Manually Count Objects tool), and the area was automatically calculated (Region Measurements tool; mm^2) within each outlined region. Vertical and horizontal lines were then drawn from the medial-most and dorsal-most borders of the FN from the ventral and lateral surfaces of the medulla to their point of intersection (Fig. 1D). A line bisecting the resultant rectangle divided the ventral and lateral count regions. The region ventral to the FN included the tissue from the medial-most borders of the FN to the ventral-most border of the FN, extending to the bisecting line and to the ventral medullary surface. In contrast, the region lateral to the FN included the tissue lateral to the bisecting line to the ventrolateral medullary surface, and dorsally to the dorsal-most border of the FN to the point where the horizontal line intersects with the dorsal-most border of FN.

As shown in Fig. 1A, beginning about 0.5 mm caudal to the FN and extending caudally, there is a distinct column of neurons at about the same dorsal–ventral plane as the FN. This column presumably is NA, which we outlined and quantified from 0.5 mm to 2.0 mm caudal to the FN. We are uncertain of the proper nomenclature for the diffuse neurons in the 0.5 mm "gap" between FN and NA, but this area was also outlined and quantified and grouped with NA. The regions ventral and lateral to NA were outlined and quantified as described above for the regions ventral and lateral to FN.

The sub-nuclei of the pontine nuclei were imaged and quantified using a similar computer-assisted technique. The rostrocaudal pontine landmark was defined as the region of peak number of Nissl-stained neurons within the KFN (pKFN). Along with the KFN, the MPBN and LPBN, which stain positive for M2 receptor (Bonis et al., 2010), encircle the superior cerebellar peduncle (SCP). Thus, the count region for each was determined using the anti-M2 receptor antibody staining (see above). The SCP was bisected (Fig. 1B), and the MPBN and LBPN outlined in "half-moon" shapes to encapsulate the boundaries of M2-stained tissues. The outlined regions were then transferred to adjacent Nissl-stained sections for quantification. Similarly, the outline of the KFN was determined using M2-staining and transferred to the adjacent Nissl-stained sections.

Two laboratory staff members completed the computer-assisted analyses, with excellent agreement between the results derived from a repeat analysis of identical images. The staff was aware of the experimental treatment in the lesion group, but strictly adhered to the objective quantification methods outlined above as not to bias results.

2.2. Manual microscopic analyses

We also performed neuron counts manually with a microscope (Olympus BX-40) fitted with a 10× eyepiece micrometer. We employed this counting method due to variation in the background

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