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INHIBITION OF THE PONTINE KÖLLIKER-FUSE NUCLEUS ABOLISHES EUPNEIC INSPIRATORY HYPOGLOSSAL MOTOR DISCHARGE IN RAT

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Abstract—The pontine Kölliker-Fuse nucleus (KF) has established functions in the regulation of inspiratory–expiratory phase transition and the regulation of upper airway patency via laryngeal valving mechanisms. Here we studied the role of the KF in the gating and modulation of eupneic hypoglossal motor activity (HNA) using the *in situ* perfused brainstem preparation, which displays robust inspiratory HNA. Microinjection of glutamate into the KF area triggered complex and often biphasic modulation (excitation/inhibition or inhibition/excitation) of HNA. Subsequent transient pharmacological inhibition of KF by unilateral microinjection of GABA-A receptor agonist isoguvacine reduced HNA and while bilateral microinjections completely abolished HNA. Our results indicate that mixed and overlapping KF pre-motor neurons provide eupneic drive for inspiratory HNA and postinspiratory vagal nerve activity. Both motor activities have important functions in the regulation of upper airway patency during eupnea but also during various oro-pharyngeal behaviors. These results have potential implications in the contribution of state-dependent modulation of KF hypoglossal pre-motor neurons during sleep–wake cycle to obstructive sleep apnea.

Key words: sleep apnea, parabrachial, upper airway, respiratory rhythm generation, breathing, pons.

INTRODUCTION

Efficient ventilation of the lungs requires the temporally coordinated contraction of pump muscles (e.g. diaphragm and abdominal internal obliques) and the upper airway muscles. The pump muscles generate pressure gradients that move air in and out of the lower airways whereas the upper airway muscles modulate airflow by determining upper airway patency and dynamically adapting airway resistance (Widdicombe, 1982; Bartlett, 1986; Sant'Ambrogio et al., 1995; Dutschmann and Dick, 2012; Smith et al., 2013). Spinal motor pools located in the cervical and thoracic ventral horn innervate the major pump muscles, while the cranial motor nuclei located in brainstem exclusively target upper airway muscles such as the tongue or laryngeal abductors (dilators) and adductor (constrictor) muscles. Rhythmic and sequential synaptic drive for individual pump or upper airway motoneurons is provided by pre-motor neuron groups, which are anatomically organized in a ponto-medullary respiratory central pattern generator that generates the three phase respiratory motor pattern (inspiration, postinspiration and expiration, see Alheid et al., 2004; Rybak et al., 2004, 2007; Smith et al., 2007, 2013; Abdala et al., 2009; Dutschmann and Dick, 2012). The location and electrophysiology of bulbo-spinal pre-motor neurons underlying the control of inspiratory and expiratory pump muscle motoneurons within the ventral horn of cervical (phrenic), thoracic (intercostal) and lumbar (abdominal) spinal cord has been thoroughly investigated (Kalia, 1981; Richter, 1982; Feldman and Ellenberger, 1988; Ezure, 1990; Monteau and Hilaire, 1991; Bianchi et al., 1995). Contrarily, the activity and physiological significance of pre-motor circuits that control upper airway patency and resistance during eupneic breathing is only recently receiving increased attention (Dutschmann and Paton, 2002; Alheid et al., 2004; Rybak et al., 2004, 2007; Dutschmann and Herbert, 2006; Smith et al., 2007; see Dutschmann and Dick, 2012). Among all upper airway muscles the hypoglossal pre-motor circuits of the tongue (Fregosi and Fuller, 1997; Kubin, 2001; Kubin and Fenik, 2004; Gestreau et al., 2005; Horner 2008) appears to be investigated best, because of the clinical relevance of its state-dependent neuro-modulation as a causative factor in obstructive sleep apnea (Remmers et al., 1978; Dempsey et al., 2010; Bailey, 2011). However, the significance of hypoglossal inspiratory and pre-inspiratory motor activity during different forms of breathing (e.g. forced breathing, eupnea, gasping) is not without controversy (St-John, 1983; Leiter and St-John, 2004; St-John and Leiter, 2009).

Anatomical studies show that the pontine Kölliker-Fuse nucleus (KF) is a major source of descending projections that target the hypoglossal motor nucleus in the caudal medulla oblongata (Luo et al., 2001; Roda et al., 2004; Ezure and Tanaka, 2006; Yokota et al., 2011; Song et al., 2012) as well as the lateral hypoglossal pre-motor area (Gestreau et al., 2005; see Fig. 3; Dutschmann and Dick, 2012). However, studies that investigate the physiological significance of these

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Abbreviations: aCSF, artificial cerebrospinal fluid; KF, Kölliker-Fuse; PNA, phrenic nerve activity; VNA, vagal nerve activity.

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anatomical connections of the KF are surprisingly sparse (Kuna and Remmers, 1999; see Gestreau et al., 2005).

In the present study, we hypothesized that the KF contributes to significant modulation of the hypoglossal motor output given its well-described role modulating another upper airway motor output, the larynx (Dutschmann and Herbert, 2006). This was investigated in the *in situ* perfused brainstem preparation of juvenile rat, which is decerebrate and thus circumvents the depressive effect of anesthesia on central respiratory-related neurons, including those that control upper airway muscles (Nishino et al., 1985; Sun et al., 2011). We used chemical stimulation and transient pharmacological inhibition to reveal that the KF provides the eupneic inspiratory motor drive for the hypoglossal motor circuit. Moreover, chemical stimulation of the KF triggers complex (usually biphasic) modulation of HNA, indicating that the KF might be a hypoglossal pre-motor population that can be utilized in the mediation of non-respiratory hypoglossal motor activities.

EXPERIMENTAL PROCEDURES

All experimental procedures were performed in accordance with the Australian code of practice for the care and use of laboratory animals. The ethics committee of the Florey Institute for Neuroscience and Mental Health at Melbourne approved the study (AEC 12-084).

Perfused-brainstem preparation

The experiments were performed using the arterially perfused brainstem preparation (Paton, 1996). Juvenile rats at postnatal days 18–24 were anaesthetized deeply in an atmosphere saturated with isofluorane (1-chloro-2,2,2-trifluoroethyl-difluoromethylether). Once the animal failed to respond to noxious pinch to the tail or a hind paw, the whole animal was transected below the diaphragm, and the rostral half was transferred into ice-cold (5 °C) artificial cerebrospinal fluid (aCSF) gassed with carbogen (95% O_2 and 5% CO_2), decerebrated at the pre-collicular level and cerebellectomized. The lungs and heart were removed (see Dutschmann et al., 2009). The left phrenic nerve was isolated and cut at the level of the diaphragm. The cervical vagal and hypoglossal cranial motor nerves were isolated and also cut for recording. The descending aorta was isolated from the ventral surface of the spinal column at the lumbar level. These initial procedures took approximately 15–25 min. The preparation was then transferred to a recording chamber. The descending aorta was cannulated and perfused using a peristaltic pump (Watson-Marlow, Wilmington, USA) with carbogen-gassed aCSF at 31 °C. The aCSF also contained ficoll (1.25%, Sigma–Aldrich, Steinheim, Germany) to maintain colloid-osmotic pressure. The perfusate contained in mM: NaCl 125, KCl 3, KH_2PO_4 1.25, $CaCl_2$ 2.5, $MgSO_4$ 1.25, $NaHCO_3$ 25, D-glucose 10 and 1.25% ficoll. The osmolarity of the perfusate was 300 ± 10 mosmol l^{-1} and the pH was 7.35 ± 0.05 when gassed with carbogen. The perfusate was filtered and passed through bubble traps to remove gas bubbles. The perfusate leaking from the preparation was collected and recirculated after re-oxygenation. Rhythmic contractions of respiratory muscles returned within 3–5 min after the onset of reperfusion. Respiratory-related movements were abolished by vecuronium bromide (0.3 μg ml^{-1}, Clifford Hallam Healthcare).

Nerve recording

As an index of respiratory activity, we recorded the phrenic nerve activity (PNA; inspiratory) and vagal nerve activity (VNA; inspiratory/postinspiratory) and hypoglossal nerve activity (HNA; inspiratory/pre-inspiratory) via suction electrodes in all experiments. Nerve activities were amplified (differential amplifier DP-311, Warner Instruments, Hamden, USA), digitized (PowerLab/16SP ADInstruments, Sydney, Australia) and stored on a computer using Chart v7.0/s software (ADInstruments). In each preparation, PNA was used to fine-tune the perfusion in order to obtain a ramping envelope of the integrated PNA with discharge duration of 1 s or shorter. Flow rates (18–22 ml min^{-1}) and perfusion pressures (40–70 mmHg) can vary.

Experimental protocol microinjections

In all experiments multi-barreled were used to perform local microinjections. The individual barrels we filled with glutamate (10 mM), isoguvacine (10 mM, GABA receptor agonist, Sigma–Aldrich) and rhodamine beads in aCSF (2%). The injected volume ranged 20–50 nl and was monitored by the movement of the liquid meniscus examined by a microscope equipped with a reticule. All pontine microinjection sites were first characterized by using glutamate microinjections to evoke respiratory modulation (e.g. a brief postinspiratory apnea). After the initial physiological characterization, the GABA-A receptor agonist isoguvacine was injected at the same locus, followed by rhodamine beads (Sigma–Aldrich) for later histological verification. The pipette was then immediately moved to the same co-ordinates on the contralateral side, and isoguvacine and dye were injected sequentially. To minimize elapsed time and washout of isoguvacine, glutamate microinjections were not performed on the contralateral side. Following the stimulation protocols, the brainstem was removed and fixed in 4% paraformaldehyde for histological analysis.

Histological analysis

After fixation of brainstems for several days in 4% paraformaldehyde–20% sucrose, serial 50-μm cryosections were prepared using a cryostat microtome. The locations of microinjections were documented on schematic drawings of coronal sections showing the parabrachial complex of the dorsolateral pons.

Data analysis

Basic breathing parameters such as duration of inspiration (T_i), postinspiration (T_{pi}) and stage 2