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INHIBITORY CONTROL OF ASCENDING GLUTAMATERGIC PROJECTIONS TO THE LAMPREY RESPIRATORY RHYTHM GENERATOR

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Abstract—Neurons within the vagal motoneuron region of the lamprey have been shown to modulate respiratory activity via ascending excitatory projections to the paratrigeminal respiratory group (pTRG), the proposed respiratory rhythm generator. The present study was performed on *in vitro* brainstem preparations of the lamprey to provide a characterization of ascending projections within the whole respiratory motoneuron column with regard to the distribution of neurons projecting to the pTRG and related neurochemical markers. Injections of Neurobiotin were performed into the pTRG and the presence of glutamate, GABA and glycine immunoreactivity was investigated by double-labeling experiments. Interestingly, retrogradely labeled neurons were found not only in the vagal region, but also in the facial and glossopharyngeal motoneuron regions. They were also present within the sensory octavolateral area (OLA). The results show for the first time that neurons projecting to the pTRG are immunoreactive for glutamate, surrounded by GABA-immunoreactive structures and associated with the presence of glycinergic cells. Consistently, $GABA_A$ or glycine receptor blockade within the investigated regions increased the respiratory frequency. Furthermore, microinjections of agonists and antagonists of ionotropic glutamate receptors and of the $GABA_A$ receptor agonist muscimol showed that OLA neurons do not contribute to respiratory rhythm generation. The results provide evidence that glutamatergic ascending pathways to the pTRG are subject to a potent inhibitory control and suggest that disinhibition is one important mechanism subserving their function. The general characteristics of inhibitory control involved in rhythmic activities, such as respiration, appear to be highly conserved throughout vertebrate evolution.

Key words: control of breathing, inhibitory amino acids, glutamatergic transmission.

INTRODUCTION

The isolated brainstem of the adult lamprey, a lower vertebrate that diverged from the main vertebrate line ~560 million years ago (Kumar and Hedges, 1998), spontaneously generates a stable and regular respiratory neuronal activity *in vitro* (fictive respiration) for at least 12 h; this activity is very similar to that underlying respiration in intact animals (Rovainen, 1977, 1983; Thompson, 1985; Russell, 1986; Bongianni et al., 1999, 2002, 2006; Mutolo et al., 2007, 2010, 2011;Martel et al., 2007; Cinelli et al., 2013, 2014). The lateral walls of its central nervous system consist of two longitudinal zones or plates: the ventral primarily motor basal plate and the dorsal primarily sensory alar plate that comprises the octavolateral area (OLA; see e.g. Nieuwenhuys, 1972; Villar-Cerviño et al., 2008). Respiratory motoneurons are located in the facial, glossopharyngeal and, especially, in the vagal nuclei (Rovainen, 1974, 1977, 1979; Guimond et al., 2003), while the putative central neural mechanisms generating the respiratory rhythmic activity are located in the paratrigeminal respiratory group (pTRG), rostral to the trigeminal motor nucleus (Mutolo et al., 2007, 2010, 2011; Cinelli et al., 2013, 2014; Bongianni et al., 2016).

Endogenously released excitatory amino acids, but not GABA and glycine, have been shown to have an essential role in the respiratory rhythmogenesis (Rovainen, 1983; Martel et al., 2007; Bongianni et al., 1999, 2006, 2016; Cinelli et al., 2013, 2014). Only GABAergic influences have a modulatory role at the pTRG level. On the other hand, GABAergic and glycinergic inputs to neurons within the vagal motoneuron region mediate changes in respiratory frequency through ascending excitatory projections to the pTRG (Cinelli et al., 2014). However, an extensive characterization of the facial, glossopharyngeal and vagal motoneuron regions is still lacking with regard to projections to the pTRG and related neurochemical markers such as glutamate, GABA and glycine.

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Abbreviations: I_1, isthmic Müller cell; MRRN, middle rhombencephalic reticular nucleus; NOMI, intermediate octavomotor nucleus; NOMP, posterior octavomotor nucleus; NTS, nucleus tractus solitarii; nVm, motor root of the trigeminal nerve; nVs, sensory root of the trigeminal nerve; nVIII, vestibular nerve; nIX, glossopharyngeal nerve; nX, vagal nerve; OLA, octavolateral area; PB, phosphate buffer; PBS, phosphate-buffered saline; PRRN, posterior rhombencephalic reticular nucleus; pTRG, paratrigeminal respiratory group; rdV, descending root of the trigeminal nerve; SL, sulcus limitans of His; VA, raw vagal nerve activity; V, trigeminal motor nucleus; VII, facial motor nucleus; IX, glossopharyngeal motor nucleus; X, vagal motor nucleus.

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The present study was performed on *in vitro* brainstem preparations of the lamprey to investigate whether retrogradely labeled neurons could be identified within the whole respiratory motoneuron column by injections of Neurobiotin into the pTRG. The presence of glutamate, GABA and glycine immunoreactivity within the projection areas was ascertained by double-labeling experiments and the responses to microinjections of bicuculline or strychnine into the same regions were investigated.

In addition, since retrogradely labeled neurons were found in the alar plate within the OLA, the distribution of glutamate, GABA and glycine immunoreactivity was investigated and microinjections of bicuculline and strychnine were also performed in this region. An attempt was made to disclose a possible role of this portion of the OLA in respiratory rhythm generation by using microinjections of ionotropic glutamate receptor agonists and antagonists as well as of the $GABA_A$ receptor agonist muscimol.

EXPERIMENTAL PROCEDURES

Ethical approval

A total of 56 young adult (12–15 cm) lampreys (*Petromyzon marinus*) were employed. All animal care and experimental procedures were conducted in accordance with the Italian legislation and the official regulations of the European Communities Council on the use of laboratory animals (directive 86/609/EEC and 2010/63/UE). The study was approved by the Animal Care and Use Committee of the University of Florence. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Anatomical experiments

Six lampreys were used. For details on animal preparation see Cinelli et al. (2013, 2014). The animals were anaesthetized with tricaine methanesulphonate (100 mg/l; MS 222, Sigma–Aldrich, St Louis, MO, USA) and transected below the gills. The isolated brain-spinal cord was mounted dorsal side up in a Sylgard-lined recording chamber perfused with a cold physiological solution. The chamber volume was 3.0 ml, and the perfusion rate was set at 2.5 ml/min. Bath temperature was set at 9–10 °C. The solution flowed from a reservoir and had the following composition (in mM): 91 NaCl, 2.1 KCl, 2.6 $CaCl_2$, 1.8 $MgCl_2$, 4 glucose, 23 $NaHCO_3$. The solution was bubbled with 95% O_2–5% CO_2 to oxygenate and maintain the pH in the bath at 7.4. The brain was exposed dorsally and a transection was made caudal to the obex. The roof of the isthmic region was cut along the midline and the alar plates were spread laterally and pinned down (see Mutolo et al., 2007, 2010, 2011; Cinelli et al., 2013, 2014).

Retrograde tracing. Unilateral microinjections (~10 nl) of Neurobiotin (20% in distilled water; Vector Laboratories, Burlingame, CA, USA) were performed by means of glass micropipettes (tip diameter 10–20 μm) and by applying pressure pulses with a Picospritzer (General Valve Corporation, Fairfield, NJ, USA) connected to the injection pipette. The inactive dye Fast Green (0.2%, Sigma–Aldrich) was added to the solution to aid visualization of the injected tracer and to verify that the injection was reasonably confined to the investigated region. The localization of the pTRG was judged by the position of the dye spot with respect to the sulcus limitans of His (SL) and the isthmic Müller cell (I_1) (Cinelli et al., 2013, 2014). The depth of the injection (~0.3 mm below the dorsal surface) was inferred from that of rhythmic extracellular neuronal activity previously recorded in each preparation (see below for further details).

Dissection and histology. After Neurobiotin injections, the brains from the six lampreys were kept continuously perfused with the physiological solution in the dark at 4 °C. To allow the retrograde transport of the tracer, a 24-h perfusion period was scheduled. Thereafter, the brains were dissected out of the surrounding tissue and used for the immunohistochemistry (double-labeling experiments). For glutamate immunohistochemistry, two brains were fixed by immersion in 4% formalin, 1% glutaraldehyde and 14% of a saturated solution of picric acid in 0.1 M phosphate buffer (PB). For GABA immunohistochemistry, two brains were fixed by immersion in 4% formalin, 0.25% glutaraldehyde and 14% of a saturated solution of picric acid in PB. For glycine immunohistochemistry, two brains were fixed by immersion in 2% formalin in PB pH 7.4 for 12–24 h. The brains were postfixed for 24–48 h, after which they were cryoprotected in 20% sucrose in PB for 3–12 h. Transverse 20-μm-thick sections were made using a cryostat, collected on gelatin-coated slides and stored at −20 °C until additional processing.

Immunohistochemistry. All primary and secondary antibodies were diluted in 1% BSA, 0.3% Triton-X 100 in 0.1 M PB. For immunohistochemical detection of glutamatergic neurons (double-labeling experiments), sections were incubated overnight with a polyclonal rabbit anti-glutamate antibody (1:600; AB133; Millipore Corporation, Billerica, MA, USA). Following a thorough rinse in 0.01 M phosphate-buffered saline (PBS) the sections were incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:250; Invitrogen, Life Technologies, Carlsbad, CA, USA) and Alexa fluor-568 conjugated streptavidin (1:1000; Invitrogen) for 3 h, rinsed in PBS and coverslipped with glycerol containing 2.5% diazabicyclanooctane (Sigma Aldrich). For the immunohistochemical detection of GABA (double-labeling experiments), sections were incubated overnight with rabbit polyclonal anti-GABA conjugated to BSA with gluteraldehyde (1:1000; NBP1-78346; Novus Biologicals, Cambridge, UK). Sections were subsequently incubated with a mixture of Alexa Fluor 488 goat anti-rabbit IgG (1:200; Invitrogen) and Alexa fluor-568 conjugated streptavidin (1:1000; Invitrogen) for 3 h. For the immunohistochemical detection of glycine (double-labeling experiments), sections were incubated