Monosynaptic Projections From the Lateral Periaqueductal Gray to the Nucleus Retroambiguus in the Rhesus Monkey: Implications for Vocalization and Reproductive Behavior

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ABSTRACT

The periaqueductal gray (PAG) is known to be essential for vocalization and reproductive behavior. The PAG controls components of these behaviors by means of projections to the nucleus retroambiguus (NRA), a group of premotor neurons in the caudal medulla oblongata. In the accompanying study (VanderHorst et al., 2000 [accompanying study]), the NRA and its lumbar-sacral projections have been identified in the rhesus monkey. The present light and electron microscopical tracing study describes the PAG-NRA pathway in primates. To locate midbrain neurons projecting to the NRA, wheat germ agglutinin horseradish peroxidase (WGA-HRP) was injected into the NRA in six monkeys. To determine the distribution pattern of PAG axons in the medulla oblongata, WGA-HRP was injected into the PAG and adjacent tegmentum in three additional monkeys. In one of these three monkeys, biotinylated dextran amine and cholera toxin subunit b were injected into the lumbar-sacral cord to retrogradely identify NRA neurons. The results show that a compact group of neurons in the medial part of the lateral PAG at the intercollicular level sends a dense projection to the NRA. The projection is bilateral with a clear ipsilateral predominance. At the ultrastructural level, there are monosynaptic contacts between PAG fibers and NRA neurons, including NRA neurons that project to the lumbar-sacral cord. The synaptic contacts were primarily asymmetrical and the labeled terminal profiles contained spherical and dense core vesicles. It is concluded that there exists a strong and direct PAG-NRA pathway in the rhesus monkey. Because NRA neurons projecting to the lower lumbar cord are included, the PAG-NRA projection is likely to be involved not only in vocalization but also in other behaviors, such as receptive posture. J. Comp. Neurol. 424:251–268, 2000.

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The periaqueductal gray (PAG) has long been known for its role in vocalization in a wide variety of species. As early as 1937, Magoun et al. showed that PAG stimulation in decerebrate animals elicits natural sounding vocalizations, whereas PAG lesions lead to mutism in cats and humans (Adametz and O’Leary, 1959; Esposito et al., 1999). Each species has its own repertoire of vocalizations, such as meowing, growling, and hissing in cats and clicking, cooing, and barking in monkeys. The various types of vocalization can be elicited by stimulation of the PAG (e.g., monkey: Jürgens and Ploog, 1970; Robinson, 1967; Larson, 1985; Larson and...
On the basis of a light microscopic study in the cat (Holstege, 1989), it was proposed that the PAG controls vocalization by means of a relay in the nucleus retroambigus (NRA). The NRA is a group of premotor neurons in the caudal medulla. It sends direct projections to thoracic and upper lumbar motoneurons involved in expiration (in the monkey: VanderHorst et al., 2000 [accompanying study]; in the cat: Holstege and Kuypers, 1982; Feldman et al., 1985; Holstege, 1989; Miller et al., 1989) and to the nucleus ambiguous containing larynx, pharynx, and soft palate motoneurons (Holstege, 1989; VanderHorst et al., 2000 [accompanying study]). Physiologic studies in cats and monkeys have shown that the NRA is involved in vocalization, although only for the activation of expiratory and laryngeal muscles and not for the orofacial component (Larson et al., 1994; Zhang et al., 1995; Shiba et al., 1997).

Apart from vocalization, the PAG has also been demonstrated to be crucial for female receptive behavior (in the rat: Sakuma and Pfaff, 1979a,b; in the cat: Bard and Macht, 1958; in the monkey: Okada et al., 1988). As is the case for vocalization, receptive behavior is species-specific and requires coordination between different groups of muscles. Recent anatomic studies suggest that the NRA might function not only as a relay nucleus between the PAG and motoneurons involved in vocalization but also in reproductive behavior. The NRA in the rhesus monkey (VanderHorst et al., 2000 [accompanying study]), cat (VanderHorst and Holstege, 1995, 1997), and hamster (Gerrits and Holstege, 1999) has been shown to project directly to different groups of lumbosacral motoneurons innervating muscles that are likely to be involved in the species-specific mating posture.

Altogether, the PAG-NRA pathway might be important for vocalization as well as for reproductive behavior. Although the relation between vocalization and the PAG in primates has been extensively studied, there is no anatomic or physiologic information available about connections between the PAG and the NRA in monkeys. Moreover, some studies suggest that the PAG controls larynx motoneurons by means of direct projections to the nucleus ambiguous (in the monkey: Jürgens and Pratt, 1979; in the rat: Ennis et al., 1997). Regarding reproductive behavior, one of the questions is whether the PAG projects to NRA neurons that send their axons to lumbosacral motoneurons. These questions are addressed in the present tracing study. The distribution of NRA-projecting neurons in the PAG, and the distribution of PAG axons terminating in the caudal medulla oblongata are described at the light microscopic level. At the ultrastructural level, it will be shown for the first time that PAG axons make monosynaptic contacts with NRA neurons, including those projecting to the lumbosacral cord.

MATERIAL AND METHODS

Surgical procedures

**Animals and general surgical procedures.** Nine adult ovariectomized rhesus monkeys (M. mulatta; age, 5–12 years; weight, 5.8–12.8 kg) were used. The protocol for this study was reviewed and approved by the Animal Care and Use Committee, University of Wisconsin, and all experiments were performed under the guidelines established by the National Institutes of Health and United States Department of Agriculture. The animals were tranquillized with ketamine hydrochloride (10 mg/kg, i.m.), intubated, and kept anesthetized under isoflurane (1.5%, i.t.). Buprenorphine hydrochloride (0.01 mg/kg, i.m.), atropine sulfate (0.04 mg/kg, i.m.), and penicillin-g procaine (40,000 units/kg, i.m.) were given before the surgery. Sterile neurosurgical techniques were used for all surgical procedures. After surgery, the animals were returned to their home cages. Three days after the tracer injections with wheat germ agglutinin-horseradish peroxidase (WGA-HRP), the animals were anesthetized with ketamine (0.1 mg/kg, i.m.) and pentobarbital (0.5 mg/kg, i.v.) and transcardially perfused with 2 liters of phosphate buffered saline (PBS; pH 7.4, 0.1 M, room temperature) followed by 2 to 3 liters of fixative containing 1.5–2% glutaraldehyde (EMS, Fort Washington, PA) and 1–1.5% paraformaldehyde (EMS) in 0.1 M phosphate buffer (pH 7.4). The brains were removed, postfixed for 1–3 hours, and stored in PBS (at 4°C). In cases M3, M4, M5, and M7, which were exclusively used for light microscopy in the present study, the tissue was stored in 25% sucrose in 0.1 M phosphate buffer (at 4°C).

**Injections into the NRA.** To identify neurons in the PAG that project to the NRA, WGA-HRP (Sigma; 2.5% dissolved in saline) was injected into the NRA and adjacent tegmentum in six monkeys (M3, M4, M5, M7, M8, and M9). The head of the animal was placed into a stereotaxic head holder, and the head was flexed at an angle of approximately 90 degrees. A midline incision was made in the neck, and the dorsal neck muscles were separated in the midline. After opening the lamina between the occipital bone and the atlas, the caudal medulla and first cervical segment were exposed. A glass micropipette was held in a microsyringe carrier, and in each animal, three to four injections of WGA-HRP (total volume 240–320 nl) were made in the left ventrolateral medulla including the NRA (2.8 to 4.2 mm lateral to the midline, and 2.9 to 3.9 mm deep) by using a pressure pump (Picospritzer II; General Valve Cooperation). To cover the entire rostrocaudal extent of the NRA, the injections were made at rostrocaudal intervals of 1 mm.

**Injections into the PAG.** To study the innervation pattern of PAG axons in the NRA, in three cases (M14, M15, and M16) one or two injections with 2.5% WGA-HRP (total volume 160–320 nl) were made into the left ventrolateral medulla including the NRA (2.8 to 4.2 mm lateral to the midline, and 2.9 to 3.9 mm deep) by using a pressure pump (Picospritzer II; General Valve Cooperation). To cover the entire rostrocaudal extent of the NRA, the injections were made at rostrocaudal intervals of 1 mm.
caudal part of the PAG. After verifying the exact location of the pipette tip with additional X-ray pictures, the pipette was pulled back, rinsed with saline, and filled with WGA-HRP, inserted again; the tracer was injected by using a pressure pump (Picospritzer II; General Valve Cooperation).

**Injections into the lumbosacral cord.** In one of the experiments (M16), 4 weeks before the injection into the PAG, the lumbosacral cord was injected with tracers to identify neurons in the NRA that project to the lumbosacral cord. The spinal cord was exposed after a dorsal laminectomy at the level of the L1-L3 vertebrae. On the left side of the L3-L6 spinal segments, multiple injections of 2% cholera toxin subunit B (CTb; List Biological Labs., CA; total volume of 6 μl) were made with a glass micropipette by using a pressure pump. The right side of the L5-S2 segments in the same animal was injected with a total volume of 10 μl of 5% biotinylated dextranamine (BDA; 3000 molecular weight; Molecular Probes, Eugene, OR) in 15 injection sites.

**Histologic procedures**

**Injection sites.** The midbrain, pons, and lower brainstem-C1 segment were blocked, and the spinal cord was cut into segments based on their dorsal rootlets. To determine the injection sites, the midbrain in cases M14, M15, and M16, the caudal medulla in M8 and M9, and the lumbar segments in M16 were cut into 50-μm (cases M14, M15, M16) or 80-μm (cases M8 and M9) sections by using a Vibratome. The caudal brainstem and the C1 segment in cases M3, M4, M6, and M7 were cut into 40-μm sections on a freezing microtome.

To visualize the WGA-HRP injection sites, every fourth (cases M3, M4, M6, and M7), fifth (M8 and M9), or tenth section (M14, M15, and M16) was incubated in diamino-benzidine (DAB; Sigma, St. Louis, MO) with 0.3% H₂O₂, mounted on glass slides, dehydrated in methanol, cleared in xylene, and cover-slipped by using DePeX (EMS) mounting medium.

For precise delineation of the BDA injection site (M16), the tissue was incubated by using the ABC technique. After incubation for 1.5 hours in Tris buffered saline (TBS, pH 7.6) containing 8 μl/ml solution A and 8 μl/ml solution B (ABC Elite Kit; Vector Laboratories, Burlingame, CA; at room temperature), the tissue was rinsed and reacted for 10–30 minutes in 0.025% DAB in TBS (pH 7.6) with 0.001% H₂O₂ (at room temperature).

To visualize CTb (M16), the tissue was preincubated for 2 hours in normal rabbit serum (NRS, Vector Laboratories; 3%; at room temperature) in 0.05 M Tris buffered saline containing 0.5% Triton X-100 (TBS⁺; pH 7.6), then incubated for 2 nights in anti-CTb (1:30,000; at 4°C; List Biological Lab.) in TBS⁻ containing 2% NRS, for 1 hour in anti-goat (1:100; at room temperature; DAKO, Carpinteria, CA) in TBS⁺ containing 2% NRS, and for another hour in PAP-goat (1:100 in TBS⁺; at room temperature; DAKO). The reaction product was visualized by using 0.025% DAB in TBS with 0.001% H₂O₂ for 2–5 minutes.

**Retrograde tracing in the PAG.** To visualize WGA-HRP in the PAG in cases M3, M4, M6, and M7, the midbrain was cut with a freezing microtome into four series of 40-μm serial sections. In cases M8 and M9, the tissue was vibratomized into six series of 80-μm, serial sections. All tissue was cut in the transverse plane. One series was incubated with tetramethylbenzidine (TMB; Sigma) and sodium nitroprusside (Fisher Scientific, Pittsburgh, PA) according to Mesulam (1982), and a second series with TMB and ammonium paratungstate (K&K Laboratories, Cleveland, OH) according to Weinberg and van Eyck (1991). Before cover-slipping, the paratungstate-treated sections were used for a Nissl stain.

The midbrain was divided into eight rostrocaudal levels with intervals of approximately 1 mm. For each level, labeled cells in one representative section were plotted into one drawing by using a Nikon microscope and Neurolucida system. In cases M4 and M6, the size of heavily labeled neurons in the lateral PAG and lightly labeled neurons in the adjacent tegmentum and ventrolateral PAG were measured by using Neurolucida. Photomicrographs were taken of representative sections by using a Nikon microscope. The slides were digitalized by using a UMAX scanner and Macintosh PowerPC, contrast and brightness were adjusted by using Adobe Photoshop 5.0, and the micrographs were labeled by using Adobe Illustrator 8.0 software.
Anterograde tracing in the NRA. In cases M14, M15, and M16, the lower brainstem and C1 segment were cut into 10 series of 50-μm sections by using a Vibratome. For light microscopic visualization, one series of consecutive sections was processed by using the TMB procedure according to Mesulam (1982). A second series was incubated according to Olucha et al. (1985) and stabilized with CoCl₂-DAB.

In case M16, two additional series were used to visualize CTb or BDA reaction products in addition to the WGA-HRP. After incubation according to Olucha et al. (1985) and stabilization of the WGA-HRP reaction product with CoCl₂ (black precipitate), CTb or BDA were visualized as a brown precipitate by using the protocols as described above.

The caudal medulla was divided into seven rostrocaudal levels extending from the obex (level 0) to 6 mm caudal to the obex (level -6). Each level consists of two 50-μm sections of a 1 of 10 series of consecutive sections (representing 1,000 μm: two times 50 μm times 10). For each level, photomicrographs were taken of representative sections by using a Nikon microscope and processed as described above.

Electron microscopy. In all midbrain injected cases, the lower brainstem and C1 segment were processed for electron microscopy. In each case, in one series of sections, WGA-HRP was reacted according to Olucha et al. (1985) and stabilized with CoCl₂. In M16, in two additional series of sections both WGA-HRP and CTb or BDA were visualized as described above, except for the concentration of Triton X-100, which did not exceed 0.02%. The tissue was osmificated in 1% osmium tetroxide (EMS) in phosphate buffer (0.1 M; pH 7.4; 1 hour), and stained in uranyl acetate (EMS; 1% in aquadest; 1 hour). After dehydration in a graded series of ethanol, the tissue was transferred to propylene oxide (10 minutes; EMS) and incubated in a mixture of Epon and propylene oxide (1:1) for 1 hour. The sections were embedded in Epon between plastic slides, left at 40°C for 1 night, and at 70°C for 2 nights. A selection was made of tissue containing anterogradely labeled fibers in the NRA ipsilateral to the injection side. In case M16, tissue was selected that contained both anterogradely labeled fibers (black) and retrogradely labeled neurons (brown). After blocking and trimming, this tissue was cut into semithin sections. Finally, ultrathin sections were cut, mounted on Butvar-coated copper grids, stained in uranyl acetate (1%; 10 minutes) and lead citrate (Reynolds solution; 10 minutes), and examined and photographed by using a JEOL electron microscope. The TMB reaction product (indicating WGA-HRP) was visible as electron dense crystalline structures. DAB reaction product (indicating CTb or BDA labeling) was electron dense but flocculent, and was often found around smooth endoplasmic reticulum or Golgi apparatus. After the electron micrographs were taken, the negatives were digitized by using a UMAX scanner and Macintosh PowerPC. Adobe Photoshop 8.0 was used to adjust brightness, contrast, and sharpness.

Subdivision of the PAG
Rostrocaudal. In the present study, the midbrain was cut in the transverse plane. Because the transverse plane in rhesus monkeys does not match the stereotaxic plane, stereotactic anterior-posterior coordinates cannot be used to indicate the different rostrocaudal levels of the PAG. To overcome this problem, and to be able to compare the different levels in the individual animals, in each of the animals the level was determined at which the commissure between the two inferior colliculi was not yet established. This level was called level zero (level 0; see Figs. 3, 5). Continuing rostrally, sections were selected representing levels one to eight at intervals of approximately 1 mm (every sixth section of a 1:4 series of 40-μm sections in cases M3, M4, M6, M7; every third section of a 1:6 series of 60-μm sections in M8 and M9; every second section of a 1:10 series of 50-μm sections for cases M14, M15, M16).

Longitudinal columns. The PAG can be subdivided into different columns (see Bandler and Shipley, 1991; Bandler and Shipley, 1994). The lateral and dorsal PAG are present throughout its entire rostrocaudal extent, whereas the dorsolateral PAG is absent caudally and well-developed rostrally. In contrast, the ventrolateral PAG is well developed caudally but absent more rostrally. In Figure 3, Nissl-stained sections have been used to demarcate the borders of the PAG. The borders between the different columns have been indicated based upon the subdivision in a previous study in macaques, in which NADPH-diaphorase was used to delineate the dorsolateral column (An et al., 1998).

RESULTS
Distribution of PAG neurons projecting to the NRA
Injection sites. In all six cases (M3, M4, M6, M7, M8, and M9), the injections into the caudal medulla oblongata involved the NRA at levels -2 to -4 (Fig. 2). At these levels, the NRA is most prominent (see VanderHorst et al., 2000 [accompanying study]). In addition, the injection sites included parts of the caudal part of the spinal trigeminal complex, the tegmentum medial to the NRA, and the white matter (Fig. 2).

Distribution of retrogradely labeled neurons in the midbrain
The PAG and adjacent tegmentum. In all cases, the majority of labeled neurons was found in the PAG and adjacent tegmentum. The densest cluster of labeled cells was located in the lateral PAG at the intercollicular level (levels 2 to 4 in Figs. 3, 4A,B,E). Within the lateral PAG, this cluster occupied a medial position. In all cases, the labeled neurons in this cluster were heavily labeled and possessed large dendrites (Fig. 4E). Their average soma size was 328 μm² (± 16 SEM; n = 45) in M4 and 377 μm² (± 21 SEM; n = 45) in M6. These large, heavily labeled neurons were distributed bilaterally, but with an ipsilateral predominance (ipsilateral: contralateral = 4:1).

Labeled cells were also found more laterally in the lateral PAG (levels 1 to 5 in Fig. 3; Fig. 4C,F), in the ventrolateral PAG (levels 1 to 3 in Fig. 3), and in the tegmentum lateral and ventrolateral to the PAG (levels 1 to 7 in Fig. 3), including the pedunculopontine nucleus (Fig. 3, level 2). In contrast to the labeled neurons in the medial part of the lateral PAG, the majority of these cells were lightly labeled (Fig. 4F), were present at all rostrocaudal levels, and were sparse in number contralateral to the injection side. The average size of the labeled neurons in these areas was 211 μm² (± 13 SEM; n = 45) in M4 and 215μm²
Although some of these neurons were multipolar, most of them were fusiform or triangular in shape and had small dendrites. They were most abundant in cases M7 and M9, in which the injections involved much of the area immediately ventromedial to the NRA. In the ventral part of the caudal PAG, a few small labeled cells were found in the area immediately adjacent to the ependyma (levels 0 and 1 in Fig. 3; Fig. 4D). More rostrally in the ventral PAG, some labeled cells were present dorsal to the oculomotor nucleus (levels 3 to 7 in Fig. 3). Labeled neurons in this area were present bilaterally and were most numerous in cases M7 and M9 with injections extending into the tegmentum medial to the NRA.

The dorsal PAG contained a few labeled neurons which were mainly located at levels 1 to 4 (Fig. 3). Occasionally, labeled neurons were found scattered throughout the dorsolateral PAG.

Remaining areas. Ventral to the caudal PAG and continuing further caudally, a group of labeled cells was...
Fig. 3. Schematic drawings of transverse sections of the midbrain at intervals of 1 mm in case M4 showing the distribution of retrogradely labeled neurons after a wheat germ agglutinin-horseradish peroxidase (WGA-HRP) injection into the nucleus retroambiguus (NRA). Each drawing is composed of 1 section, and each dot represents 1 labeled neuron. Large dots indicate densely labeled neurons, whereas the small dots indicate lightly labeled neurons (see Fig. 4). Note the compact group of labeled neurons in the lateral periaqueductal gray at levels 2–4. BC, brachium conjunctivum; BIC, brachium; BP, brachium pontis; C, nucleus Cajal; DR, dorsal raphe nucleus; CP, posterior commissure; CSN, central superior nucleus; CUN, cuneiforme nucleus; D, nucleus of Darkshewitsch; IC, inferior colliculus; IPN, interpeduncular nucleus; LGB, lateral geniculate body; LL, lateral lemniscus; MGB, medial geniculate body; ML, medial lemniscus; MLF, medial longitudinal fascicle; nIV, trochlear nerve; nLL, nucleus of the lateral lemniscus; nIII, oculomotor nerve; PBN, parabrachial nucleus; PC, pedunculus cerebri; PON, pontine nuclei; PUL, pulvinar; RL, rostral linear nucleus; RN, red nucleus; SC, superior colliculus; SN, substantia nigra; xBC, decussation of the brachium conjunctivum; III, oculomotor nucleus.
found in the ventrolateral part of the ventral pontine tegmentum (levels 0 and 1 in Fig. 3). In contrast to the labeled neurons in the PAG, these cells were predominantly located contralateral to the injection site. Also contralaterally, labeled neurons were present in the magnocellular part of the red nucleus, whereas a few scattered labeled neurons were found in the deep layers of the superior colliculus. Ipsilateral to the injection and caudal to the PAG, some labeled neurons were found in the locus coeruleus and parabrachial nucleus (Fig. 3, level 0). Occasionally, a few lightly labeled MesV neurons were observed.

**Projection from the PAG and adjacent tegmentum to the caudal medulla**

**Injection sites.** In two cases (M15 and M16), the injection sites were located within the PAG (Fig. 5). In M15, it involved the ventrolateral part of the caudal PAG (levels 2 and 3), and extended into the lateral PAG at level 4. In M16, the injection was larger and occupied...
the lateral and ventrolateral parts of the PAG at levels 3 to 7. It also involved the dorsolateral PAG at levels 6 and 7. In one case, M14, the core of the injection site was located ventrolateral to the PAG at levels 4 to 5 (Fig. 5).

**Distribution of anterogradely labeled fibers in the nucleus retroambiguus and adjacent tegmentum.** In M16, with a large injection in the lateral and ventrolateral PAG slightly extending into the laterally adjacent tegmentum, a heavy projection was found in the NRA (Fig. 6). This projection was bilateral, but with a clear ipsilateral preponderance. Rostrocaudally, it was present between the obex and 5 mm caudal to the obex and was largest 1 to 3 mm caudal to the obex. Anterogradely labeled fibers were observed in close proximity to NRA neurons that were labeled after tracer injections into the lumbosacral cord (Fig. 7A,B). However, at levels 2 to 3 mm caudal to the obex, the part of the NRA just lateral to the lumbosa-
Fig. 6. Low magnification photomicrographs of transverse sections at different levels of the lateral part of the caudal medulla oblongata ipsilateral to the periaqueductal gray injection site (M16). Note that the entire rostrocaudal extent of the nucleus retroambiguus (NRA) receives a dense anterograde projection. Red dots in the drawings represent retrogradely labeled cells in the photomicrographs. Cu, cuneate nucleus; ECU, external cuneate nucleus; G, gracile nucleus; IO, inferior olivary complex; LRN, lateral reticular nucleus; ML, medial lemniscus; NRA, nucleus retroambiguus; NTS, nucleus of the solitary tract; P, pyramidal tract; Vspin caud, caudal nucleus of the spinal trigeminal complex; Xd, dorsal vagal nucleus; XII, hypoglossal nucleus. Scale bar = 500 μm.
Fig. 7. A,B: Photomicrographs of the nucleus retroambigus (NRA) at 3 mm caudal to the obex in M16, showing retrogradely labeled NRA-lumbosacral neurons (brown reaction product) and anterogradely labeled periaqueductal gray (PAG) axons (black reaction product). In A, note that the density of anterogradely labeled fibers is higher in the lateral part of the NRA, than in its medial part where the NRA-lumbosacral neurons are located. The higher magnification in B shows anterogradely labeled PAG axons in close proximity to retrogradely labeled NRA-lumbosacral neurons (arrowheads). The arrows point at retrogradely labeled neurons that project to the PAG (black reaction product). C,D: Polarized light photomicrographs of anterogradely labeled fibers 2 mm caudal to the obex ipsi- (C) and contralateral (D) to the injection site in M15. Note that the anterograde projection in M15 is almost exclusively confined to the NRA, whereas very few retrogradely labeled neurons are present. The anterograde projection is stronger ipsilaterally than at the contralateral side. E,F,G: Polarized light photomicrographs of the ambiguus complex 4 mm rostral to the obex and ipsilateral to the PAG injection in cases M14, M15, and M16, respectively. The outer dashed line represents the boundaries of the ambiguus complex, and the inner dashed circle indicates the compact formation. In M14, a strong anterograde projection is present in the ventral medullary medial tegmentum, but the ambiguus complex receives hardly any labeled fibers. In M15, sparse projections are present in the ambiguus complex and the adjacent areas. In M16, the ambiguus complex and adjacent lateral tegmentum receive a moderate projection, whereas the projection to the ventral medullary medial tegmentum is sparse. IO, inferior olive; LRN, lateral reticular nucleus; VMMT, ventral medial medullary tegmentum; Vspin caud, caudal nucleus of the spinal trigeminal complex. Scale bars = 100 μm in A,B; 900 μm in C (applies to C–G).
cral cord-projecting neurons appeared to receive an even stronger projection (Fig. 7A).

In addition to labeled fibers in the NRA, some were present in the tegmentum medial to the NRA, dorsal to the NRA, in the area extending dorsomedially between the NRA and the nucleus of the solitary tract (NTS), in the commissural nucleus of the NTS, and in the caudal tip of the inferior olive. These projections were less dense than the projection to the NRA (Fig. 6) and were almost entirely limited to the side. No labeled fibers were present in the lateral reticular nucleus.

In M15, with a smaller injection restricted to the ventrolateral and lateral caudal PAG, the projection to the lower brainstem was not as dense as in M16 but was more specific. Labeled fibers were found almost exclusively in the NRA (Fig. 7C,D). Similar to M16, this projection was bilateral, with an ipsilateral predominance. It extended from 1 to 4 mm caudal to the obex. In M14, with an injection just ventrolateral to the PAG, some labeled fibers were found in the NRA bilaterally. However, compared with the injections involving the PAG (M15 and M16), the projection to the NRA was weak and was not distinguished from the projection to the tegmentum medial and dorsomedial to the NRA. Similar to M16, the caudal pole of the olivary complex contained some anterogradely labeled fibers.

**Retrogradely labeled neurons in the ambiguous complex at the level of the NRA.** In all three cases, a few retrogradely labeled neurons were found in the NRA ipsi- and contralateral to the injection site. In M14 and M16, with injections involving the lateral part of the lateral PAG or the adjacent tegmentum, numerous retrogradely labeled neurons were present contralaterally in the medial tegmentum and in the superficial layer of the caudal spinal trigeminal complex. Ipsilaterally, a few labeled neurons were present in a band extending from the NTS to the area ventromedially to the NRA, and dorsal to the NRA. In contrast to cases M14 and M16, in case M15, with an injection involving more medial parts of the PAG, the caudal medulla contained only a few labeled neurons.

**Distribution of anterogradely labeled fibers in the ambiguous complex and adjacent tegmentum.** In cases M15 and M16, with injections involving the PAG, labeled fibers were present in the ambiguous complex (see Discussion section) ipsilateral to the PAG injection site. Rostrocaudally, the projection included the entire complex from the obex (Fig. 6) to the caudal pole of the facial nucleus. In both cases, the projection from the PAG to the ambiguous complex was less dense then the PAG-NRA projection (for M16: compare Fig. 6 obex and Fig. 7G representing 4 mm rostral to the obex with Fig. 6 level –2; for M15: compare Fig. 7C with Fig. 7F). Also in contrast to the projection to the NRA, very few labeled fibers were present in the contralateral ambiguous complex. In case M16, with an injection involving more rostral and dorsal parts of the PAG than in M15, the projection to ambiguous complex was denser than in M15 (Fig. 7F,G).

In M14, with an injection just lateral to the caudal PAG, no clear projection was present in the ambiguous complex, but numerous anterogradely labeled fibers were present in the ventral part of the medullary medial tegmentum (Fig. 7E).

**Electron microscopy.** In M15 and M16, the PAG-NRA projection was examined at the ultrastructural level. Anterogradely labeled terminal profiles were found in the NRA of both cases, ipsi- and contralateral to the injection site (Figs. 8, 9). Labeled terminal profiles were filled with primarily round vesicles and contained a few dense core vesicles (Fig. 8A,B). They formed asymmetrical synaptic contacts with proximal dendrites, distal dendrites, and occasionally with somata (Figs. 8, 9). No axo-axonal contacts were found.

Some terminals contained pleomorphic vesicles (Fig. 8C), or large numbers of dense core vesicles in addition to round vesicles (Fig. 8D). These terminals formed symmetrical contacts with proximal dendrites. Occasionally, reaction product was present in small myelinated axons. In M16, anterogradely labeled terminal profiles were found to contact retrogradely labeled dendrites of NRA neurons that send projections to the lumbosacral cord (Fig. 9; see also legends).

**DISCUSSION**

The PAG-NRA pathway in the rhesus monkey

**PAG neurons that project to the NRA are mainly located in the medial part of the lateral PAG.** The retrograde study showed that injections involving the NRA densely labeled a compact group of relatively large neurons in the medial part of the lateral PAG at the intercollicular level. Injections involving not only the NRA but also extensive parts of the tegmentum medial to the NRA labeled neurons (mostly small in size) laterally in the lateral and ventrolateral PAG, and in the adjacent tegmentum. Strong anterograde projections from the midbrain to the NRA were found after injections involving the medial part of the lateral and ventrolateral PAG.

Combining the results of the antero- and retrograde tracing studies, it can be concluded that a compact group of relatively large neurons in the lateral PAG at the intercollicular level sends a strong and specific projection to the NRA. Relatively small neurons in the lateral and ventrolateral PAG and the adjacent tegmentum, in all likelihood, give rise to a more diffuse projection to the medullary medial tegmentum, although some of these fibers may terminate in the NRA. Findings in an earlier study in the macaque describing PAG projections to the medial medulla oblongata (Chung et al., 1983) support this finding.

The borders of the cluster of PAG-NRA neurons in the rhesus monkey are more restricted compared with tracing studies in other species. In the cat (Holstege, 1989) and rat (Ennis et al., 1997; Holstege et al., 1997), PAG neurons that project to the NRA and surrounding area were found to be dispersed throughout the entire lateral PAG and adjacent tegmentum. However, tracer injections limited to the NRA labeled a circumscribed group of neurons in the lateral PAG in the cat (VanderHorst and Holstege, 1996).

The differences in cell size between NRA-projecting neurons in the lateral PAG and other neurons in the PAG and adjacent tegmentum that project to the caudal medulla oblongata are in line with a study in the rat (Chen and Aston-Jones, 1996). Also in agreement with previous studies in other species is that PAG-NRA projections in the rhesus monkey are clearly more substantial to the ipsilateral than to the contralateral side (4:1) (Holstege, 1989; Chen and Aston-Jones, 1996; Holstege et al., 1997). However, there exist also a few differences. In the present study, no projections were found to the lateral
reticular nucleus after PAG injections (in contrast to Mantyh, 1983, in the monkey), and the NRA injections labeled only very few neurons in the supraoculomotor area (in contrast to Chen and Aston-Jones, 1996; Ennis et al., 1997, in the rat).

**The PAG-NRA projection involves the entire NRA.** The NRA in the rhesus monkey extends between 1–5 mm caudal to the obex, but is most distinct between 2 and 4 mm caudal to the obex (see VanderHorst et al., 2000 [accompanying study]). The present study shows that the

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**Fig. 8.** A,B: Electron photomicrographs of wheat germ agglutinin-horseradish peroxidase (WGA-HRP)–labeled terminal profiles in the nucleus retroambiguus of M15 (A) and M16 (B,C,D). In A and B, the terminal profiles form asymmetrical contacts with dendrites and contain large numbers of mainly spherical vesicles and an occasional dense core vesicle (small arrow in B). Note the postsynaptic densities.

C: A small terminal profile filled with pleomorphic vesicles forms a symmetrical contact with a proximal dendrite. D: A large terminal profile containing large numbers of dense cored and small round vesicles makes a symmetrical contact with a proximal dendrite. d, dendrite; arrowheads, synaptic contacts; white asterisks, WGA-HRP labeling. Scale bar = 1 μm in D (applies to A–D).
Fig. 9.  
A,B: Electron photomicrographs of wheat germ agglutinin-horseradish peroxidase (WGA-HRP)–labeled terminal profiles in the nucleus retroambiguus of M16. In A, the terminal profile contains large numbers of spherical vesicles and contacts a cholera toxin subunit b (CTb)–labeled dendrite (black asterisk). In B, a small terminal profile filled with round vesicles opposite the origin of a proximal dendrite. B is a magnification of the box in C. 
D: A large, densely labeled terminal profile containing round vesicles in close contact to a dendrite heavily labeled with CTb. A clear synaptic contact is not present. 
E: Two labeled terminal profiles next to a CTb-labeled proximal dendrite. One of the terminals forms a synaptic contact (arrowhead) and contains mainly round vesicles. d, dendrite; arrowheads, synaptic contacts; white asterisks, WGA-HRP labeling; black asterisks, CTb labeling in dendrites of NRA neurons; er, endoplasmatic reticulum; n, nucleus. Scale bars = 1 μm in B,E (applies to A,B,D,E); 10 μm in C.
entire rostrocaudal extent of the NRA receives a projection from the PAG. The projection includes NRA neurons that project to the lumbosacral cord, as demonstrated in the present study, and in all likelihood also NRA neurons that send their axons to larynx motoneurons in the nucleus ambiguus (VanderHorst et al., unpublished observations).

These findings of the entire rostrocaudal extent of the NRA receiving input from the PAG is in line with the PAG-NRA projection described in the cat (Holstege, 1989; VanderHorst and Holstege, 1996) and with the figures presented in the study by Jürgens and Pratt (1979) in the monkey. They showed a bilateral projection from the PAG to the area just dorsomedial to the lateral reticular nucleus. This area corresponds to the NRA, although the horizontal sections used in their study may have been a factor in the confusion between nucleus retroambiguus and nucleus ambiguus.

The existence of monosynaptic PAG-NRA projections. In the present study, PAG-NRA terminals formed monosynaptic contacts with NRA neurons that were retrogradely labeled after tracer injections into the lumbosacral cord. The majority of PAG axon terminal profiles contained primarily spherical vesicles, a few dense cored vesicles, and formed asymmetrical synaptic contacts with somata, proximal and more distal dendrites. These terminals might have an excitatory effect on NRA neurons. This hypothesis is supported by physiologic studies on vocalization-related NRA neurons in the cat and guinea pig (Zhang et al., 1995; Shiba et al., 1997). Injection of excitatory amino acids in the NRA was found to elicit vocalization, whereas PAG-induced vocalization could be abolished by transection of the medulla 1 mm caudal to the obex, thus transecting the PAG-NRA pathway (Zhang et al., 1995). Similarly, Shiba et al. (1997) reported that PAG-induced vocalization was abolished by bilateral injections of high doses of kainic acid into the NRA. In addition, Shiba et al. (1997) identified NRA neurons that projected to the contralateral nucleus ambiguous and that received input from the PAG.

However, not all terminal profiles contained round vesicles and formed asymmetrical contacts. Terminals with pleomorphic vesicles and symmetrical contacts might have modulatory or inhibitory effects on NRA neurons. The terminals with a large number of dense-cored vesicles may contain peptides. Because the PAG is known to contain neurons immunoreactive for peptides such as substance P, vasoactive intestinal polypeptide, and enkephalin (e.g., Moss and Basbaum, 1983; Moss et al., 1983), any of these peptides might be involved. This type of terminal might have a modulatory effect on NRA neurons. A double-labeling study at the ultrastructural level is necessary to determine the content of the synaptic vesicles.

In addition to the NRA dendrites that contained retrograde label, numerous dendrites that were postsynaptic to PAG terminals were not labeled. This finding may be because not all NRA neurons were labeled, the retrograde label from the cord injections did not completely fill NRA dendrites and/or dendrites other than those of NRA neurons received projections from the PAG. In summary, we provide evidence that there are monosynaptic projections from the PAG to NRA neurons, but there may exist projections to dendrites of other neurons.

Do somatic motoneurons in the nucleus ambiguus receive direct input from the PAG? A major point of discussion in the literature is whether ambiguous motoneurons receive direct input from the PAG. The ambiguous complex contains somatic motoneurons, cardiovascular motoneurons, as well as interneurons (Delgado-Garcia et al., 1983; Yoshida et al., 1984, 1985; Hopkins and Armour, 1998). Moreover, it is coextensive with the rostral ventral respiratory group and is invaded by dendrites of A1 and C1 cell groups (Hopkins et al., 1996).

With respect to the somatic motoneurons innervating laryngeal muscles, the intrinsic laryngeal muscles are innervated by motoneurons in the caudal ambiguous complex (Yoshida et al., 1984, 1985), whereas motoneurons innervating the cricothyroid muscle (an extrinsic laryngeal muscle) are present more rostrally, dorsomedial to the compact formation at 4–6 mm rostral to the obex (Yoshida et al., 1984, VanderHorst et al., unpublished observations). The laryngeal motoneurons do not form a compact group but are distributed among interneurons.

The results of the present study showed that rostral to the NRA in the ambiguous complex (compare “level obex” in Fig. 6 of the present study and Fig. 1 of VanderHorst et al., 2000 [accompanying study], and see Fig. 7F,G) the density of the PAG projection decreased but did not diminish. In contrast to the PAG-NRA projection, this projection is weak (see Fig. 6) and is almost exclusively present ipsilaterally. These observations are in good agreement with earlier reports in the squirrel monkey (Mantyh, 1983) and rat (Ennis et al., 1997). In other words, the projection from the PAG to the ambiguous complex is sparser than the PAG-NRA and the NRA-ambiguous projections (Holstege, 1989; VanderHorst et al., unpublished observations). This stresses the importance of the PAG-NRA-ambiguous pathway, which is involved in vocalization (see below).

PAG neurons might project directly to somatic motoneurons in the ambiguous complex, but such connections have not yet been demonstrated. Another option is that the PAG projection to this area of the medulla oblongata involves interneurons that control laryngeal vocal cord, orofacial, and swallowing movements (see Jürgens and Pratt, 1979; Jürgens, 1998; Yajima and Larson, 1993), play a role in respiration (by means of the rostral ventral respiratory group; see Feldman, 1986), or a role in heart (Inui and Nosaka, 1993; Standish et al., 1994, Ennis et al., 1997). An ultrastructural study will be necessary to determine the characteristics of the projection from the PAG to motoneurons and interneurons in the ambiguous complex.

Does the NRA contain neurons that project to the PAG? In addition to anterogradely labeled fibers in the NRA, a few retrogradely labeled neurons were found at both sides in the NRA after PAG injections. These findings suggest that there is a reciprocal connection between the PAG and the NRA. However, the tracer WGA-HRP can be transported transneuronally. Therefore, it cannot be excluded that neurons in the NRA were labeled by means of transneuronal transport from heavily labeled PAG-NRA fibers.

Functions of the PAG-NRA pathway

The PAG is involved in a wide variety of emotional functions such as aggressive and defensive behavior, blood pressure control, analgesia, receptive posture, quiescence, tachypnea, micturition, and vocalization (see Magoun et al., 1937; Mayer et al., 1971; Liebeskind et al., 1973; Sakuma and Pfaff, 1979a,b; Zhang et al., 1990; Bandler and Depaulis, 1991; Carrive and Bandler, 1991; Lovick,
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1993; Blok and Holstege, 1994; Bandler and Shipley, 1994). Combination of these functions results in different types of stereotypical, species-specific behavior. For example, receptive behavior is characterized not only by receptive posture, immobility, and mating calls but also by less overt reactions such as analgesia and changes in blood pressure. Aggressive behavior is accompanied with cardiovascular changes and various types of vocalization.

Different parts in the PAG are involved in the control of these emotional responses (see Bandler and Depaulis, 1991; Bandler and Shipley, 1994). The ventrolateral PAG has a role in immobility, quiescence, hypotension, brady-cardia, and opioid-dependent analgesia, whereas the lateral PAG is known to mediate flight-flight, hypertension, tachycardia, and nonopiod analgesia. Similarly, the efferent connections, afferent connections, or both, between the PAG and forebrain structures such as the prefrontal cortex (see An et al., 1998), ventromedial hypothalamus (see Canteras et al., 1994), medial preoptic area (see Veening et al., 1991), spinal cord (see Yezierski, 1988; VanderHorst et al., 1996), solitary complex (Bandler and Tork, 1987), and parabrachial nucleus (Krouth et al., 1998) are restricted to distinct parts within the PAG. The finding that the PAG-NRA neurons form a compact cluster in the medial part of the lateral caudal PAG agrees with the concept that different parts in the PAG are involved in the control of different emotional responses and further supports the idea of a distinct mediolateral organization in the monkey PAG.

The PAG controls the different components of emotional behavior by means of projections to different nuclei in the brainstem such as the rostral and caudal ventrolateral medulla for blood pressure control (Carrive et al., 1988; Lovick, 1993), Barrington’s nucleus for micturition (Blok and Holstege, 1994) and the ventral part of the pontomedullary tegmentum for level- and gainsetting mechanisms (review see Lovick, 1993; Mason and Leung, 1996).

The NRA is another cell group in the brainstem that receives extensive input from the PAG. Because the NRA projects to distinct sets of somatic motoneurons, the PAG-NRA pathway is involved in stereotypical emotional motor behavior that requires the activation of distinct sets of striated muscles. Examples of such behavior are emotionally related changes in respiration, vocalization, and reproductive posture.

Respiration. The NRA has long been known to be involved in expiration and expiration-related activities such as vomiting and other forms of straining-related activities (Merrill, 1970, 1974; Miller et al., 1985, 1987, 1995). In this context, the NRA receives input from respiratory-related neurons in the brainstem (Feldman, 1988; Smith et al., 1989; Gerrits and Holstege, 1996). In addition, the PAG is able to affect respiration. During aversive and defensive reactions evoked by stimulation in the PAG, changes in the respiratory pattern have been documented (see Davis et al., 1996a). The PAG in cats and monkeys contains not only neurons that are related to vocalization or a combination of vocalization and respiration but also neurons that are related exclusively to respiration. In contrast to the vocalization-related sites, the sites from which changes in respiration were elicited by microinjection of excitatory amino acids were widely distributed throughout the PAG and the adjacent tegmentum (Davis et al., 1996a; Larson, 1991; Zhang et al., 1994). Possibly, some of the small PAG-NRA neurons serve a function-related exclusively to respiration. However, separate pathways might be involved in the PAG control of expiration and inspiration (Shiba et al., 1997). High dose kainic acid injections into the NRA in cats abolished phasic activation of expiratory abdominal muscles after PAG stimulation, which indicates that the PAG-NRA pathway serves expiration. PAG-induced inspiration was not affected, and might be controlled by respiratory neurons in more rostral parts of the medulla (Merrill, 1974).

Vocalization, including mating calls. Vocalization is a modified form of respiration, i.e., an increased inspiratory effort, followed by a controlled and sustained expiratory effort with laryngeal and orofacial modification of the expiratory flow. Vocalization elicited in the PAG is natural sounding and requires a high degree of coordination between laryngeal, expiratory, orofacial, and tongue muscles (Jürgens and Pratt, 1979; Larson, 1985; Zhang et al., 1994). In contrast to PAG-induced vocalizations, vocalizations elicited in the NRA are not natural sounding, because of lack of involvement of orofacial and tongue muscles (Zhang et al., 1995). This illustrates the function of the PAG in the integration of species-specific behavior and indicates that the NRA is only involved in one of the components of vocalization, i.e., premotor control of expiratory and laryngeal vocal cord muscles. It remains unclear how the PAG coordinates the activation of orofacial and tongue muscles during vocalization. A possibility is by means of a pathway from the lateral PAG and adjacent tegmentum to other brainstem areas, such as to motor- or interneurons in the ambiguus complex (Jürgens and Pratt, 1979; Ennis et al., 1997). Stimulation in this area has been shown to elicit orofacial and tongue activation (Zhang et al., 1995). Another possibility is that the PAG-NRA pathway sends collateral fibers to this area.

Each species has its own repertoire of vocalizations. Macaques use at least 13 unvoiced harsh noises such as barking, growling, and squeaking, and even more voiced calls, such as cooing (e.g., Napier and Napier, 1967; Erwin, 1975). In the cat, microstimulation experiments suggest that small groups of neurons in the PAG are involved in the different types of vocalization (Zhang et al., 1994). Vocalized vocalizations, involving the activation of thyroarytenoid and cricoarytenoid muscles together with expiratory abdominal and intercostal muscles, could be elicited mainly in the caudal, lateral PAG. Unvoiced vocalizations, involving the posterior cricoarytenoid, thyroarytenoid, and orofacial muscles, were evoked from a more rostral and slightly dorsolateral region of the PAG (Zhang et al., 1994). These results have been confirmed in the guinea pig (Kyuhou and Gemba, 1998). In the cat, the PAG-NRA pathway has been demonstrated to be involved in voiced vocalization (Zhang et al., 1995). Transection of the medulla just rostral to the NRA abolishes the activity of the thyroarytenoid and cricoarytenoid muscles (involved in voiced vocalization). Because this procedure could not completely eliminate activation of the posterior cricoarytenoid and orofacial muscles (predominantly involved in unvoiced vocalization), and stimulation in the NRA did not evoke unvoiced vocalizations (Zhang et al., 1995), a separate PAG-medullary pathway might be involved in unvoiced vocalization.

In macaques, the main area from which vocalization has been elicited by electrical stimulation or in which neuronal activity was recorded during vocalization (Larson, 1985, 1991; Larson and Kistler, 1986) is a restricted area
of approximately 1 mm² in the medial half of the caudal, lateral PAG (according to the subdivision in An et al., 1998, and the present study). Apart from this area, only a few dispersed vocalization-related cells were found throughout the PAG and in the adjacent tegmentum. The location of the PAG-NRA neurons in the present study is in line with these physiologic results in macaques. However, in addition to the caudal, lateral PAG, also the intermediate, dorsolateral PAG seems to be involved in vocalization in macaques (Larson, 1985; Larson and Kistler, 1986). In these studies, no distinction was made between voiced and unvoiced vocalization sites. On the basis of the vocalization experiments in the cat, the intermediate, dorsolateral PAG is involved in unvoiced vocalization (Zhang et al., 1994). However, the presence of vocalization-related neurons in the dorsolateral PAG is not in line with the very small numbers of PAG neurons in the dorsolateral PAG that project to the nucleus retroambiguus area.

Vocalization forms, together with facial expression, an important way to communicate emotions. Like other species, humans have their own species-specific repertoire of vocalizations such as laughing, crying, and groaning. These vocalizations serve nonverbal emotional vocal expression and remain present after bilateral lesions of the facial motor cortex or corticospinal tract (see Jürgens and Zwirner, 1996). This finding suggests that the PAG-NRA pathway described in the cat (VanderHorst and Holstege, 1995) might be the posture displayed during mating. Similar to the monkey which part of the NRA is involved in vocalization, the PAG-NRA pathway in humans would be involved in nonverbal vocalizations. In addition, this pathway might mediate emotional intonation, motivation or initiation to communicate, and vocal intensity (Larson, 1985; Jürgens, 1998). Apart from the nonverbal emotional vocal expression, humans make use of verbal expression that requires the neocortex (speech). At this point, it is not clear whether the PAG also plays a role in nonemotional vocalization (Jürgens, 1998; Davis et al., 1996).

**Submitive and reproductive behavior** The PAG-NRA projection described in the cat (VanderHorst and Holstege, 1996) involves the entire rostrocaudal extent of the NRA, similar to the monkey. However, in the cat vocalization-related NRA neurons are located between 1 and 4 mm caudal to the obex (Zhang et al., 1995), whereas the NRA in the cat extends until 8 mm caudal to the obex (VanderHorst and Holstege, 1995). This finding indicates that the PAG-NRA pathway may serve another function apart from vocalization. Although it is not known in the monkey which part of the NRA is involved in vocalization, the PAG-NRA pathway in the monkey might also serve more than one function.

Another function that requires the PAG-NRA pathway might be the posture displayed during mating. Similar to vocalization, mating behavior is an example of highly stereotypical, species-specific behavior that requires the coordinated activation of a set of striated muscles. The PAG is as crucial for female receptive behavior in rats (Sakuma and Pfaff, 1979a,b), as it is for vocalization in a wide variety of species. However, only very limited information is available about the role of the midbrain in receptive behavior in monkeys.

The NRA in the rhesus monkey does not only project to expiratory and laryngeal motoneurons involved in vocalization but also to motoneurons innervating axial, ilio- soas, and pelvic floor muscles. This set of muscles might serve the receptive posture in female monkeys. In other species, such as cat and hamster, the NRA projects to sets of motoneurons which are likely to be involved in mating behavior in these species (VanderHorst and Holstege, 1995, 1997; Gerrits and Holstege, 1999; VanderHorst et al., 2000 [accompanying study]).

In the rhesus monkey, the posture displayed during mating serves not only reproduction, but is also part of social interactions in general. Both males and nonestrous females use the posture to show submissiveness (presenting behavior; Napier and Napier, 1967). Although the function of this type of behavior is completely different from mating, it can be viewed as a form of emotional expression between members of the same species. As such, it serves a similar function as vocalization. Physiologic studies will be necessary to test this hypothesis.

In females, reproductive behavior and vocalizations related to mating (Oda and Masataka, 1995 in macaques) are (partly) dependent on the estrous cycle. This finding leads to the question whether the PAG-NRA pathway is affected by estrogen. In the cat, in which receptive behavior is completely estrogen dependent, the PAG contains numerous estrogen receptor-alpha immunoreactive neurons that project to the NRA (VanderHorst et al., 1997). At present, a study in the rhesus monkey is under way to determine the presence of estrogen receptors in PAG-NRA neurons in primates.

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