Retroambiguus Projections to the Cutaneus Trunci Motoneurons May Form a Pathway in the Central Control of Mating

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Gerrits, Peter O., Chris Vodde, and Gert Holstege. Retroambiguus projections to the cutaneus trunci motoneurons may form a pathway in the central control of mating. J. Neurophysiol. 83: 3076–3083. 2000. Our laboratory has proposed that the nucleus retroambiguus (NRA) generates the specific motor performance displayed by female cats during mating and that it uses direct pathways to the motoneurons of the lower limb muscles involved in this activity. In the hamster a similar NRA-projection system could generate the typical female mating posture, which is characterized by lordosis of the back as well as elevation of the tail. The present study attempted to determine whether this elevation of the tail is also part of the NRA-mating control system. The basic assumption was that elevation of the tail is a function of the cutaneus trunci muscle (CTM), which was verified by bilateral tetanic stimulation of the lateral thoracic nerves innervating the CTM. It resulted in upward movement of the tail to a position similar to the tail-up position during the lordosis posture. Retrograde tracing results showed that CTM motoneurons are located in the ventral and ventrolateral part of the C6–C8 ventral horn, those innervating the tail region ventrolateral to those innervating the axillary region. Anterograde tracing studies showed that NRA fibers terminate bilaterally in both parts of the CTM motoneuronal cell groups. Electron microscopical studies revealed that labeled NRA terminals make monosynaptic contacts with retrogradely labeled dendrites of CTM motoneurons. Almost all of these terminal profiles had asymmetric synapses and contained spherical vesicles, which suggests an excitatory function. The observation that 15% of the labeled NRA terminals make more than one synaptic contact with a retrogradely labeled CTM motoneuronal dendrite within the same section indicates how powerful the NRA-CTM projection is. The results indicate that during mating the NRA not only could generate the lordosis posture but also the elevation of the tail.

INTRODUCTION

In the study of the CNS control of mating behavior in mammals, the female hamster is particularly interesting, because in estrous she displays a very distinctive receptive behavior. After stimulation of the flanks, lower back, or perineum, she exhibits a rigid tonic immobility and sustained lordosis posture, consisting of elevation of the pelvis, elongation of the body, straightening of the back, tensing up the skin, and elevation of the tail. This posture lasts for several minutes, even when the animal remains untouched by the male hamster or the experimenter (Carter 1985; own observations). This behavior can be elicited in estrus females during a time period of ~12–20 h provided that no mating takes place (Frank and Fraps 1945; Kent 1968).

Recently, in cat (VanderHorst and Holstege 1995, 1997a,b) and hamster (Gerrits and Holstege 1999), a distinct pathway from the nucleus retroambiguus (NRA) to motoneurons of the muscles performing the receptive posture has been demonstrated. The NRA is a rostrocaudally oriented column of premotor interneurons located in the ventrolateral tegmentum of the caudal medulla. This nucleus has been described in humans (Olszewski and Baxter 1954), cats (Holstege 1989; Merrill 1970), rats (Ellenberger and Feldman 1990; Holstege et al. 1997), songbirds (Wild 1993, 1997), and hamster (Gerrits and Holstege 1999).

In the hamster the NRA projects to discrete motoneuronal cell groups of the upper lumbar cord innervating the iliopsoas muscle (Gerrits and Holstege 1999) but, in contrast to cat, not to the sacral cord where, among others, some of the motoneuronal cell groups innervating the tail muscles are located. At first glance, this is surprising because elevation of the tail is an important part of the lordosis posture, and similar to the cat one would expect the NRA to project to tail muscle motoneurons also. However, in the hamster tail elevation might be generated by the cutaneus trunci muscle (CTM). The CTM, also called cutaneus maximus in the cat (Crouch 1969), is a thin skeletal muscle just beneath the skin covering very large portions of the trunk. It is found in most mammals, but not in primates. In the hamster the CTM is especially thick at the tail base and in the axillary region (Fig. 1). The muscle is innervated by branches of the lateral thoracic nerve. Tracing studies in other mammals have shown that the CTM motoneurons are located in a distinct cell group in the lower cervical cord. In the cat they were found at the level of C5–T1 (Holstege and Blok 1989; Holstege et al. 1987); in the dog at the level of C7–T3 (Krogh and Towns 1984); and in the rat at the level of C6–T1 (Baulac and Meinginger 1981; Haase and Hrycyszyn 1985). In the cat CTM motoneurons receive direct afferents from the lumbar cord (Giovanelli Barilari and Kuijpers 1969), from the dorsolateral pontine tegmentum and from the NRA (Holstege and Blok 1989).

In the present study it was investigated whether the elevation of the tail, which represents an important part of the lordosis posture, is also controlled by the NRA. Therefore it had to be demonstrated that the CTM indeed generates tail elevation, then the location of the CTM motoneurons was determined, and subsequently the NRA projections to the CTM motoneurons were studied on the light as well as electronmicroscopic level. The results indicate that in the hamster the NRA indeed
controls tail elevation similar to the control of the lumbar trunk muscles.

M E T H O D S

The surgical procedures, pre- and postoperative care, and handling and housing of the animals were in accordance with the protocols approved by the Faculty of Medical Sciences of the University of Groningen. A total of 16 adult female golden hamsters (Mesocricetus auratus), weighing 100–120 g, was used. During surgery the animals were anesthetized with chloral hydrate (400 mg/kg ip).

Cutaneus trunci muscle stimulation

To find out whether the CTM generates tail elevation, in two experiments the lateral thoracic nerves innervating the various parts of the CTM were stimulated ipsi- and bilaterally. The experiments were carried out on anesthetized hamsters (chloral hydrate (400 mg/kg ip)). After a rostrocaudal midline incision over the back of the animal the left and right axillary region were exposed, and part of the CTM, including the various branches of the lateral thoracic nerve, were carefully dissected and isolated from the overlying skin. Bipolar contact electrodes were placed ipsi- or bilaterally onto the lateral
thoracic nerve branches, which were tetanically stimulated (50 Hz; 1 ms; L/R 145 μA). Before and during bilateral stimulation, photographs of the tail position were made.

Retrograde tracing study

To determine the spinal cord location of CTM motoneurons, in eight animals injections of the retrograde tracer horseradish peroxidase (HRP, Sigma type VI, 10% in distilled water) were placed into two parts of the CTM (tail and axilla). In five animals (H154, H157, H181, H187, and H188), after a small superficial incision of the skin, four injections of 5 μl HRP were placed into the CTM at the dorsal surface of the base of the tail directly underneath the skin. In three cases (H156, H189, and H190), after a rostrocaudal midline incision of the back of the animal the axillary region was exposed, and two injections of 5 μl HRP were carefully placed into the axillary CTM. A thin plastic voile was placed underneath the CTM to prevent HRP leakage into the underlying muscles.

After a 24-h survival time, the animals were reanesthetized with an overdose of Nembutal (0.7 ml of 6% pentobarbital sodium, ip), and transcardially perfused with 300 ml of heparinized saline, followed by 300 ml of fixative containing 2% glutaraldehyde, 1% paraformaldehyde, and 4% sucrose in 0.1 M phosphate buffer, pH 7.2–7.4, at room temperature. The spinal cords were removed, postfixed for an additional hour, and stored overnight in 25% sucrose in 0.1 M phosphate buffer at 4°C. The next day, the lower cervical and upper thoracic segments (C6 – T2) were frozen in an isopentane bath (–25°C) and cut on a cryostat microtome into 50-μm transverse sections. Every other section was processed serially using the Mesulam tetramethylbenzidine (TMB) procedure (1982). All sections were mounted on coated slides, dehydrated, and coverslipped with Permount mounting medium.

Anterograde tracing study

In six cases (H169, H171, H193, H194, H195, and H196), injections containing a total of 20–40 nl 2.5% wheat germ agglutinin–horseradish peroxidase (WGA-HRP) were placed into the area of the NRA. The NRA injections were placed after exposure of the caudal medulla between the foramen magnum and the first cervical vertebrae. Because the NRA in the hamster is known to send its axons almost exclusively through the contralateral cord, in four cases (H193, H194, H195, and H196), before the injection, an ipsilateral C6 hemisection was made to interrupt all ipsilaterally descending reticulospinal path-
ways, which do not originate from the NRA (Gerrits and Holstege 1999). After a survival time of 24 h the animals were deeply anesthetized with an overdose of Nembutal (0.7 ml of 6% pentobarbital sodium, ip), and transcardially perfused with 300 ml of heparinized saline at 37°C, followed by 300 ml of fixative containing 1% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at room temperature. Brains and spinal cords were removed and post-fixed for an additional hour. The C6 – T2 segments were cut on a vibratome into 60 μm transverse sections. The sections were incubated with TMB and ammonium molybdate overnight (Olucha et al. 1985) and subsequently stabilized with DAB-cobalt chloride. To determine the extent of the injection sites, the medulla oblongata and rostral C1 spinal segment were cut into 50-μm transverse sections on a cryostat. These sections were incubated using a standard DAB procedure. The selected cervical and thoracic segments were used for both light and electron microscopy. At the light microscopic level, the location and distribution of retrogradely labeled CTM neurons and anterogradely labeled terminals were examined with a Zeiss Axioplan light microscope equipped with polarized darkfield illumination. In all cases photomicrographs were taken from representative sections.

Electron microscopy

The light microscopic results strongly suggested the existence of a direct NRA-CTM motoneuronal projection. To determine the precise nature of this connection, WGA-HRP injections into the NRA were combined with injections of 20 μl cholera toxin B-subunit (CTB; Vector Laboratories, Burlingame, CA, 1% in distilled water) into different parts of the CTM. Because the rate of retrograde transport of CTB and WGA-HRP is different, two-step surgery was necessary. First, cholera toxin B-subunit was injected into the CTM, and 2 wk later WGA-HRP was injected into the NRA and adjoining reticular formation.

In three cases (H169, H171, and H193), 20 μl CTB was carefully placed into the CTM at the tail base, and in one case (H195) into the axillary part of the CTM.

The NRA was injected with a total of 20–40 nl 2.5% WGA-HRP. In two of the four cases (H193 and H195), before the injection, a C6 hemisection ipsilateral to the injection site was made.

Following tissue processing and stabilization of the TMB reaction product with DAB-cobalt chloride (see also the anterograde tracing
The vibratome sections were rinsed in Tris-buffered saline (TBS), pH 7.4 and blocked with 5% normal rabbit serum in TBS containing 0.03% Triton X-100 (TBS1). The sections were incubated overnight at 4°C in a solution of TBS1 containing the primary antibody goat anti-CTB (1:10,000; List Biological Laboratories, Campbell, CA) and 1% normal rabbit serum. The next day the sections were transferred to the secondary antibody biotinylated rabbit anti-goat IgG (1:200; DAKO, Glostrup, Denmark) in TBS1 and 1% normal rabbit serum for 1 h at room temperature, and then placed in the third antibody ABC-reagent (Vectastain Elite kit PK6100) in TBS+ solution. Subsequently, they were incubated with diaminobenzidine (DAB; Sigma), osmificated, stained in 1% uranylacetate in distilled water, dehydrated in a graded series of ethanol, and finally embedded in Epon between dimethyldichlorosilane-coated glass slides.

Only regions (n = 13) that at the light microscopical level contained retrogradely labeled CTM motoneurons were sampled for ultrastructural analysis. The samples were glued on an Epon stub. Single thin sections of ~60 nm thickness were cut with a diamond knife. At the ultrastructural level all labeled profiles were photographed at a magnification of ×20,000 using a Philips 201 electron microscope and afterward classified.

**RESULTS**

**CTM stimulation**

Bilateral tetanic stimulation of the lateral thoracic nerves innervating the CTM resulted in upward movement of the tail to a position identical to the position during the lordosis posture characteristic of estrus (Fig. 2A). During stimulation the tail remained in an elevated position. Unilateral stimulation led to lateral deviation of the tail toward the side that was stimulated. Figure 2, B and C, shows the tail positions in rest and during bilateral tetanic stimulation.

**Retrograde tracing results**

**TAIL CTM INJECTIONS.** Multiple injections of HRP were bilaterally placed at the base of the tail. In all cases a similar distribution pattern of retrogradely labeled motoneurons was found.

The great majority of them was located bilaterally in the ventrolateral part of ventral horn in the C7 and C8 segments of the cervical cord close to the border between the gray and white matter and partly within the white matter (Figs. 3B and 4, ●). Occasionally a few labeled cells were found in the most caudal C6 or most rostral T1 segments.

**AXILLARY CTM INJECTIONS.** In all cases retrogradely labeled motoneurons were found ipsilaterally to the side of the injection ventrally in the ventral part of ventral horn in the C7 and C8 spinal segments (Figs. 3A and 4, ○), medial to the region with the CTM motoneurons innervating the tail.

In conclusion, all CTM motoneurons are located ventrolaterally in the ventral part of ventral horn in the C7–C8 spinal segments, but those innervating the tail part of the muscle are located lateral to those innervating the axillary part.

**TABLE 1.** Nucleus retroambiguus labeled terminals in CTM motor nucleus

<table>
<thead>
<tr>
<th>Hamster</th>
<th>Total Number of Labeled Profiles</th>
<th>T+D+</th>
<th>T+D–</th>
<th>T+D+ With Asymmetric Synapses</th>
<th>T+D+ With Not Identifiable Synapses</th>
<th>T+D+ With Multiple Asymmetric Synapses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail CTM</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>H169</td>
<td>72</td>
<td>55 (76)</td>
<td>17 (24)</td>
<td>43 (78)</td>
<td>12 (22)</td>
<td>10 (18)</td>
</tr>
<tr>
<td>H171</td>
<td>71</td>
<td>51 (72)</td>
<td>20 (28)</td>
<td>41 (80)</td>
<td>10 (20)</td>
<td>8 (16)</td>
</tr>
<tr>
<td>H193</td>
<td>44</td>
<td>38 (86)</td>
<td>6 (14)</td>
<td>29 (76)</td>
<td>9 (24)</td>
<td>5 (13)</td>
</tr>
<tr>
<td>Axillary CTM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>H195</td>
<td>63</td>
<td>51 (81)</td>
<td>12 (19)</td>
<td>39 (76)</td>
<td>12 (24)</td>
<td>6 (12)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are percentages. T+D+, wheat germ agglutinin–horseradish peroxidase (WGA-HRP) labeled nucleus retroambiguus (NRA) terminals making contact with cholera toxin B-subunit (CTB) labeled dendrites; T+D–, WGA-HRP labeled NRA terminals making contact with unlabeled dendrites; CTM, cutaneus trunci muscle.
Anterograde tracing results

LIGHT MICROSCOPY. In six cases (H169, H171, H193, H194, H195, and H196) injections of WGA-HRP were made in the caudal medulla oblongata. All these injections involved the NRA and its adjoining tegmentum, lateral funiculus, and parts of the caudal spinal trigeminal complex (Fig. 5).

To interrupt the ipsilaterally descending fibers from the caudal medulla, in four cases (H193, H194, H195, and H196) the injections were preceded by C6 hemisections. The hemisections were complete or almost complete (Fig. 5). Because retrograde tracing studies have demonstrated that contralateral projections from the caudal medulla to the lower cervical cord (cat, Holstege and Kuijpers 1982) or to the upper lumbar cord (cat, VanderHorst and Holstege 1995; hamster, Gerrits and Holstege 1999) originate almost exclusively from the NRA, it is assumed that the labeled fibers found caudal to the level of the hemisection are derived from the NRA, or from the few cells in the tegmentum between the NRA and solitary nucleus.

In all cases with injections of WGA-HRP in the NRA and adjoining reticular formation NRA fibers crossed the midline at the level of the caudal medulla and descended contralaterally in the lateral and ventrolateral funiculi.

At the level of C7–C8 some labeled fibers entered the gray matter to terminate in the ventral horn, but particularly the area of the CTM motoneuronal cell group. The projection involved the medial as well as lateral CTM motoneuronal cell group (Fig. 6). A similar, but less dense projection was found on the ipsilateral side. The strength of the ipsilateral projection was estimated at ~25% of the contralateral one. The ipsilateral projection must have been derived from contralaterally descending fibers that recrossed at the level of the CTM, because virtually no ipsilaterally descending fibers were found in the white matter below the level of the C6 hemisection. NRA projections were not only present to the CTM motoneuronal cell group, but also to a small motoneuronal cell group in the medial part of the ventral horn. It is not known which muscles these motoneurons innervate.

Electron microscopy

To demonstrate that the NRA fibers make direct contact with the CTM motoneurons, and to find out the nature of the projections, in four cases (H169, H171, H193, and H195) the WGA-HRP injections in the NRA were combined with CTB injections in different parts of the CTM muscle (Fig. 5). In two of the cases (H193 and H195), before the WGA-HRP injections, a hemisection was made at the level C6.

TAIL CTM. In cases H169, H171, and H193, respectively 72, 71, and 44 WGA-HRP labeled NRA terminal profiles were found, of which ~77% had synapses on CTB labeled dendrites of tail CTM motoneurons (Fig. 7, A and B). About 78% of these synaptic contacts were of the asymmetric type (Fig. 7, A...
and B, Table 1). The other 22% of the contacts could not be identified as being symmetric or asymmetric. About 16% of the labeled NRA terminals were observed to make multiple (2 or 3) asymmetric synaptic contacts with retrogradely labeled motoneuronal dendrites within the same ultra thin section (Fig. 7B). The labeled NRA terminal profiles with asymmetric synapses always contained numerous small round vesicles, and in some cases also a few dense core vesicles.

Of 5 (2.6%) of the 195 labeled NRA terminal profiles making contact with labeled dendrites, an additional synaptic contact with an unlabeled dendrite was observed. Labeled NRA terminals on retrogradely labeled or unlabeled somata, and axonotic contacts were not found.

In case H193, with a C6 hemisection ipsilateral to the NRA injection, a similar synaptic distribution pattern was found with WGA-HRP labeled NRA terminals on CTB labeled dendrites of tail CTM motoneurons (Table 1), which means that the labeled terminals are derived from contralaterally descending NRA axons.

**AXILLARY CTM.** In case H195 with a C6 hemisection ipsilateral to the NRA injection, a total of 63 WGA-HRP labeled NRA terminal profiles was observed, of which 51 (81%) had synaptic contact with CTB labeled dendrites of axillary CTM motoneurons (Table 1). Of these 51 synapses, 39 (76%) were asymmetric. About 12% of the labeled NRA terminals had multiple (2 or 3) asymmetric synaptic contacts with retrogradely labeled motoneuronal dendrites.

In summary, within the CTM motoneuronal nucleus the great majority of the NRA terminals were found to have direct contact with the CTM motoneuronal dendrites. The finding that the great majority of these contacts had asymmetric synapses and contained round vesicles, whereas no symmetric contacts with flat vesicles were observed, strongly suggests that the NRA-CTM motoneuronal pathway is of an excitatory nature.

**DISCUSSION**

The CTM, also called cutaneous maximus in the cat (Crouch 1969), is best known for the reflex that would protect the skin from irritant stimuli (Theriault and Diamond 1988). The muscle does not contain muscle spindles, but instead receivesafferent projections from the overlying skin (Theriault and Diamond 1988). Because the muscle extends over large thoracic and abdominal regions, some of these skin afferents enter the spinal cord at lumbar levels. Therefore there exists proprioosseal pathways to the CTM motoneurons from many thoracolumbar segments (Giovanelli Barilari and Kuijipers 1969; Matsushita and Ueyama 1973). Such projections do not originate from the cervical cord (Holstege and Blok 1989), because the muscle does not lie underneath cervical dermatomes. In the rat (Nixon et al. 1984; Theriault and Diamond 1988) and guinea pig (Blight et al. 1990) stimulation of the skin itself, or the cutaneous nerve innervating a dermatome overlying the CTM, results in a bilateral contraction of a specific portion of the CTM, just rostral to the dermatome of the stimulated nerve. The fact that only a portion of the muscle reacts on stimulation of a particular part of the skin suggests a somatotopical subdivision of the motoneuronal nucleus. The present results show that the CTM motoneurons innervating the caudal part of the muscle are located lateral to the motoneurons innervating the rostral part of the CTM, and these results are in agreement with a somatotopical organization of the CTM motor nucleus.

The present results in the hamster, as well as earlier results obtained in the cat (Holstege and Blok 1989), have demonstrated NRA projections to CTM motoneurons. Because the NRA is known to be active during expiration (Bainton and Kirkwood 1979; Merrill 1970, 1974), vomiting (Miller et al. 1987, 1995), and vocalization (Holstege 1989; Jürgens and Pratt 1979; Zhang et al. 1992), the question arises, whether the NRA-CTM pathway plays a role in these activities. Holstege and Blok (1989) suggested that the NRA-CTM projections in the cat might be involved in abdominal straining functions. The more recent findings of a critical role for the NRA in generating the motor activity during mating in the cat suggests a similar role for the NRA in the hamster. This idea is further supported by the present finding that the NRA has a direct projection to the CTM motoneurons that elevate the tail and tense up the skin. Whether abdominal straining also takes part in mating behavior remains to be determined.

The NRA-CTM motoneuronal pathway is the output system of large parts of the limbic system, but especially of the periaqueductal gray (PAG). The PAG maintains direct projections to the NRA (Holstege 1989), and via this pathway it can generate vocalization (Zhang et al. 1992), mating behavior (Pfaff et al. 1994), and perhaps also other behaviors such as vomiting. Figure 8 illustrates the role of the CTM within the framework of mating behavior.

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