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DIENCEPHALIC AND MESENCEPHALIC INFLUENCES ON PONTO-MEDULLARY RESPIRATORY CONTROL IN NORMOXIC AND HYPOXIC CONDITIONS: AN *IN VITRO* STUDY ON CENTRAL NERVOUS SYSTEM PREPARATIONS FROM NEWBORN RAT

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Abstract—We investigated the effects of the diencephalon and mesencephalon on the central respiratory drive originating from ponto-medullary regions in normoxic and hypoxic conditions, using central nervous system preparations from newborn rats. We used two approaches: 1) electrophysiological analysis of respiratory frequency and the amplitude of inspiratory C4 activity and 2) immunohistochemical detection of Fos protein, an activity-dependent neuronal marker. We found that, in normoxic conditions, the mesencephalon moderated respiratory frequency, probably by means of an inhibitory effect on ventral medullary respiratory neurons. Diencephalic inputs restored respiratory frequency. Moreover, O_2-sensing areas in the diencephalon (caudal lateral and posterior hypothalamic areas) and mesencephalon (ventrolateral and dorsolateral periaqueductal gray) seem to increase the amplitude of respiratory bursts during adaptation of the central respiratory drive to hypoxia. In contrast, decrease in respiratory frequency during hypoxia is thought to be mediated by a cluster of ventral hypothalamic neurons. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: caudal hypothalamus, central O_2 detection, low O_2 level, Fos protein.

The breathing of mammals is controlled by a neuronal network in the ponto-medullary region (Bianchi et al., 1995; Richter and Spyer, 2001). The homeostasis of the organism is maintained by continually adjusting central respiratory drive in response to peripheral and central inputs (Von Euler, 1986; Bianchi et al., 1995). Breathing is irregular in newborn mammals, with a mixture of apneic, bradypneic, eupneic and tachypneic episodes (Mortola, 1984; Hilaire and Duron, 1999). Apnea and bradypnea induce hypoxia, resulting in respiratory depression characterized in the newborn by a transitional increase in ventilation followed by a severe decrease (Blanco et al., 1984; Neubauer et al., 1990). The initial increase in respiration results mainly from an increase in the activity of peripheral O_2-chemoreceptors (Darnall et al., 1991; Fung et al., 1996), and involves structures located in the caudal part of the hypothalamus (Waldrop and Porter, 1995). The mechanisms responsible for the secondary decrease—known as hypoxic respiratory depression (HRD)—operate in the central nervous system (CNS) (Vizek et al., 1987) in the brainstem areas (Dillon et al., 1991; Mitra et al., 1993; Okada et al., 1998; Bodineau et al., 2000a).

It has been suggested that the central mechanisms involved in the hypoxic respiratory response depend on the existence of O_2-sensing neurons. Such neurons have been described in the rostral ventrolateral medulla (Nolan and Waldrop, 1993; Sun and Reis, 1994; Kawai et al., 1999; Mazza et al., 2000; Neubauer and Sunderram, 2004), an area involved in HRD (Dillon et al., 1991; Mitra et al., 1993). In ponto-medullary–spinal cord (PMS) preparations, which contain these O_2-sensing neurons acting in absence of peripheral chemoreceptors influence, the adaptation of the central respiratory drive to hypoxia is characterized by a respiratory depression (Kawai et al., 1995; Okada et al., 1998; Bodineau et al., 2000a, 2001, 2003, 2004; Kato et al., 2000; Cayetanot et al., 2001). These results suggest that medullary O_2-sensing neurons play a key role in the development of HRD. O_2-sensing neurons have also been found in the caudal part of the hypothalamus (Dillon and Waldrop, 1992; Horn and Waldrop, 1997; Horn et al., 1999) and in the periaqueductal gray (PAG; Kramer et al., 1999). As the caudal part of the hypothalamus is involved in the increase in respiratory drive in response to hypoxia (Waldrop and Porter, 1995), hypothalamic O_2-sensing neurons may have an excitatory effect on respiration. However, this hypothesis remains to be tested. Although the PAG has been shown to influence respiratory control (Horn and Waldrop, 1998), no data are available concerning the possible influence of O_2-sensing neurons on hypoxic respiratory adaptation. We therefore carried out this study to characterize further the contribu-

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Abbreviations: ANOVA, analysis of variance; BS, brainstem–spinal cord; ∫C4, amplitude of the integrated C4 burst activity; cLH, caudal part of the lateral hypothalamic area; CNS, central nervous system; CSF, mock cerebrospinal fluid; DBS, diencephalon–brainstem–spinal cord; DLPAG, dorsolateral part of the periaqueductal gray; FLI, Fos-like immunoreactive; HRD, hypoxic respiratory depression; LPB, lateral subnucleus of the parabrachial nucleus; MPB, medial subnucleus of the parabrachial nucleus; Pa, paraventricular nucleus of the hypothalamus; PAG, periaqueductal gray; PH, posterior hypothalamic area; PMS, ponto-medullary–spinal cord; PP, parapyramidal group; Rf, respiratory frequency; Rf×∫C4, index of central respiratory output; RTN, retrotrapezoid nucleus; VLM, ventrolateral reticular nucleus of the medulla; VLPAG, ventrolateral part of the periaqueductal gray; VRG, ventral respiratory group; VZ, ventral zone of the hypothalamus.

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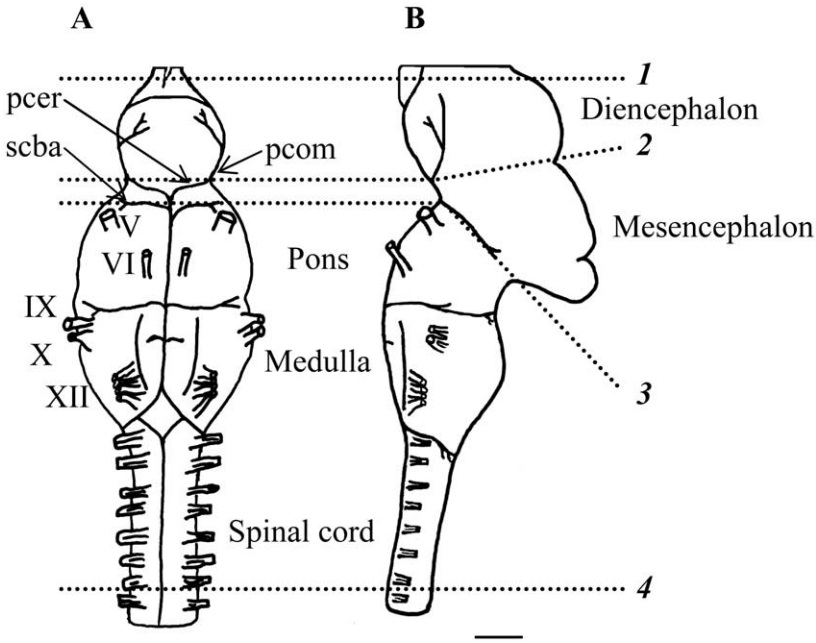

Fig. 1. Schematic diagram showing the various levels of rostrocaudal transection on ventral (A) and lateral (B) views of CNS preparations from newborn rats. Cuts were made at: *1*, the rostral extremity of the optic chiasm for the DBS preparations; *2*, the level of the intersection between posterior cerebral (pcer) and posterior communicating arteries (pcom) for the BS preparations; *3*, rostral to the fifth cranial nerves at the level of the superior cerebellar arteries (scba) and caudal edge of inferior colliculi for the PMS preparations; *4*, between the seventh and eighth cervical spinal roots. Scale bar=2 mm.

tion and role of the caudal hypothalamic and PAG mechanisms involved in hypoxic respiratory adaptation in the newborn. To achieve this end, we needed to analyze hypoxic respiratory adaptation in the absence of inputs from peripheral O_2-chemoreceptors. We used three types of *in vitro* deafferented CNS preparations differing in rostral extension: PMS, brainstem–spinal cord (BS) and diencephalon–BS (DBS) preparations. In parallel, we analyzed respiratory parameters and *c-fos* expression to identify areas presenting changes in neuronal activity in response to hypoxia. Indeed, as *c-fos* expression is dependent on neuronal activity (Morgan et al., 1987), Fos protein detection is classically used as a marker of the central pathway involved in specific physiological responses (Erickson and Millhorn, 1991; Horn et al., 2000; Bodineau and Larnicol, 2001; Okada et al., 2002; Saadani-Makki et al., 2004).

EXPERIMENTAL PROCEDURES

Experiments were performed on 114 CNS preparations from newborn (0–3 days old) Sprague–Dawley rats (Janvier, Le Genest St Isle, France). The regional animal ethics committee approved the experimental protocol, which conformed to the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Surgery

Newborn rats were placed under deep anesthesia and PMS (n=49), BS (n=32) and DBS (n=33) preparations were dissected out as previously described (Suzue, 1984; Okada et al., 1998). The three types of preparation differed only in the rostral cut (Fig. 1). This cut was made rostral to the fifth cranial nerves at the level of the superior cerebellar arteries and caudal edge of inferior colliculi for PMS (Suzue, 1984), at the level of the intersection between posterior cerebral and posterior communicating arteries for BS (Okada et al., 1998) and at the level of the rostral extremity of the optic chiasm for DBS (Okada et al., 1998) preparations. In all cases, the caudal cut was made between the seventh and eighth cervical spinal roots (Fig. 1) and the cerebellum was removed. The preparations were placed in a recording chamber (volume=4 ml) with the ventral surface upward. They were continuously superfused, at 26 °C at a rate of 7.5 ml/min, with a control mock cerebrospinal fluid (control-CSF; 130.0 mM NaCl, 5.4 mM KCl, 0.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 26.0 mM $NaHCO_3$, and 30.0 mM D-glucose) saturated with O_2 and adjusted to pH 7.4 by bubbling with 95% O_2, 5% CO_2.

Experimental protocols

The hypoxia test was performed by replacing the control-CSF by a solution of the same composition bubbled with 95% N_2, 5% CO_2 (hypoxic-CSF). In this condition, the central chemosensitivity to CO_2 or pH was not affected. We carried out two experimental protocols: an analysis of respiratory parameters (protocol 1) and an analysis of *c-fos* expression (protocol 2).

Protocol 1: respiratory parameter measurement. After completion of the surgical procedure, PMS (n=25), BS (n=16) and DBS (n=17) preparations were superfused with

Control-CSF for 1 h 30 min;

Control-CSF (30 min) → hypoxic-CSF (30 min) → control-CSF (30 min).

The electrical activity of a C4 ventral root was recorded using a suction electrode, filtered (10–3000 Hz), amplified (×5000), integrated (time constant 100 ms) and digitized through a spike 2 data analysis system (CED, Cambridge, UK), with a sampling frequency of 1010 Hz. The respiratory frequency (Rf) was defined as the frequency of the bursts recorded from a C4 ventral root, as described by Suzue (1984). As previously reported, Rf×∫C4 (am-