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ScienceDirect
Neuroscience Research 56 (2006) 322–331
Neuroscience Research
www.elsevier.com/locate/neures

# Involvement of medullary GABAergic and serotonergic raphe neurons in respiratory control: Electrophysiological and immunohistochemical studies in rats

Ying Cao [a,b], Kiyoji Matsuyama [a], Yutaka Fujito [a,*], Mamoru Aoki [a,1]

[a] *Department of Physiology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan*
[b] *Institute of Physiology, Shandong University School of Medicine, Jinan, PR China*

Received 16 May 2006; accepted 2 August 2006
Available online 8 September 2006

**Abstract**

In the present study we first examined the possible involvement of the putative neurotransmitters γ-aminobutyric acid (GABA) and serotonin (5-HT) in raphe-induced facilitatory or inhibitory effects on the respiratory activity of rats. Secondly, we investigated the possibility of spinal projections of GABAergic and serotonergic neurons from the medullary raphe nuclei to the phrenic motor nucleus (PMN). We observed that an intravenous (i.v.) injection of (+)-bicuculline, a $GABA_A$ receptor antagonist, significantly reduced respiratory inhibition induced by electrical stimulation of the raphe magnus (RM) or the raphe obscurus (RO). On the other hand, an i.v. injection of methysergide, a broad-spectrum 5-HT receptor antagonist, significantly reduced the respiratory facilitation induced by electrical stimulation of the raphe pallidus (RP) or RO. By using a combined method of retrograde tracing with Texas Red injected into the PMN region at segments C4 and C5 and immunohistochemical labeling, we observed that glutamic acid decarboxylase (GAD; a GABA synthesizing enzyme) immunopositive and Texas Red double labeled neurons were predominantly localized in the RM, and additionally in the RO. However 5-HT immunopositive and Texas Red double-labeled neurons were predominantly localized in the RP, and additionally in the RO and RM. These findings suggest that RM-, or RO-induced inhibitory effects, are transmitted, at least in part, to the PMN via a direct GABAergic descending pathway. The RP-, or RO-induced facilitatory effects in rats however, are transmitted via a serotonergic descending pathway.


*Keywords:* Raphe nuclei; GABA; Serotonin; Bicuculline; Methysergide; Respiration

## 1. Introduction

It is well known that in mammals the medullary raphe nuclei are involved in respiratory control. Previous studies by others, and by us, have shown that electrical or chemical stimulation of the raphe magnus (RM) produces strong inhibitory effects on respiratory activity (Lalley, 1986a; Wang et al., 1988; Aoki and Nakazono, 1992; Cao et al., 2006). Stimulation of the raphe pallidus (RP) however, produces facilitatory effects on respiratory activity (Lalley, 1986a; Cao et al., 2006). Our recent study has shown that stimulation of the raphe obscurus (RO) induced either an inhibitory or a facilitatory effect depending on the stimulation sites (Cao et al., 2006). Although it is well known that the caudal raphe nuclei contain serotonergic (5-HT) neurons, other neurons containing different neurotransmitters or modulators, such as gamma-aminobutyric acid (GABA), glycine, substance P, thyrotropin releasing hormone (TRH), leucin-enkephalin (LEU-enk), and methionine-enkephalin (MET-enk), have also been observed (Kachidian et al., 1991; Holmes et al., 1994; Stamp and Semba, 1995). Previous studies have provided evidence that GABA and 5-HT are the main putative transmitters in respiratory control (Fuller, 1980; Mueller et al., 1982; Chebib and Johnston, 1999). It has also been suggested that in cats GABA is involved in RM-induced respiratory inhibition (Aoki and Nakazono, 1992; Aoki et al., 1995; Song and Aoki, 2001), whereas 5-HT is involved in RP-induced respiratory facilitation (Lalley, 1986b; Holtman et al., 1987; Schmid et al., 1990; Haxhiu et al., 1998).

Although previous studies, performed almost exclusively on cats, have demonstrated that GABA and 5-HT are the main

* Corresponding author. Tel.: +81 11 611 2111; fax: +81 11 644 1020.
*E-mail address:* fujito@sapmed.ac.jp (Y. Fujito).
[1] Present address: Department of Physical Therapy, Faculty of Human Science, Hokkaido Bunkyo University, Eniwa, Hokkaido 061-1408, Japan.

0168-0102/$ – see front matter 
doi:10.1016/j.neures.2006.08.001

putative transmitters in the caudal raphe nuclei responsible for respiratory control, there is little information about the putative transmitters for raphe-induced respiratory facilitation and inhibition in rats.

In the present study therefore, we first attempted to determine in rats the putative transmitters responsible for raphe-induced inhibitory and facilitatory effects on respiratory activity. We examined the effects of a specific $GABA_A$ receptor antagonist, (+)-bicuculline on RM-induced respiratory inhibition, and the effects of a broad spectrum 5-HT receptor antagonist, methysergide, on RP-induced respiratory facilitation. Secondly, and based on these pharmacological studies, we attempted to determine if there are any spinal projections of GABAergic and 5HT neurons to the phrenic motor nucleus (PMN). For this purpose, we used a combined method of retrograde tracing and immunohistochemical labeling. A part of the present data has been previously published in abstract form (Cao et al., 2004, 2005).

## 2. Materials and methods

A total of 19 (13 for the electrophysiological study and 6 for the immunohistochemical study) adult Wistar rats of either sex weighing 300–500 g were used. All of the surgical and animal care methods conformed to the Guidelines for the Use of Animals of the International Brain Research Organization. The experimental protocol was approved by the Sapporo Medical University Animal Care and Use Committee (Sapporo, Japan).

### 2.1. Electrophysiology

#### 2.1.1. Surgery

In all animals ($n = 13$) anesthesia was induced with halothane, and this was followed by an intramuscular injection of ketamine (80–110 mg/kg) in combination with an intraperitoneal injection of xylazine (2–4 mg/kg). Surgical anesthesia was confirmed if a strong pinch of a hind paw did not evoke a withdrawal reflex. Atropine sulfate (0.1 mg/kg) was given hypodermically to reduce the animal's salivation. The level of anesthesia was carefully evaluated throughout the experiments. A supplemental dose of ketamine (30 mg/kg h) was given if any noxious mechanical stimulus elicited a withdrawal reflex or increased the respiratory frequency. The body temperature of each animal was monitored via a rectal thermometer and maintained at 36–37 °C with an external heating device (a heating pad or heating lamp). The femoral vein was cannulated for intravenous administration of solutions and drugs. The head of the rat was fixed in a stereotaxic apparatus (Paxinos and Watson, 2005) and the dorsal surface of the brainstem was exposed by occipital craniotomy and by removing the posterior portion of the cerebellum. Bleeding was controlled and kept to a minimum by using cotton-tipped applicators, bone wax, gel foam and cautery. All of the exposed tissues were covered with dimethyl polysiloxane oil.

During the entire experiment each animal breathed spontaneously and the end-tidal $CO_2$ was monitored with an infrared $CO_2$ analyzer (1H26, NEC-Sanei, Tokyo, Japan). When an animal was properly anesthetized the end-tidal $CO_2$ was measured as 5.0–5.7%. The animal's chest was suspended with vertebral clamps attached to the C7 and L3 superior spinous processes and to the stereotaxic frame.

#### 2.1.2. Stimulation and drug injection

Monopolar tungsten electrodes (shaft diameter 150 μm, impedance 2–7 MΩ) which were insulated except for the tip were used for stimulation. The electrodes were inserted into the medulla oblongata, by a dorsal approach, at a forward angle of 20° from the vertical and on the midline. The electrode positions for stimulating the caudal raphe nuclei (RM, RP and RO) were sterotaxically adjusted by using the obex as the reference point (Cao et al., 2006). Train pulse stimuli (100 Hz, 100–200 pulses, 0.2 ms pulse width, 10–30 μA) via a digital stimulator (PG4000, Neuro Data Instruments, New York, NY, USA) were applied for 4–6 times at intervals of 10–15 s (Lalley, 1986a; Aoki and Nakazono, 1992; Lalley et al., 1997; Cao et al., 2006). The respiratory movements were measured with an abdominal pneumograph. The pneumogram signals (bandpass 0.5–300 Hz) and the stimulation pulses were stored on a computer with Powerlab software (AD instruments, Mountain View, CA, USA).

The peak amplitude and the peak-to-peak intervals (from which the respiratory frequency was calculated) of 6–10 successive respiratory movements before electrical stimulation were averaged. The same variables of the respiratory movements, but during the stimulation period (1 or 2 s) from 3 to 5 stimulation trials, were then pooled and averaged. The stimulation effects were evaluated as the ratios (percentages) of the values (amplitude and frequency) during the stimulation to those before the simulation. The drug effects were quantified by comparing the stimulation effects after the drug application with those before the drug application.

Student's *t*-test and paired *t*-test were used for statistical analysis of the data, with a significance level of $P < 0.05$. All of the values are expressed as the mean ± S.E.

#### 2.1.3. Intravenous drug administration

The following two drugs were intravenously administrated: (+)-bicuculline (MP Biochemicals, Aurora, OH, USA, 0.2 mg/kg) as a $GABA_A$ receptor antagonist and methysergide (Sigma, St. Louis, MO, USA, 1.5 mg/kg) as a 5-HT receptor antagonist. All of the chemicals were dissolved in physiological saline with the following concentration: (+)-bicuculline, 0.25 mg/ml; methysergide, 1.5 mg/ml.

#### 2.1.4. Histological identification of stimulation sites

At the end of each experiment the stimulation sites were marked by passing DC current (cathodal, 20 μA for 30 s) through the electrodes. The animals were killed by an intraperitoneal injection of a lethal dose of pentobarbital (100 mg/kg). After overnight fixation in 50% formalin the brainstem was removed and cut into serial transverse, or sagittal, 50 μm sections on a cryotome. The sections were then mounted on gelatin-coated glass slides, stained with 1% cresyl violet, dehydrated and cover slipped. All stained sections of the brainstem were examined under a light microscope. The location of each of the effective stimulation sites was determined with the aid of a stereotaxic atlas of the rat brain (Paxinos and Watson, 2005).

### 2.2. Immunohistochemistry

#### 2.2.1. Tracer injections in the cervical cord and colchicine injections in the brainstem

Experiments were performed on six adult rats of either sex weighing 300–400 g. Each animal was initially anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). A laminectomy was performed to expose the cervical segments (C4 and C5) of the spinal cord, and the dura mater was cut open. The rat was then fixed in a stereotaxic apparatus using nontraumatic ear bars. A glass micropipette (external tip diameter ~30 μm) was connected to a nanoliter pump (Nanoliter 2000, WPI, Sarasota, FL) which was mounted on a micromanipulator (SM-11, Narishige, Tokyo, Japan). The pipette was filled with a retrograde fluorescent tracer consisting of a 10% solution of dextran Texas Red dissolved in saline (3000 MW; Molecular Probe Inc., Eugene, OR). Although phrenic motoneurons are distributed in the ventral portion of the ventral horn at segments C3–C6, they are concentrated at segments C4 and C5 (Goshgarian and Rafols, 1981; Dobbins and Feldman, 1994). Therefore, in this study, the injection pipette was introduced vertically into the ventral portion of the left ventral horn at segments C4 and C5, i.e. 0.5–0.8 mm lateral to the midline and 1.8–2.0 mm from the dorsal surface. In each animal, injections of 200 nl of tracer (40 nl × 5) were made at five sites at 0.5 mm intervals along the spinal neuraxis. After the injections the muscles and skin were sutured and closed. None of the animals exhibited any abnormal behavior during their recovery.

In all of the animals with the retrograde tracer injections colchicine was injected to enhance the perikaryal immunostaining of GAD or 5-HT containing neurons in the brainstem. One week after the tracer injections the animals were anesthetized with pentobarbital (50 mg/kg, i.p.) and then fixed in a stereotaxic apparatus. A small craniotomy was made in the calvarium to expose the dorsal surface of the cerebellum and brainstem. A glass micropipette (tip diameter