The role of the nucleus retroambiguus in the neural control of respiration, vocalization and mating behavior
Boers, José
Chapter 3

Ultrastructural evidence for direct excitatory retroambiguus projections to cutaneus trunci and abdominal muscle motoneurons in the cat

ABSTRACT

The nucleus retroambiguus (NRA) is a group of interneurons, located laterally in the caudal medulla oblongata. The NRA controls abdominal pressure in the framework of respiration, vomiting, vocalization, probably parturition, and, in all likelihood mating behavior. The NRA exerts this control because of its projections to motoneurons to the nucleus ambiguus in the lateral medulla (innervating pharynx, larynx), and spinal cord (innervating cutaneus trunci, intercostal, abdominal, pelvic floor, and lower limb muscles). The question is what the nature is of these NRA-motoneuronal projections. In this study we have determined the ultrastructure of the NRA-motoneuronal projections, and especially those to the abdominal external oblique (AEO) and cutaneus trunci muscle (CTM).

In four cats 0.1% cholera toxin subunit b (CTb) was injected in the AEO and CTM to retrogradely label their motoneurons in the spinal cord. 2.5% WGA-HRP was injected into the NRA to anterogradely label its contralaterally descending fibers to the AEO and CTM motoneurons. In order to prevent anterograde labeling of ipsilaterally descending systems not originating from the NRA, prior to the NRA injection, a hemisection was made at the level of C2. The results indicate that at the ultrastructural level, the great majority (60-74%) of the anterogradely labeled NRA-terminal profiles makes monosynaptic contacts with retrogradely CTb-labeled dendrites of the AEO and the CTM motoneurons. The great majority (86-95%) of the NRA terminal profiles made asymmetric synaptic contacts and 79-84% contained round vesicles. These results demonstrate that there exist direct, presumably excitatory, projections from NRA to AEO and CTM motoneurons.

INTRODUCTION

The mesencephalic periaqueductal gray (PAG) in mammals is an integrator of basic survival behavior of the individual as well as of the species. In order to produce such behavior, it sends fibers to premotor interneurons in the caudal brainstem (see Holstege, 1991 for review) and, to a more limited extent, to premotor interneurons in the spinal cord (Mouton and Holstege, 1994). An important example of the role of the PAG in survival behavior is the way it controls abdominal pressure. Abdominal pressure control is necessary for motor activities as vocalization, vomiting, forced expiration, parturition, and defecation.

The amount of abdominal pressure is determined by the action of the muscles constituting the abdominal wall and pelvic floor. According to lightmicroscopical
findings there is only one cell group, the nucleus retroambiguus (NRA), that has direct specific access to the motoneurons of these muscles (Fig. 1) (Holstege and Kuypers, 1982; Feldman et al., 1985; Holstege and Tan, 1987; Holstege, 1989; Holstege, 1991). The NRA is a cell group located laterally in the most caudal medulla oblongata.

The diaphragm is one of the muscles that has a strong effect on abdominal pressure, especially when operating in conjunction with the abdominal muscles, though its primary role is usually regarded as the production of negative intrathoracic pressure during inspiration. Its motoneurons, located in the phrenic nucleus, are also under strong control of the caudal brainstem, in particular, of the lateral solitary nucleus and the most rostral portion of the NRA. In an ultrastructural study in the rat, Ellenberger and Feldman (Ellenberger and Feldman, 1988) showed that fibers originating from the rostral NRA make direct contact with the phrenic nucleus.

**Fig. 1.** Overview of the PAG-NRA-motoneuronal pathway involved in abdominal straining activities.
motoneurons, especially via synapses on their dendrites. Ellenberger et al. (1990) also demonstrated that these direct projections were of an excitatory nature, because they found asymmetric synapses with round vesicles in the presynaptic element.

Other muscles that play a role in abdominal pressure control are the abdominal muscles, perhaps the overlying thin cutaneus trunci muscle (CTM), and the muscles of the pelvic floor. Holstege and coworkers (Holstege and Kuypers, 1982; Holstege and Tan, 1987; Holstege, 1989) have shown at the lightmicroscopical level that the NRA has direct projections to these motoneurons. The question is whether these projections are the same as those to the phrenic nucleus motoneurons. In the present ultrastructural study in the cat we have determined the exact nature of the NRA projections to the CTM and abdominal muscle motoneurons. Abdominal muscles are innervated from many thoracic and upper lumbar segments. The present study concentrated on L2 (used previously in a number of light microscopical studies), as well as T8, to allow for comparison with physiological studies that have involved this segment (see Kirkwood et al., 1999 for refs).

**MATERIAL AND METHODS**

The surgical procedures, pre- and postoperative care, and handling and housing of the animals were carried out according to the protocols approved by the University Medical Center 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.

**NRA injections combined with injections in cutaneus trunci and abdominal external oblique muscles**

To retrogradely label the motoneurons in the spinal cord, in one cat (2439) the cutaneus trunci muscle was injected with a total of 200 µl 0.1% cholera toxin subunit b (CTb) and the abdominal external oblique (AEO) muscle was superficially injected with 300 µl CTb. In one other cat (2428) only the cutaneus trunci muscle, and in two other cats (2440 and 2483) only the AEO muscle was injected. The CTb was injected unilaterally, in many portions and in different parts of the two muscles, using a 50 µl Hamilton syringe.

Three days after the CTb injections, single injections of 60–100 nl 2.5% (cases 2428, 2439 and 2440) to 10% (case 2483) WGA-HRP were made in the NRA, contralateral to the muscle injections, using the same anesthetic protocol as previously. Light microscopical studies (Holstege et al., 1987; Holstege, 1989; Vanderhorst and Holstege, 1995) have revealed that in cat the NRA sends the great majority of its fibers through the contralateral spinal cord, while other tegmental cell groups medially adjoining the NRA project ipsilaterally through the
cord. In order to be certain that the anterogradely labeled fibers and terminals are derived from the NRA, the ipsilaterally descending fibers were interrupted via an ipsilateral hemisection at the level of C2. This was done in the same surgical session just prior to the NRA injection.

After a survival time of three days, the animals were deeply anesthetized with an overdose of Nembutal (160 mg/kg pentobarbital sodium, i.p.). Subsequently, they were transcardially perfused with 200 ml of heparinized saline, followed by 2l of 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 a 25% sucrose solution. The caudal half of C8 (of cases 2428 and 2439), the rostral one fourth of L2 (of cases 2439 and 2440) and the rostral one third of T8 (of case 2483) were selected for electron microscopy. These parts of the segments were 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) and ammoniummolybdate overnight (Olucha et al., 1985). After stabilization of the TMB reaction product with diaminobenzidine (DAB)-cobalt, the sections were rinsed with Tris-buffered saline (TBS) 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 goat anti-CTb (1:5.000) 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 (nrs; for one hour at room temperature), and transferred to a TBS solution containing avidin-biotin-complex-peroxidase (ABC, Vectastain, Vector; 1:200) and 1% Triton X-100. Subsequently, the sections were incubated in DAB (Sigma), osmificated, stained ‘en bloc’ in 1% uranylacetate in aquadest, dehydrated in graded series of alcohol, and finally embedded in Epon between dimethyl dichlorosilane-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 (Fig. 2). These sections were examined and photographed with a Philips 201 electron microscope. Care was taken to ensure that the same terminal profile was not counted more than once by means of screening the ultrathin sections at different levels, and at intervals of 1 µm.

The tissue with the injection site and hemisection was frozen in an isopentane bath. To determine the injection sites, the caudal medulla and segment C1 were cut on a freezing microtome into 40 µm sections, and processed using a standard diaminobenzidine (DAB) procedure. To verify whether the hemisections were complete, the segments containing the hemisection were cut into 40 µm sections, mounted on slides, dehydrated, and coverslipped. The injection sites and hemisections were examined using a Zeiss Axioplan microscope.
RESULTS

The injections of WGA-HRP in the lateral part of the caudal medulla involved the NRA and adjoining parts of the lateral tegmental field (Fig. 3). To interrupt the ipsilaterally descending fibers from the caudal medulla, hemisections were made at the level of C2. In cases 2428, 2439 and 2483 the hemisections were complete or almost complete, but in 2440 the medial part of the ventral funiculus remained intact. None of the hemisections extended across the midline (Fig. 3).

In case 2428 the NRA injection was combined with CTb injections in CTM. In this case in the caudal half of C8 retrogradely labeled motoneurons were found in the ventrolateral part of the ventral horn. In case 2439 the NRA injection was combined with CTb injections in the CTM as well as in the AEO. In this case in the caudal C8 segment retrogradely labeled motoneurons were observed in the ventrolateral ventral horn and in rostral T8 and L2 in the lateral part of the ventral horn. In the other two cases (2440 and 2483) the NRA injection was combined with injections in the AEO. Also in these cases retrogradely labeled motoneurons were present in the lateral ventral horn at the rostral T8 and L2. The retrogradely labeled motoneurons were found exclusively ipsilateral to the side of the muscle injection. The WGA-HRP injections in the NRA resulted in anterogradely labeled fibers in the region of all the motoneuronal cell groups investigated.

At the ultrastructural level, the TMB reaction product appears as black crystalline deposits in the NRA terminals, and the DAB reaction product of the CTb-immunohistochemistry appears as small amorphic deposits in the cytoplasm of CTM and AEO motoneuronal dendrites. In each animal about 100 anterogradely labeled terminal profiles per segment were studied (Table 1). In order to collect such a high number of labeled terminals, different numbers of grids had to be investigated, ranging from 7 in case 2439 (external oblique at the level of L2), to 77 in case 2428 (cutaneus trunci).
**Cutaneus trunci**

Within the confines of the CTM motor nucleus at C8 in cases 2428 and 2439, 68-71% of the NRA terminals made synaptic contacts with retrogradely labeled dendrites of CTM motoneurons (Table 1; Fig. 4D), and 16-18% with unlabeled dendrites. In 13-14% of the terminals the abundant WGA-HRP labeling obscured the synapses. Only two axo-somatic contacts were found (case 2428). The great majority (86-93%) of the labeled terminals made asymmetric synaptic contacts with labeled dendrites, and only 7-14% made symmetric synaptic contacts (Table 2). Of the anterogradely labeled terminals contacting retrogradely labeled CTM motoneuronal dendrites most (81-84%) contained round vesicles (Table 2). Only a few terminals (2-12%) contained pleiomorphic vesicles, and in the remaining 8-14% the type of the vesicles was not clear, again because of strong WGA-HRP labeling. In 18-21% of the labeled terminals making asymmetric synaptic contacts with round vesicles, dense core vesicles were also observed (Table 2).

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**Fig. 3.** Drawings showing the injection sites in the caudal medulla, and the hemisections at the level of C2 in cases 2428, 2439, 2440, and 2483. Cu: cuneate nucleus; G: gracile nucleus; NRA: nucleus retroambiguus; P: pyramidal tract; V spin. caud.: spinal trigeminal complex; pars caudalis.
Abdominal oblique

In case 2483 in segment T8, within the confines of the external oblique motoneuronal cell group about two thirds of the anterogradely labeled NRA terminals made one or more synaptic contacts with retrogradely CTb labeled motoneuronal dendrites (Table 1; Fig. 4C). The great majority (94%) of these terminals made asymmetric, and only 6% symmetric synaptic contacts (Table 2). Most terminals (81%) contained round vesicles and only 7% contained exclusively pleiomorphic vesicles. Terminals with flat vesicles were not found (Table 2). Of the labeled terminals containing round vesicles, making asymmetric synaptic contacts, 15% contained dense core vesicles also. Axo-somatic contacts were not observed.

In cases 2439 and 2440 in L2 (Figs. 4A and B) a similar synaptic distribution pattern of anterogradely labeled NRA terminals on CTb labeled dendrites of the external oblique motoneurons was found as in T8. Of the terminals 60-74% made synaptic contacts with labeled dendrites (Table 1). The great majority (93-95%) of these contacts was asymmetric, and only 5-8% symmetric. Most of the terminals (81-87%) contained round vesicles, 7-8% round as well as dense core vesicles, and 6-13% pleiomorphic vesicles (Table 2). As in case 2483 no axo-somatic contacts were found.

These results indicate that the great majority (79-84%) of the labeled NRA terminals, contacting labeled dendrites of the CTM and AEO, made asymmetric synaptic contacts and contained round vesicles, strongly suggesting that these contacts are of an excitatory nature. Only a limited number (1-6%) of the labeled terminals made symmetric synaptic contacts and contained pleiomorphic vesicles, which might represent inhibitory contacts, although labeled terminals with flat vesicles, that go together with an inhibitory function, were never found.

### Table 1. Labeled NRA terminals in the motoneuron group of the CTM and the external oblique.

<table>
<thead>
<tr>
<th>Case</th>
<th>Number of grids studied</th>
<th>Number of labeled terminals</th>
<th>Number of labeled terminals contacting dendrites</th>
<th>Number of labeled terminals contacting unlabeled dendrites</th>
<th>Number of labeled terminals with unclear synapses</th>
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<tr>
<td>Cutaneus trunci C8 2428</td>
<td>77</td>
<td>99</td>
<td>67 (68%)</td>
<td>18 (18%)</td>
<td>14 (14%)</td>
</tr>
<tr>
<td>Cutaneus trunci C8 2439</td>
<td>36</td>
<td>101</td>
<td>72 (71%)</td>
<td>16 (16%)</td>
<td>13 (13%)</td>
</tr>
<tr>
<td>External oblique T8 2483</td>
<td>26</td>
<td>96</td>
<td>62 (64%)</td>
<td>21 (22%)</td>
<td>13 (14%)</td>
</tr>
<tr>
<td>External oblique L2 2439</td>
<td>7</td>
<td>98</td>
<td>73 (74%)</td>
<td>17 (17%)</td>
<td>8 (8%)</td>
</tr>
<tr>
<td>External oblique L2 2440</td>
<td>25</td>
<td>104</td>
<td>62 (60%)</td>
<td>33 (32%)</td>
<td>9 (9%)</td>
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</table>
Fig. 4. Electron photomicrographs of anterogradely WGA-HRP-labeled NRA terminal profiles (asterisks) making asymmetrical synaptic contacts (arrows) with retrogradely CTb-labeled dendrites (arrowheads) of the external oblique motoneurons in L2 in cases 2439 (A) and 2440 (B), and T8 in case 2483 (C), and of the cutaneus trunci muscle motoneurons in C8 in case 2428 (D). The labeled terminals contain many round vesicles and some mitochondria. Scale bar = 0.5 µm.
Fig. 4. (continued)
<table>
<thead>
<tr>
<th>Case</th>
<th>Labeled terminals contacting labeled dendrites</th>
<th>Terminals with asymmetric synapses</th>
<th>Terminals with symmetric synapses</th>
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<td></td>
<td>Labeled terminals</td>
<td>Terminals with asymmetric synapses</td>
<td>Terminals with symmetric synapses</td>
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<td></td>
<td>contacting labeled dendrites</td>
<td>Round + dense core vesicles</td>
<td>Pleiomorphic vesicles</td>
</tr>
<tr>
<td>Cutaneus trunci C8</td>
<td>2428 67 41 (61%) 12 (18%) 1 (1%) 4 (6%)</td>
<td>3 (5%) 1 (1%) 5 (8%)</td>
<td></td>
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<tr>
<td>Cutaneus trunci C8</td>
<td>2439 72 43 (60%) 15 (21%) 4 (6%) 5 (7%)</td>
<td>0 (0%) 4 (6%) 1 (1%)</td>
<td></td>
</tr>
<tr>
<td>External oblique T8</td>
<td>2483 62 41 (66%) 9 (15%) 1 (2%) 7 (11%)</td>
<td>0 (0%) 3 (5%) 1 (2%)</td>
<td></td>
</tr>
<tr>
<td>External oblique L2</td>
<td>2439 73 53 (73%) 5 (7%) 7 (10%) 4 (5%)</td>
<td>1 (1%) 2 (3%) 1 (1%)</td>
<td></td>
</tr>
<tr>
<td>External oblique L2</td>
<td>2440 62 47 (76%) 5 (8%) 2 (3%) 4 (6%)</td>
<td>2 (3%) 2 (3%) 1 (2%)</td>
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DISCUSSION

The results of this study indicate that the contralaterally descending fibers from nucleus retroambiguus (NRA) make direct, presumably excitatory, contacts with AEO and CTM motoneurons. Previous light microscopical studies (Holstege and Kuypers, 1982; Ellenberger and Feldman, 1988; Holstege, 1989) have shown terminations concentrated particularly within certain motor nuclei. The new data demonstrate that these terminations include a very high percentage of direct connections to motoneurons, at least within the regions investigated. These contacts are likely of an excitatory nature. Thus, the NRA projections to motoneurons of the abdominal external oblique (AEO) at both thoracic and lumbar levels, as well as to motoneurons of cutaneus trunci (CTM), have the same properties as those already described to the motoneurons of the diaphragm and some hindlimb muscles (Ellenberger et al., 1990; VanderHorst and Holstege, 1998).

It cannot be excluded that there was CTb leakage either from the AEO to the CTM or from the CTM to the AEO. However, light microscopical studies have revealed that the AEO motoneurons are located between the T5 and L3 segments, and the CTM motoneurons in the C8 and T1 segments (Holstege et al., 1987; Miller, 1987). Since these cell groups do not overlap rostrocaudally, such leakage cannot have affected the results. Another possibility is eventual leakage to the latissimus dorsi muscle, which might cause a problem, because the latissimus dorsi motoneurons are located in the ventral horn near the CTM motoneurons (Holstege et al., 1987; Hörner and Kümmel, 1993). However, the CTb injections in the CTM were made from the ventral side, in those parts of the muscle that were exposed when the skin was opened for injections into the AEO. This procedure makes it unlikely that the CTb in these parts of the CTM has leaked into the latissimus dorsi muscle, because at the site of the injection the CTM does not cover the latissimus dorsi.

Significance of terminations on AEO motoneurons

The present results relate to the direct connections of expiratory bulbospinal neurons (EBSNs). Caudal to the obex EBSNs are numerous and the great majority of them is located within the confines of the NRA (Merrill, 1970). One might expect, therefore, that a large part of the NRA-AEO motoneuronal projections originates from these EBSNs. In a physiological study, Merrill and Lipski (Merrill and Lipski, 1987) searched for direct connections between EBSNs and thoracic motoneurons, but found very few examples. On the other hand, other physiological studies [5;21] did find strong direct connections from EBSNs to motoneurons. These projections include the expiratory motoneurons with axons in the internal intercostal nerve at T8, as shown by cross-correlation (Cohen et al., 1985; Kirkwood, 1995), a result later extended to motoneurons innervating individual expiratory muscles at T8 and L1 (Miller et al., 1985; Kirkwood and Road JD, 1995), including, in particular, the AEO at T8. More recently, in a systematic spike-triggered averaging study (Annisimova et al., 2001) EBSNs have again shown strong connections to the motoneurons of AEO and of other expiratory muscles. Whatever the reason for
the failure of the connections to show up in the measurements of Merrill and Lipski (1987), the positive results of the other studies are closest to the present results. In addition to their role in expiration, the same NRA neurons probably play an important role in producing the strong contractions during vomiting or other forms of abdominal straining (Miller et al., 1987). Nevertheless, neurons other than EBSNs cannot be excluded to play a role also: it has recently been shown that a considerably fraction of NRA neurons which project to the lumbar spinal cord are not active during expiration and have non-respiratory function (Boers et al., 2003; Kirkwood and Ford, 2004).

The positive evidence to date of strong direct connections to motoneurons makes the projection from the NRA quite different from the descending projections of other motor systems, known mostly for their involvement in limb control (e.g. red nucleus and motor cortex) or the projections controlling posture in general, including the position of the head and eyes in space (brainstem regions, such as pontine and medullary medial dorsal tegmentum). These systems project mainly to the local premotor interneurons and, except for a few vestibulospinal (Rapoport et al., 1977) and reticulospinal projections (Peterson et al., 1979; Alstermark et al., 1987), they do not include direct connections to motoneurons. In this respect Holstege and Kuypers (1982) and Feldman et al. (1985) suggested that the NRA cells, (considered by Feldman et al. to also include the more rostrally located inspiratory population), could be thought of as premotor interneurons, analagous to those in the spinal cord. They suggested that they were located in the medulla rather than in the spinal cord because the peripheral afferent fibers that monitor functions such as lung volume, airway pressure or abdominal pressure, run in cranial nerves that terminate in the caudal medulla (Loewy and Burton, 1978). With respect to the role of premotor interneurons Jankowska (2001) stressed their multifunctional role in a variety of networks, including the integration of both central and peripheral inputs. An additional justification for a caudal medullary location of these premotor interneurons could be the integration required for the control of motoneurons at very widely different levels of the neuraxis. For the NRA this includes cranial to sacral levels. For instance, in vomiting, both the glottis and the anal and urethral sphincters should be held shut (see discussion in Miller et al., 1995). It should also be kept in mind that, like some spinal interneurons, the neurons of the NRA may be involved in rhythm or pattern generation. Such pattern generators, for instance for respiration, can be reconfigured for other motor acts, such as vomiting (Grélot et al., 1996). Reconfiguration for different actions is a central function, for which a rostral location is appropriate.

Like all motoneurons, those of the abdominal muscles receive their afferents from different sources, depending on the function in which they participate, such as control of posture or control of abdominal pressure. With respect to postural control, abdominal muscle motoneurons receive afferents from muscle spindles as well as from local interneurons that receive afferents from other neurons in the spinal cord (propriospinal connections), and from certain regions in the brainstem.
NRA projections to abdominal motoneurons

For their function in the control of abdominal pressure in the context of respiration, vocalization, vomiting and probably also defecation, parturition and the kyphosis posture during lactation, this study shows that the abdominal motoneurons receive a large component of their afferent input directly from neurons in the NRA.

Significance of terminations on CTM motoneurons

It is not immediately clear what the meaning is of the NRA-CTM motoneuronal projection. In the female golden hamster this projection is thought to play a role in raising the tail during the lordosis posture (Gerrits et al., 2000a). In the cat the CTM does not have such a strong effect on the tail, which makes such a function for the NRA-CTM motoneuron projection in this animal unlikely. In how far the CTM, or indeed the abdominal muscle motoneurons, play a role in the female receptive posture during mating remains unclear.

With respect to the control of abdominal pressure, the CTM is so thin that one might not expect a great deal of power from this muscle to change abdominal pressure. It is thought that this muscle is involved in protecting the skin from irritant stimuli, like insects or heat (Theriault and Diamond, 1988), through shivering movements. It is unknown whether the NRA regulates this activity, because the CTM motoneurons also receive afferents from a spinal reflex system that originates from cutaneous afferents. They enter the spinal cord at various thoracic and lumbar levels, and reach the motoneurons of the CTM via interneuronal relays at these spinal levels (Giovanelli Barilari and Kuypers, 1969; Matsushita and Ueyama, 1973). This afferent organization might explain that only part of the CTM is involved in shivering movements of the skin (Krogh and Denslow, 1979; Theriault and Diamond, 1988; Holstege and Blok, 1989).

Another interesting feature of the CTM is that it receives afferents from the lateral parabrachial nuclei. The function of this pathway remains to be elucidated, but it is interesting that the abdominal muscle motoneurons do not receive such afferents (Holstege and Kuypers, 1982; Holstege and Blok, 1989). Perhaps, the CTM has a not yet identified function in which the abdominal muscles do not play a role.

Conclusion

The descending projections from the NRA to motoneurons at a wide variety of spinal levels are dominated by direct connections, an unusual arrangement. The functions that are likely to be sustained by these connections can all be included in the category of survival behavior. The directness of the input may be an indication of the importance of these behaviors (Grélot et al., 1996; Annisimova et al., 2001).