Ascending projections from spinal cord and brainstem to periaqueductal gray and thalamus
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Chapter 2

Nucleus retroambiguus projections to the periaqueductal gray in the cat

Esther-Marije Klop, Leonora J. Mouton and Gert Holstege


ABSTRACT

The nucleus retroambiguus (NRA) of the caudal medulla is a relay nucleus by which neurons of the mesencephalic periaqueductal gray (PAG) reach motoneurons of pharynx, larynx, soft palate, intercostal and abdominal muscles and several muscles of the hindlimbs. These PAG-NRA-motoneuronal projections are thought to play a role in survival behaviors, such as vocalization and mating behavior. The present combined antero- and retrograde tracing study in the cat tried to determine whether the NRA, apart from the neurons projecting to motoneurons, also contains cells projecting back to the PAG. After injections of WGA-HRP in the caudal and intermediate PAG labeled neurons were observed in the NRA with a slight contralateral preponderance. In contrast, after injections in the rostral PAG or adjacent deep tectal layers no or very few labeled neurons were present in the NRA.

After injection of $[^3H]$-leucine in the NRA, anterograde labeling was present in the most caudal ventrolateral and dorsolateral PAG, and slightly more rostrally in the lateral PAG, mainly contralaterally. When the $[^3H]$-leucine injection site extended medially into the medullary lateral tegmental field, labeling was found in most parts of the PAG, as well as in the adjoining deep tectal layers. No labeled fibers were found in the dorsolateral PAG and only a few were found in the rostral PAG.

Because the termination pattern of the NRA fibers in the PAG overlaps with that of the sacral cord projections to the PAG, it is suggested that the NRA-PAG projections play a role in the control of motor functions related to mating behavior.

INTRODUCTION

The motor system can be subdivided into the somatic and the emotional motor system (EMS; Holstege, 1997). The pathways belonging to the EMS originate in brain regions that take part in, or are related to, the limbic system. One of the key structures of the EMS is the periaqueductal gray (PAG), which is involved in basic survival behaviors. Electrophysiological or chemical stimulation in the PAG can elicit motor activities including changes in blood pressure and heart rate (Bandler et al., 1991; Bandler and Keay, 1996; Lovick, 1996), respiration (Davis and Zhang, 1991; Lovick, 1993), micturition (Blok and Holstege, 1996), vocalization (Kanai and Wang, 1962; Jürgens and Pratt, 1979; Larson, 1985; Bandler et al., 1991; Jürgens and Lu, 1993, Jürgens, 1994) and mating behavior (Sakuma and Pfaff, 1979; Pfaff et al., 1994). The anatomical
framework for the PAG control of vocalization and mating behavior are its projections to the nucleus retroambiguus (NRA; Holstege, 1989), a cell group in the ventrolateral portion of the most caudal medulla oblongata (Fig. 1; Olszewski and Baxter, 1954; Taber, 1961; Merrill, 1970). The NRA, in turn, projects to the brainstem motoneurons of the pharynx, larynx, and soft palate in the nucleus ambiguus (Holstege, 1989), as well as to the spinal cord motoneurons of the internal intercostal and abdominal muscles (Merrill, 1970; Holstege and Kuypers, 1982; Feldman et al., 1985; Miller et al., 1985; Merrill and Lipski, 1987; Billig et al., 1999; Boers and Holstege, 1999; Kirkwood et al., 2000; VanderHorst et al., 2000). The NRA also projects to cervical and lumbosacral motoneurons (hamster: Gerrits and Holstege, 1999; Gerrits et al., 2000; cat: VanderHorst and Holstege, 1995; monkey: VanderHorst et al., 2000b) involved in eliciting species specific mating postures. Thus, the NRA contains premotor interneurons projecting to distinct sets of motoneuronal cell groups. The PAG gains access to these motoneurons involved in vocalization and mating posture via a relay through the NRA. The question arises whether the NRA exclusively contains premotor interneurons, or also neurons projecting to non-motoneuronal cell groups such as the PAG, where it receives so many afferents from.

In order to investigate the possibility of the existence of NRA-PAG projections, a combined retro- and anterograde tracing study was done. Because neurons in the caudal medullary tegmentum adjacent to the NRA also project to the PAG (in rat: Loewy et al., 1981; Beitz, 1982 and 1989; Herbert and Saper, 1992; in rabbit: Meller and Dennis, 1986; in monkey: Mantyh, 1982), in an anterograde tracing study of an NRA-PAG projection, it is important that such cells not be involved in the NRA injections. Furthermore, the anterograde tracer cannot be one that also is transported retrogradely. Therefore, the tracer [3H]-leucine was used, that exclusively anterogradely labels fibers and terminals, and which also has the advantage that there is no uptake of tracer by fibers of passage.

Figure 1. Brightfield photomicrographs showing the nucleus retroambiguus (NRA) in a Nissl-stained section. Bar represents 250 µm.
MATERIALS AND METHODS

Retrograde tracing study

Surgical procedures

A total of 8 female cats was used. The surgical procedures, pre- and postoperative care, as well as the handling and housing of the animals followed protocols approved by the Faculty of Medicine of the University of Groningen. The animals were initially anesthetized with intramuscular ketamine (Nimatek, 0.1 ml/kg) and xylazine (Sedamun, 0.1 ml/kg), and subsequently ventilated with a mixture of O₂, N₂O (1:2) and 1-2% halothane, while ECG and body temperature were monitored. In 7 cases 40-200nl 2.5% wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP) in saline was injected into the PAG, and in one control case (2338) approximately 150 nl WGA-HRP was injected in the deep layers of the superior colliculus lateral to the PAG. Injections were made using a glass micropipette with a pneumatic picopump (World Precision Instruments PV830). All injections were made stereotaxically, using Berman’s (1968) atlas. In order to reach the PAG (2367 and 2385) or the tectum (2338) in 3 cases, the pipette went through the superior colliculus and in three other cases through the inferior colliculus (2300, 2401, and 2411). In two cases (2471 and 2479) the PAG was reached via the cerebellum.

Following a survival time of 3 days the animals were initially anesthetized with intramuscular ketamine (Nimatek, 0.1ml/kg) and xylazine (Sedamun, 0.1ml/kg), followed by an overdose (6-10ml) of intraperitoneal 6% pentobarbital sodium. Subsequently, they were perfused transcardially with 2 liters of 0.9% saline at 37°C, immediately followed by 2 liters of 0.1M phosphate buffer (pH 7.4), containing 4% sucrose, 1% paraformaldehyde and 2% glutaraldehyde.

Histological procedures

After perfusion the brain and spinal cord were removed, post-fixed for two hours and stored overnight in 20% sucrose in phosphate buffer at 4°C. Subsequently, the brainstem was frozen in an isopentane bath (-55°C) and cut on a cryostat microtome into 40µm frozen sections. Of these, every fourth section was incubated according to the tetramethyl benzidine (TMB) method (Mesulam, 1978; Gibson et al., 1984). All sections were mounted on chromalum-gelatine coated slides, dried, dehydrated and coverslipped with Permount mounting medium. In order to define the extent of the injection site, an extra series of sections was incubated with diaminobenzidine (DAB). The injection sites were plotted using a drawing tube connected to a Zeiss brightfield stereomicroscope.

Quantification and visualization of retrogradely labeled neurons in the caudal medulla

In order to determine the exact location and number of the labeled neurons in the NRA after a WGA-HRP injection in the PAG or tectum, in each case the retrogradely labeled NRA neurons were plotted and counted, using a drawing tube connected to a Zeiss Axioplan with darkfield polarized illumination. In addition to the retrogradely labeled neurons in the NRA, the locations of the
other retrogradely labeled cells in the caudal medulla were plotted at five different rostrocaudal levels caudal to the obex (between P 15.0 and P 18.5 according to Berman’s (1968) atlas). The plottings were made with the aid of the Neurolucida System of MicroBrightField, Inc. (Colchester, USA).

Anterograde tracing study
Surgical procedures
In 7 female cats (cases 1019, 1183, 1471, 1472, 1571, 1683, 1684) the caudal brainstem, involving the lateral tegmental field and/or the NRA, was injected using the autoradiographic tracer L-[4,5-³H]-leucine (specific activity > 100 Ci/mmol). The injections were made stereotaxically using a Hamilton microsyringe fitted with a 22-gauge needle. In all cases 0.25-0.5 µl containing 50 µCi [³H]-leucine was injected over a period of 5 minutes, after which the needle was left in place for an additional 30 minutes to minimize the spread along the needle track. After a survival period of six weeks (Holstege, 1979) the animals were deeply anesthetized with 6-10ml of 6% intraperitoneal pentobarbital and perfused with saline followed by 4% paraformaldehyde.

Histological procedures
The brain and spinal cord were postfixed in 4% paraformaldehyde for at least 1 week, after which they were cut into 25µm transverse frozen sections. One series of every tenth section was mounted, coated with Ilford G5 emulsion by dipping, and stored in the dark at 5°C for 3 months (Cowan et al., 1972; Holstege, 1979). Subsequently the material was developed with Kodak D19 at 16°C, fixed and counterstained with cresyl violet. The injection area in all experiments was defined as that area in which the silver grains over the cell bodies were either as numerous as, or more numerous than, the surrounding neuropil (Holstege et al. 1977 and 1979).

Visualization of the anterogradely labeled fibers in the PAG
Silver grains representing anterogradely labeled fibers and nerve terminals in the PAG were studied using a Wild darkfield stereomicroscope. Darkfield photomicrographs were taken using a Zeiss Axiocam digital photocamera, attached to a Zeiss stereomicroscope, using Axiovision software. These digital photomicrographs were further processed using Adobe PhotoShop software.

RESULTS
Retrograde tracing study
Injection sites in the PAG
In a total of 8 cases injections were made in the PAG and/or adjacent deep tectal areas (Fig. 2). In the drawings of Fig. 2 the core of the injections is shown in black. In three of these cases (2367, 2385 and 2411) the injection sites involved large parts of the PAG, in case 2367 the rostral PAG, and in cases 2385 and 2411 the intermediate and caudal PAG.
In 4 of the 8 cases smaller injections were placed in the PAG at the intermediate and/
or caudal level, involving the dorsolateral (case 2479) and lateral and/or ventrolateral parts (cases 2300, 2401 and 2471). In 2300 and 2401, the injections extended slightly into the laterally adjoining deep tectal layers. In a control case 2338, an injection was made, almost exclusively involving the deep layers of the superior colliculus at the level of the rostral and intermediate PAG with only very limited involvement of the lateral PAG.

Retrogradely labeled neurons in the medulla oblongata caudal to the obex
In the case with the large injection site involving the rostral PAG (2367) some labeled cells were found in the medial and lateral tegmental field, but almost none in the NRA (Figs. 3, 5 and Table 1). A few labeled neurons were found at this level bilaterally in the commissural nucleus of the nucleus of the solitary tract (Fig. 3, case 2367: C).

In case 2385 with a large injection site in the caudal and intermediate PAG retrogradely labeled neurons were observed in the dorsal column nuclei and medially in the tegmentum, especially at the most caudal medullary levels (Fig. 3, case 2385: C, D and E). This distribution pattern was found bilaterally, with a contralateral preponderance.
Figure 3. Drawings of five transverse sections of the medulla oblongata at the levels between P15.0 and P18.5. One dot represents one WGA-HRP labeled cell. Each drawing represents one 40 µm transverse section. The caudal spinal trigeminal nucleus together with the tegmentum in the medulla caudal to the obex were subdivided into different laminae according to Gobel and Hockfield (1977). CU, cuneate nucleus; GR, gracile nucleus; IO, inferior olive; LRN, lateral reticular nucleus; LTF, lateral tegmental field; NRA, nucleus retroambiguus; NTS, nucleus of the solitary tract; P, pyramidal tract; spV, spinal trigeminal nucleus; XII, hypoglossal nucleus.
Figure 3 (continued)
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Table 1: Numbers and percentages of labeled neurons in the NRA

<table>
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<tr>
<th>case</th>
<th>ipsilateral number</th>
<th>ipsilateral percentage</th>
<th>contralateral number</th>
<th>contralateral percentage</th>
<th>total</th>
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<td>37.9%</td>
<td>18</td>
<td>62.1%</td>
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<tr>
<td>2367</td>
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<td>1</td>
<td>20.0%</td>
<td>5</td>
</tr>
<tr>
<td>2385</td>
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<td>34.3%</td>
<td>67</td>
<td>65.7%</td>
<td>102</td>
</tr>
<tr>
<td>2401</td>
<td>41</td>
<td>41.4%</td>
<td>58</td>
<td>58.6%</td>
<td>99</td>
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<td>2411</td>
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<td>35</td>
<td>56.5%</td>
<td>62</td>
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<td>2479</td>
<td>17</td>
<td>51.5%</td>
<td>16</td>
<td>48.5%</td>
<td>33</td>
</tr>
<tr>
<td>control injection</td>
<td>2338</td>
<td>0 (-)</td>
<td>0</td>
<td>(-)</td>
<td>0</td>
</tr>
</tbody>
</table>

Neurons were also found in the lateral part of the tegmentum, including the NRA (Figs. 3, 4 and 5). When the injection was limited to the ventrolateral PAG (case 2401), a similar distribution pattern was found, except that only a small number of neurons was present in the medial tegmentum. After a very small PAG injection (case 2471), exclusively involving the medial portion of the ventrolateral part of the PAG, in the caudal medulla labeled neurons were only observed in the NRA (Fig. 5 and Table 1) and not in the adjoining tegmental field. In the tectum injected control case (2338), retrogradely labeled neurons were found in the lateral part of the dorsal column nuclei, and a few in the tegmentum, but none in the NRA (Figs. 3, 5 and Table 1).

In summary, in all cases, except for the tectum injected case, labeled neurons were present in the NRA, bilaterally with a slight contralateral preponderance. After a very small injection in the ventrolateral PAG labeled cells were exclusively present in the NRA.

Figure 4. Drawing and darkfield photomicrograph of a transverse section of the caudal brainstem (P 16.8) in case 2385. The photomicrograph represents the area indicated by the gray box in the drawing of the medulla oblongata and shows labeled neurons in the nucleus retroambiguus after a WGA-HRP injection in the caudal and intermediate PAG. Bar represents 200 μm. CU, cuneate nucleus; GR, gracile nucleus; NRA, nucleus retroambiguus; NTS, nucleus of the solitary tract; spV, spinal trigeminal nucleus.
Figure 5. Drawings of labeled neurons in the contra- and ipsilateral nucleus retroambiguus after WGA-HRP injections in the periaqueductal gray and/or the tectum. Note that one drawing of the nucleus retroambiguus represents six consecutive 40µm sections (of a 1:4 series). One dot represents one labeled neuron.
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Anterograde tracing study

Injection sites

Figures 6 and 7 show the [3H]-leucine injection sites in the caudal brainstem. In two cases (1571 and 1683), although the bulk was in the white matter, the injection sites extended medially into the NRA, but not into the medially adjoining tegmentum. In four cases (1471, 1183, 1019 and 1684) the injections in the lateral tegmental field extended laterally into the NRA. In one control case (1472) the injection site did not involve the NRA. In cases 1471 and 1472, the injection sites extended also into the cuneate and the caudal spinal trigeminal nuclei.

Figure 6. Brightfield photomicrographs showing the [3H]-leucine injection sites in the caudal medulla oblongata (cases 1471, 1472 and 1683). Bar represents 1 mm.

Labeled fibers in the PAG

The injections involving substantial portions of the tegmentum resulted in stronger projections to the PAG than the ones limited to the NRA. The strongest PAG projections were found in case 1471, in which labeled fibers were distributed to all parts of the intermediate PAG, except its dorsolateral and ventromedial regions. Labeled fibers were also found in the adjoining deep tectal layers (Fig. 8 left). The other cases with large injections in the tegmental field involving the NRA (cases 1019 and 1183) showed a similar, but weaker projection pattern. Also, in control case 1472 (Fig. 8 right) a similar, somewhat weaker projection pattern was present, possibly because the NRA and the cell group medial to the NRA were not injected. In the cases with white matter injections extending into the NRA, but not in its adjoining regions (cases 1683 and 1571), rather weak projections were found (Figs. 9-10). In these two cases labeled fibers were observed to terminate in the ventromedial and dorsolateral parts of the most caudal PAG near its central border and in the lateral part of the caudal-intermediate PAG. Further rostrally in the PAG no labeled fibers were found. When the injection extended further medially into the lateral tegmental field (case 1684), labeled fibers were also found in the more rostral lateral and dorsomedial parts of the PAG.

In conclusion, the anterograde tracing results show that the NRA contains neurons that project to the caudal and intermediate PAG. In the caudal PAG, NRA fibers terminated exclusively in small regions of its ventrolateral and dorsolateral parts, and in the intermediate PAG in its lateral part. Larger injections in the caudal medulla resulted in projections to larger PAG regions and adjoining deep tectal layers, but not to the dorsolateral PAG.
NRA injections:

1571

1683

1684

Tegmental injections involving the NRA:

1019

1183

1471

Control injection

1472

Figure 7. Drawings of the location of the [3H]-leucine injection sites in the 7 cases used in the anterograde tracing study
Figure 8. Darkfield photomicrographs of the periaqueductal gray and adjoining deep tectal layers after injections of [3H]-leucine in the cases 1471 and 1472. Bar represents 1 mm.
Figure 9. Darkfield photomicrographs of the caudal periaqueductal gray after injection of [3H]-leucine in case 1683. Bar represents 1 mm.
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DISCUSSION

The nucleus retroambiguus (NRA) is known to contain neurons that receive afferents from the periaqueductal gray (PAG) and project to motoneurons particularly involved in vocalization (Holstege, 1989), vomiting (Miller et al., 1985), respiration (Merrill and Lipski, 1987) and lordosis posture (VanderHorst and Holstege, 1995). The present results demonstrate for the first time that the NRA also contains cells that project to other structures than motoneuronal cell groups, in casu the PAG.

The retrograde tracing results of this study, as well as those of others (Meller and Dennis (1986) in the rabbit, Beitz (1982) and Herbert and Saper (1992) in the rat and Mantyh (1982) in the monkey) show that the NRA is not the only source of caudal medullary afferents to the PAG, because the PAG also receives afferents from cells medial to the NRA.

It means that interpretation of the anterograde results must take into account that some of the injection sites in the NRA involve some PAG projecting cells in medially adjoining tegmental cell groups. However, in cases 1571 and 1683 the injection sites
NRA-PAG projections

are mainly located in the white matter lateral to the NRA, involving the NRA itself, but not the more medially located parts of the tegmentum. In these two cases well defined termination patterns were observed, but of limited strength. The reason for the relatively weak projections are the small injections and the limited number of NRA cells projecting to the PAG. The retrograde study revealed that, in a series of 1:4, a maximum of 67 contralateral NRA cells were found to project to the PAG, while, according to VanderHorst and Holstege (1995), a maximum of 334 contralateral neurons in the NRA projected to the lumbosacral cord. Apparently, approximately five times as many NRA cells project to lumbosacral cord as to the PAG.

The results also indicate that in almost all cases the contralateral NRA projection to the PAG is stronger than the ipsilateral one (Table 1), which is also the case for the NRA projections to motoneurons (Holstege, 1989; VanderHorst and Holstege, 1995).

Figure 11. Schematic representation of projections possibly involved in mating behavior. The nucleus retroambiguus-periaqueductal gray pathway, described in the present study, is shown in black.
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The possibility exists that the injection sites in case 1571, 1683 and 1684 involve some of the catecholaminergic cells of the A1 cell group that project to the PAG. However, Herbert and Saper (1992) in the rat showed that only a small portion of these cells projects to the PAG. Moreover, in the cat, the majority of these catecholaminergic cells is located more rostrally in the caudal medulla than the three injection sites (Jones and Friedman, 1983; Blessing et al., 1980). Furthermore, according to Jones and Friedman (1983) and Blessing et al. (1980) the catecholaminergic neurons are located ventrolaterally to the NRA, while the retrograde tracing results show that most of the PAG projecting neurons are located in the NRA or medially to it. In conclusion, a catecholaminergic projection to the PAG may take part in the projections demonstrated in this paper, but its role seems to be of minor importance.

The projection from the NRA to the PAG is limited to exclusively the ventrolateral and dorsolateral parts of the caudal PAG and the lateral part of the caudal half of the intermediate PAG. This projection pattern overlaps with the PAG projections from the various parts of the spinal cord. However, most of the spinal projections also terminate in the deep collicular layers and more rostral parts of the PAG (cat: Wiberg and Blomqvist, 1984b; Björkeland and Boivie, 1984; monkey: Wiberg et al., 1987). The only spinal projection pattern in the PAG that precisely corresponds with that of the NRA is the projection from lamina I and the lateral parts of laminae V and VII of the sacral cord (see Fig. 18A in VanderHorst et al., 1996). This sacral region receives genital and pelvic floor afferents and one might speculate that both the sacral cord and NRA afferents to the PAG play a role in the control of motor functions related to mating behavior (Fig. 11).

The projection pattern of the NRA fibers does not overlap extensively with the PAG cell groups from where the descending motor output systems originate (Holstege, 1989). It suggests that interneurons within the PAG play a role in relaying the ascending afferent information to the motor output systems in the PAG. Perhaps this ascending information is integrated with the strong descending projections from the many limbic areas and the prefrontal cortex, before it influences the motor systems in the PAG.