The role of the nucleus retroambiguus in the neural control of respiration, vocalization and mating behavior
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Chapter 4

Direct projections from the nucleus retroambiguus to cricothyroid motoneurons in the cat

ABSTRACT

Vocalization can be elicited by stimulation in the periaqueductal gray (PAG). Lightmicroscopical tracing and physiological studies have revealed that the PAG uses the nucleus retroambiguus (NRA) as a relay to excite the vocalization muscle motoneurons. Direct NRA projections have been demonstrated to pharyngeal and abdominal wall muscle motoneurons, but not to laryngeal motoneurons. In two cats 0.1% cholera toxin subunit b was injected in the cricothyroid muscle of the larynx to retrogradely label its motoneurons, and 2.5% WGA-HRP was injected into the NRA to anterogradely label its fibers. The electronmicroscopical results indicate that the NRA fibers make monosynaptic contacts with cricothyroid motoneuronal dendrites. Almost all NRA terminal profiles had asymmetrical synapses and contained mostly round or pleiomorphic vesicles, which strongly suggests that the NRA-cricothyroid motoneuronal projection is an excitatory pathway.

INTRODUCTION

Vocalization can be elicited by electrical or chemical stimulation in the caudal periaqueductal gray (PAG; rat: Yajima et al., 1980; cat: Zhang et al., 1994; monkey: Larson and Kistler, 1984) as well as in various parts of the limbic system (Robinson, 1962). However, only lesions in the PAG result in total muteness (Adametz and O'Leary, 1959), indicating that the PAG is the final integrator of vocalization. Vocalization is the production of sound by inducing an airflow from the thorax along the vocal cords in the glottis opening. This airflow is the result of an increase in abdominal and thoracic pressure caused by simultaneous contraction of internal intercostal, abdominal, and pelvic floor muscles. The position of the vocal cords is determined by laryngeal muscles as cricothyroid and lateral cricoarytenoid. Although the motoneurons of these laryngeal muscles are thought to be part of the nucleus ambiguous in the medulla oblongata, they do not form a clearly recognizable cell group, but lie somewhat scattered within the lateral tegmental field of the medulla (Gacek, 1975; Holstege et al., 1983; Okubo et al., 1987). The PAG does not project directly to any of the vocalization motoneurons, but uses the nucleus retroambiguus (NRA) as relay (Holstege, 1989; Zhang et al., 1995). According to anterograde and retrograde tracing studies (Holstege, 1989; Vanderhorst and Holstege, 1996) these PAG-NRA projecting neurons are located in the lateral and ventrolateral parts of the caudal half of the PAG and in the laterally adjoining tegmentum. Also the dorsal PAG contains a few NRA projecting neurons. The NRA is the only cell group that has been demonstrated lightmicroscopically to project...
to all motoneuronal cell groups involved in vocalization (Holstege, 1989), although NRA projections to laryngeal muscle motoneurons have never been demonstrated anatomically. The reason is that such a NRA-laryngeal motoneuronal projection can only be demonstrated at the ultrastructural level, with retrograde tracing of motoneurons combined with anterograde tracing of NRA fibers. In this study the cricothyroid was selected as the laryngeal muscle of which the motoneurons were labeled retrogradely, because when injecting this muscle, spillage of tracer to other laryngeal or pharyngeal muscles is highly unlikely. Moreover, the cricothyroid plays an important role in the production of sound; i.e. it tenses the vocal cords and increases the anteroposterior diameter of the glottis opening, and thus changes the resistance to airflow (Patrickson et al., 1991).

**MATERIALS AND METHODS**

The surgical procedures, pre- and postoperative care, and handling and housing of the animals occurred according to the protocols approved by the faculty of Medicine of the University of Groningen. The animals were anesthetized with an initial dose of ketamine (0.1 ml/kg; i.m.) and xylazine hydrochloride (0.1 ml/kg; i.m.) and subsequently artificially ventilated under a mixed halothane-nitrous oxide anesthesia.

To retrogradely label the cricothyroid muscle motoneurons in the brainstem, in two cats (2451 and 2457) the muscle was injected unilaterally with a total of 30 µl 0.1% cholera toxin subunit b (CTb) using a 50 µl Hamilton syringe. Three days after the CTb injections, single injections of 100 nl 2.5% WGA-HRP were made in the NRA. Because of the contralateral preponderance of the NRA projection (Holstege, 1989), the WGA-HRP injection in the NRA was made contralateral to the CTb injection in the cricothyroid muscle. After a survival time of three days, the animals were deeply anesthetized with an overdose of Nembutal (6% pentobarbital sodium, i.p.). Subsequently, they were transcardially perfused with 2l of heparinized saline, followed by 2l fixative consisting of 2% glutaraldehyde and 1% paraformaldehyde in a 0.1M phosphate buffer (pH 7.4). The brains and spinal cords were removed and postfixed for 2 hours and stored in phosphate buffered saline (PBS). The brainstem was cut on a vibratome into 60 µm transverse sections. Every third section was incubated with tetramethylbenzidine (TMB) and ammoniummolybdate overnight (Olucha et al., 1985). After stabilization of the TMB reaction product with diaminobenzidine (DAB)-cobalt, the sections were rinsed with PBS and blocked with 5% normal rabbit serum in TBS containing 0.03% Triton X-100 (TBS+). Subsequently, the sections were incubated in a solution of TBS+ containing the primary antibody, goat anti-CTb (1:5000), and 1% normal rabbit serum (overnight; at 4°C). Next, the sections were incubated in the second antibody, biotinylated rabbit anti-goat IgG (1:200) in TBS+ and 1% normal rabbit serum, (for one hour; at room temperature), and transferred to a TBS solution containing avidin-biotin-complex-peroxidase (ABC, Vectastain, Vector; 1:200) and 1% Tx-100. Subsequently, the sections were incubated in DAB (Sigma), osmificated,
stained ‘en bloc’ in 1% uranylacetate in aquadest, dehydrated in a graded series of alcohol, and finally embedded in Epon between dimethyldichlorosilane-coated glass-slides. Those parts of the sections that contained anterogradely labeled fibers as well as retrogradely labeled motoneurons were selected, and cut into ultrathin sections. These sections were examined and photographed with a Philips 201 electron microscope. At the ultrastructural level, the TMB-Olucha reaction product appears as black crystalline deposits in the NRA terminals, and the DAB reaction product of the CTb-immunohistochemistry as small amorphic deposits in the cytoplasm of the cricothyroid motoneuronal dendrites.

To determine the injection sites, the caudal medulla and first cervical segment were cut on a freezing microtome into 40 µm sections, and processed using a standard diaminobenzidine (DAB) procedure. The sections were mounted on slides, dehydrated, coverslipped, and examined using a Zeiss Axioplan microscope.

RESULTS

CTb injections in the cricothyroid muscle resulted in retrogradely labeled motoneurons somewhat scattered in the medullary ventrolateral tegmental field as has been described previously (Gacek, 1975; Holstege et al., 1983; Okubo et al., 1987). The injections of WGA-HRP in the lateral part of the caudal medulla involved the caudal NRA and adjoining parts of the lateral tegmental field (Fig. 1).

These injections resulted in anterogradely labeled fibers bilaterally in the lateral tegmental field, including the region of the contralateral nucleus ambiguus containing retrogradely labeled cricothyroid motoneurons.

At the ultrastructural level, in case 2451 106 and in case 2457 101 anterogradely labeled terminal profiles were studied. Within the selected region with retrogradely labeled cricothyroid motoneurons in cases 2451 and 2457, respectively 83 and 88% of the labeled terminals made synaptic contacts with retrogradely labeled

![Fig. 1. Schematic drawings showing the injection sites in the caudal medulla in cases 2451 and 2457. CU: cuneate nucleus; G: gracile nucleus; NRA: nucleus retroambiguus; P: pyramidal tract; V spin. caud.: spinal trigeminal complex, pars caudalis.](image-url)
Fig. 2. Electron photomicrographs of anterogradely WGA-HRP-labeled NRA terminal profiles (asterisks) making asymmetrical synaptic contacts (arrows) with retrogradely CTb-labeled dendrites (arrowheads) of the cricothyroid motoneurons in cases 2451 (A) and 2457 (B). Scale bar = 0.5 μm.
**Table 1.** Type of synapses and vesicles in the labeled NRA terminals.

<table>
<thead>
<tr>
<th>Case</th>
<th>T+D+</th>
<th>asymmetric synaps</th>
<th>symmetric synaps</th>
<th>not identifiable synaps</th>
<th>round vesicles</th>
<th>pleiomorphic vesicles</th>
<th>round and dense core vesicles</th>
<th>pleiomorphic and dense core vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>2451</td>
<td>88 (100)</td>
<td>73 (83)</td>
<td>3 (3)</td>
<td>12 (14)</td>
<td>38 (43)</td>
<td>50 (57)</td>
<td>15 (39)</td>
<td>10 (20)</td>
</tr>
<tr>
<td>2457</td>
<td>89 (100)</td>
<td>74 (83)</td>
<td>4 (4)</td>
<td>11 (12)</td>
<td>34 (38)</td>
<td>55 (62)</td>
<td>12 (35)</td>
<td>16 (29)</td>
</tr>
</tbody>
</table>

T+D+: anterogradely labeled NRA terminals contacting retrogradely labeled cricothyroid dendrites. Values in parentheses are percentages.

dendrites of the cricothyroid motoneurons, and respectively 17 and 12% with unlabeled dendrites. In both cases 83% of the labeled terminals had asymmetric synapses (Fig. 2). Only about 3% had symmetric synapses, and in about 13% of the terminals the type of synaps was not identifiable (Table 1). About 60% of the labeled terminals contained pleiomorphic vesicles, and the remaining 40% round vesicles (Fig. 2). Of the labeled terminals a considerable number (20-39%) contained dense core vesicles also (Table 1).

**DISCUSSION**

The size of the NRA in transverse sections is very limited and the WGA-HRP injections inevitably involve not only the NRA itself, but also the medially adjacent tegmentum. It means that from these results alone it cannot be concluded that the labeled terminals on the cricothyroid motoneuronal dendrites originate exclusively from the NRA, because the cells medial to the NRA might also give rise to these motoneuronal projections. However, studies of Zhang et al. (1994) have demonstrated that stimulation of cells in the NRA resulted in strong electromyographic activity of the cricothyroid muscle, but stimulation in the medially adjacent region did not. Combining these stimulation results with the present anatomical observations demonstrates the existence of a direct excitatory NRA-cricothyroid motoneuronal projection.

The present ultrastructural results are in line with the observations regarding NRA projections to other motoneurons, such as those of the cutaneus trunci muscle (Boers et al. in preparation) the abdominal external oblique (Boers and Holstege, 1999) and the lumbosacral muscles involved in mating posture control (Vanderhorst and Holstege, 1997b). In all these projections the NRA labeled terminals, similar to the terminals on the cricothyroid motoneurons, had almost exclusively asymmetric synapses on motoneuronal dendrites. Apparently, all NRA-motoneuronal projections are excitatory.

These results fit the concept of the PAG using the NRA to excite the motoneurons involved in vocalization, respiration, and probably also vomiting (Miller et al., 1987) and mating behavior (Vanderhorst and Holstege, 1997b).