Ascending projections from spinal cord and brainstem to periaqueductal gray and thalamus
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Chapter 3

Two parts of nucleus prepositus hypoglossi project to two different subdivisions of the dorsolateral periaqueductal gray in cat

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

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ABSTRACT

The dorsolateral column of the mesencephalic periaqueductal gray (PAG) is a separate part of the PAG. Its afferent sources, efferent targets and neurochemical properties differ from the adjacent PAG columns. The dorsolateral PAG is thought to be associated with aversive behaviors, but it is not yet understood how these behaviors are brought about.

In order to further elucidate its function, in the present study we investigated which brainstem regions project to the dorsolateral PAG. Wheatgerm agglutinin-horseradish peroxidase (WGA-HRP) injections involving the dorsolateral PAG, but extending into the lateral part, resulted in many retrogradely labeled cells in the pontine and medullary tegmentum bilaterally. However, it was concluded that these neurons were labeled from the lateral PAG, because no anterograde labeling was found in the dorsolateral PAG after a large injection into the tegmentum. Retrogradely labeled cells were also found in the nucleus prepositus hypoglossi (PPH), mainly contralaterally. Injections of \(^{3}H\)-leucine or WGA-HRP in the PPH resulted in anterogradely labeled fibers in the dorsolateral PAG. Two separate distribution patterns were found. The caudal and intermediate PPH projected to a small region on the dorsolateral edge of the dorsolateral column, while the supragenual PPH distributed labeled fibers to all other parts of the dorsolateral PAG, except the area on the dorsolateral edge. These separate PPH projections suggest that there exist two subdivisions within the dorsolateral PAG. The present findings suggest a role for the dorsolateral PAG in the oculomotor system.

INTRODUCTION

The mesencephalic periaqueductal gray (PAG) plays a pivotal role in the output of the limbic system. It is well known for its role in nociception control (Mayer et al., 1971; Liebeskind et al., 1973; Vanegas and Schaible, 2004) and emotional motor output (Holstege et al., 2004). Stimulation of the PAG in animals can elicit several integrated defensive behaviors as aggression or freezing (Bandler and Depaulis, 1991; Carrive, 1993), but also more specific motor functions as vocalization (Kanai and Wang, 1962), adopting mating posture (Sakuma and Pfaff, 1979) and micturition (Blok and Holstege, 1996).

The PAG receives many afferents from major parts of the prefrontal cortex (Shipley et al., 1991), but also from the hypothalamus (Mantyh, 1982; Berk and Finkelstein, 1982; Holstege, 1987; Shipley et al., 1991), amygdala (Hopkins and Holstege, 1978; Mantyh, 1982), bed nucleus of the stria terminalis (Holstege et al., 1985) and several other limbic structures. Other afferents originate from large parts of brainstem and...
spinal cord (Mantyh, 1982; Bjorkeland and Boivie, 1984; Bandler and Tork, 1987; Yezierski and Mendez, 1991; Herbert and Saper, 1992; Mouton and Holstege, 2000). In turn, the PAG projects very strongly to the ventromedial tegmentum of caudal pons and medulla, with neurons that project to dorsal and ventral parts of the spinal gray matter throughout the length of the spinal cord. These PAG-medial brainstem-spinal cord projections are involved in level setting systems, including nociception control. In addition, the PAG projects to more laterally located brainstem structures involved in more specific behaviors (for review, see Holstege et al., 2004).

The dorsolateral PAG (PAGdl) differs from the adjacent columns of the PAG. PAGdl has been defined as a wedge-shaped column that has a significant presence in the rostral and intermediate parts of the PAG and gradually diminishes in the caudal parts (Bandler et al., 1991; Bandler and Shipley, 1994). PAGdl has frequently been identified by the presence of numerous nicotinamide dinucleotide phosphate diaphorase (NADPH-d) positive cells (fig. 1; Mizukawa et al., 1989; Herbert and Saper, 1992; Carrive and Paxinos, 1994; Floyd et al., 2000). In contrast to the dorsomedial, lateral and ventrolateral parts of the PAG, PAGdl does not receive afferents from most limbic regions, such as amygdala (Hopkins and Holstege, 1978) and bed nucleus of the stria terminalis (Holstege et al., 1985), brainstem or spinal cord (Wiberg and Blomqvist, 1984b; Illing and Graybiel, 1986; Mouton and Holstege, 2000) and does not give rise to the descending projections to brainstem and spinal cord (Illing and Graybiel, 1986; Yezierski and Mendez, 1991; Cowie and Holstege, 1992). On the other hand, some distinct brain areas project exclusively or mainly to PAGdl, such as areas 10m, 25 and 32 of the caudal medial prefrontal cortex (Hardy and Leichnetz, 1981; Illing and Graybiel, 1986; An et al., 1998; Freedman et al., 2000; Floyd et al., 2000) and the ventromedial hypothalamic nucleus (Canteras et al., 1994; Roeling et al., 1994), or receive input from only this part of the PAG, such as the cuneiform nucleus (Redgrave et al., 1988).

Besides its distinct afferent and efferent projection systems, PAGdl also differs from other parts of the PAG with respect to its neurochemistry. For example, NADPH-d (fig. 1) and cholecystokinin (Liu et al., 1994) are specifically present in PAGdl, while cytochrome oxidase (Conti et al., 1988) is found in almost all parts of the PAG, except PAGdl.

Functionally, PAGdl has been associated with defensive and aversive responses, such as threat and confrontational behaviors (rostral PAGdl) or flight (caudal PAGdl) accompanied by changes in heart rate, blood pressure and regional blood flow (for review, see Keay and Bandler, 2001; Vianna and Brandao, 2003), but it is not known how PAGdl coordinates these behaviors. In order to further elucidate its function, we have investigated which pontine and medullary regions specifically project to PAGdl. From the results it appeared that the only region in pons and medulla that projects to PAGdl is the nucleus prepositus hypoglossi (PPH). Projections from this nucleus were, therefore, investigated further.

**MATERIALS AND METHODS**

A total of seventeen cats was used. The surgical procedures, pre- and postoperative
PPH projections to dorsolateral PAG

WGA-HRP injections in the mesencephalon

Surgical procedures

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 \( \text{O}_2 \) and \( \text{N}_2\text{O} \) (1:2) and halothane (1-2%), while ECG and body temperature were monitored. In three cases injections of 40-120nl 2.5% WGA-HRP were made in the dorsal parts of the PAG. In one control case (2182) 100nl was injected into the ventrolateral PAG and in another control case (2338) approximately 150nl WGA-HRP was injected into the deep tectal layers laterally adjoining the PAG. Injections were made using a glass micropipette with a pneumatic picopump (World Precision Instruments PV830) or a Hamilton syringe (case 2182). All injections were made stereotaxically using Berman’s (1968) atlas. In two cases (2395 and 2338), the PAG or tectum was approached dorsally and the pipette passed through the superior colliculus. In cases 2182 and 2300 the PAG was approached dorsolaterally through the inferior colliculus and in case 2479 the pipette passed through the cerebellum and fourth ventricle. After a survival period of three days the animals were initially anesthetized with intramuscular ketamine (Nimatek, 0.1 ml/kg) and xylazine (Sedamun, 0.1ml/kg), followed by an overdose (6-10ml) of intraperitoneal 6% pentobarbital sodium.

Figure 1. NADPH-d staining according to the methods of Carrive and Paxinos (1994) in the dorsal midbrain. Note that NADPH-d is present in the dorsolateral PAG (PAGdl), but also in the nucleus Edinger-Westphal (EW), the supraoculomotor cap (Su3C), the deep layers of the superior colliculus (SC), the dorsal raphe nucleus (DR), and the laterodorsal tegmental nucleus (LDT). Bar represents 1mm.
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 brains were removed, post-fixed for two hours and stored overnight in 25% sucrose in phosphate buffer at 4°C. Subsequently, the brainstem was cut into 40µm frozen sections of which every fourth or every fifth (case 2182) section was incubated according to the tetramethyl benzidine (TMB) method, dehydrated and coverslipped. An extra series of sections of the area containing the PAG was incubated with diaminobenzidine (DAB) in order to define the extent of the injection sites. The injection sites were plotted using a drawing tube connected to a Zeiss brightfield stereomicroscope.

**Visualization of the retrogradely labeled cells**

Retrogradely labeled neurons in pons and medulla were studied using darkfield polarized illumination. Labeled neurons in the PPH were plotted in each case using a drawing tube connected to a Zeiss Axioplan. Photomicrographs were taken with a digital camera and minor adjustments in contrast and brightness were made using Adobe PhotoShop software.

**WGA-HRP control injection in the pontine and medullary tegmental field**

In one case (2593) a control injection was made in the pontine and medullary tegmental field. A total of approximately 700nl WGA-HRP was injected at different levels of the brainstem. Injections were made under visual guidance after removing the occipital bone overlying the cerebellum while the head of the cat was tilted, and after shifting the caudal part of the cerebellum slightly upwards, in order to visualize the dorsal surface of the brainstem. The survival time in this case was 72 hours. All other surgical and histological procedures were the same as in the WGA-HRP injected cases used in the retrograde tracing experiments described above.

**[3H]-leucine injections in PPH**

**Surgical procedures**

In six cats injections of L-[4,5-3H]-leucine (specific activity > 100 Ci/mmol) were made in the PPH and adjoining parts of the dorsal brainstem. The cats were anesthetized using pentobarbital, while heart rate and body temperature were monitored. The injections of 0.25-0.5µl containing 50 µCi [3H]-leucine were made stereotaxically using Berman’s (1968) atlas with a Hamilton microsyringe fitted with a 22-gauge needle. The tracer was injected over a period of 5 minutes, after which the needle was left in place for an additional 30 minutes. After a survival period of one day (case 1499), one week (case 1498) or two to six weeks (cases 1129, 1181, 1452 and 1061) the animals were deeply anesthetized with pentobarbital and perfused transcardially with saline followed by 4% paraformaldehyde.
Histological procedures
The brainstems were postfixied in 4% paraformaldehyde for at least one week, after which they were cut into 25µm 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 three months (Cowan et al., 1972; Holstege et al., 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; Holstege et al., 1979).

Visualization of the anterogradely labeled fibers in the midbrain
Silver grains representing anterogradely labeled fibers and nerve terminals in the midbrain were studied using darkfield illumination. Darkfield photomicrographs were made with a digital camera, attached to a Zeiss Stemi SV 11 stereomicroscope. These digital photomicrographs were further processed using Adobe PhotoShop software.

WGA-HRP injections in the PPH
Surgical procedures
In five cases (2536, 2539, 2538, 2541 and 2543) injections with a total of approximately 150nl WGA-HRP were made at different levels of the PPH. The injections were made under visual guidance after removing the occipital bone overlying the cerebellum while the head of the cat was tilted, and after shifting the caudal part of the cerebellum slightly upwards, in order to visualize the floor of the fourth ventricle. The survival time in these cases was 48 hours. All other surgical, histological, microscopic and photographic procedures were the same as in the WGA-HRP injected cases used in the retrograde tracing experiments described above.

RESULTS
Injection sites in the mesencephalon
In cases 2300, 2395 and 2479 WGA-HRP injections involved PAGdl (figs. 2 and 3). In all three cases the injections also extended into the lateral PAG, although in case 2395 only to a limited extent. In case 2479 the injection was within the confines of the PAG, but in cases 2300 and 2395 the injection site extended into the adjoining parts of the deep tectum. In two control cases the injection sites did not involve PAGdl, but the ventrolateral part of the intermediate and caudal PAG extending into the cuneiform nucleus and inferior colliculus (control case 2182) or the deep tectal layers extending slightly into the rostral lateral and dorsolateral PAG (control case 2338).

Labeled neurons in the tegmental field of pons and medulla
In three cases with injections involving PAGdl, but also the lateral PAG (cases 2300, 2395 and 2479), retrogradely labeled cells in the caudal pons and medulla were found bilaterally in the medial and lateral tegmentum. At caudal medullary levels labeled neurons were found in the solitary, gracile and cuneate nuclei, mainly contralaterally.
Figure 2. Drawings of the location of the WGA-HRP injection sites in the 5 cases used in the retrograde tracing study. The cores of the injection sites are indicated in black. IC, inferior colliculus; P, pyramidal tract; PAG, periaqueductal gray; PP, pes pedunculi; RN, red nucleus; SC, superior colliculus; SN, substantia nigra; III, oculomotor nucleus; IV, trochlear nucleus.

Figure 3. Brightfield photomicrographs showing the WGA-HRP injection sites in tectum (case 2338) and periaqueductal gray (case 2395). Aq, midbrain aqueduct; MGN, medial geniculate nucleus; PAG, periaqueductal gray; SC, superior colliculus; III, oculomotor nucleus; IV, trochlear nucleus. Bar represents 1 mm.
In cases with spread to the tectum (2300 and 2395) some labeled cells were also found bilaterally in the spinal trigeminal nucleus.

No anterogradely labeled fibers in PAGdl in control case 2593
After an control injection covering large parts of the pontine and medullary tegmental field and involving the solitary, gracile, cuneate and spinal trigeminal nuclei almost no labeled fibers were found in PAGdl, while the other columns of the PAG contained many labeled fibers and neurons (fig. 4). It was, therefore, concluded that the retrogradely labeled cells found in the tegmental field of pons and medulla in cases 2300, 2395 and 2479 were labeled from the lateral PAG and not from PAGdl.

Labeled neurons in the PPH
Besides retrogradely labeled neurons in the pontine and medullary tegmental field, in all cases with mesencephalic injections, including the control cases (2182 and 2338), retrogradely labeled cells were found in the PPH (table 1), a cell group located in the dorsal part of the medulla and pons, in the floor of the fourth ventricle. The most rostral subnucleus of the PPH is called the nucleus supragenualis nervi facialis (Taber, 1961; Brodal, 1983), and is located dorsally and laterally from the genu of the facial nerve (fig. 5). Retrogradely labeled neurons were found throughout the rostrocaudal extent of the contralateral PPH in all cases, including its supragenual part (arrows in fig. 6, and fig. 7). In case 2479, with an injection in the central and not in the peripheral part of the dorsolateral PAG, few labeled neurons were found in the caudal PPH. Ipsilaterally in the PPH only a very limited number of retrogradely labeled cells were found, with the exception of the tectum injected control case (2338), in which many labeled neurons were also observed in the ipsilateral PPH.

Figure 4. Darkfield photomicrographs of antero- and retrograde labeling in the periaqueductal gray (PAG) after a large WGA-HRP injection in the pontine and medullary tegmental field in case 2593. In the drawings the core of the injection site is indicated in dark gray. In the photomicrographs the borders of the dorsolateral PAG with its adjacent columns are indicated with dashed lines. Note that the dorsolateral PAG is almost completely devoid of labeling. Bar represents 1500μm.
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In six cases [3H]-leucine injections were made in PPH and in the adjoining dorsal pontine and/or medullary tegmentum (figs. 8 and 9). The smallest injections (cases 1129 and 1181) involved the area surrounding the genu of the facial nerve, and extended into the abducens nucleus.

In case 1452 the main part of the injection was in the caudal part of the dorsal tegmental nucleus, and extended into the dorsal pontine tegmentum. Caudally, the injection involved the nucleus supragenualis, but, unlike cases 1129, 1181, 1498 and 1499, not the abducens nucleus. Relatively large injections were made in cases 1498 and 1499, involving the dorsal tegmentum of pons and rostral medulla mainly unilaterally. The center of these injections was around the genu of the facial nerve.

In the last case (1061), the small injection site was located more caudally than in the other five cases and involved the caudal two thirds of the PPH, but not the nucleus supragenualis.

Labeled fibers in PAGdl and other parts of the midbrain

No labeled fibers were found in the dorsolateral column of the PAG in case 1061, with a small injection only involving the caudal parts of the PPH (fig. 10 and table 2). In all other cases, in which the supragenualis was involved in the injection, a distinct projection was found to PAGdl, exclusively on the contralateral side (figs 11, 12 and table 2). Even when the injections in the supragenual area were very small (cases 1129 and 1181), labeled fibers were found in PAGdl (fig 13). In none of the cases the distribution pattern in PAGdl reached the border of the aqueduct. Except in case 1452 with the most rostral injection site, a small region on the dorsolateral edge of the PAGdl was consistently devoid of labeled fibers (figs. 11, 12, 13 and table 2). This region is indicated with a dashed line in figures 11, 12 and 13, and will be referred to

### Table 1. Extent of the injection sites in the dorsal mesencephalon with regard to the involvement of PAGdl, PAGdldl, tectum and PAGvl (left) and the relative density of retrogradely labeled cells in PPH (right) after WGA-HRP injections.

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Table 1. Extent of the injection sites in the dorsal mesencephalon with regard to the involvement of PAGdl, PAGdldl, tectum and PAGvl (left) and the relative density of retrogradely labeled cells in PPH (right) after WGA-HRP injections.

contra, contralateral; ipsi, ipsilateral; PAGdl, dorsolateral periaqueductal gray; PAGdldl, dorsolateral part of PAGdl; PAGvl, ventrolateral PAG, PPH, nucleus prepositus hypoglossi.
Figure 5. Brightfield photomicrographs of Nissl stained sections of the rostrocaudal extent (from A to F) of the supragenual PPH, the confines of which have been indicated by dashed lines. MVN, medial vestibular nucleus; PPH, nucleus prepositus hypoglossi; gVII, genu of the facial nerve; nVII, facial nerve; VI, abducens nucleus. Bar represents 500 μm.
Figure 6. Drawings of labeled neurons in the nucleus prepositus hypoglossi (PPH) located in dorsal pons and medulla, after WGA-HRP injections covering the dorsolateral and lateral periaqueductual gray. Note that one drawing represents three consecutive 40µm sections (of a 1:4 series). One dot represents one labeled neuron. Arrows point to the retrogradely labeled cell group in the supragenual part of the PPH. Schematic drawings on the left show the approximate level of the brainstem. gVII, genu of the facial nerve; PPH, nucleus prepositus hypoglossi; IV, fourth ventricle. Bar represents 750µm.
as PAGdl.

In addition to labeled fibers in PAGdl, in all cases labeled fibers terminated ipsilaterally in parts of the PAG bordering the aqueduct ventrolaterally. This terminal location differs from the ventrolateral PAG column (PAGvl) as defined by Bandler (Bandler et al., 1991; Bandler and Shipley, 1994), and occupies a more medial region at caudal PAG levels and continues further rostrally than PAGvl (Bandler et al., 1991; Bandler and Shipley, 1994). Actually, the termination area of these PPH fibers corresponds much better with the location of the NADPH-d staining ventrolateral to the aqueduct (fig. 1). Some labeled fibers were also distributed to the intermediate and deep layers of the superior colliculus, mainly contralaterally. Although this projection was not very strong in the cases with the smallest injections in the nucleus supragenualis (table 2), one might conclude that the projection to PAGdl is always accompanied by a weaker projection to the adjoining part of the deep tectum. Interestingly, in case 1061 with a small injection in the caudal PPH and no labeled fibers in PAGdl, the projection to the superior colliculus was rather strong (fig. 10 and table 2). Projections from the caudal PPH to the superior colliculus have been described in earlier studies (McCrea and Baker, 1985; Corvisier and Hardy, 1997)
Figure 7. Photomicrographs using darkfield polarized illumination showing retrogradely labeled cells in the contralateral supragenual PPH just lateral to the genu of the facial nerve in cases 2300, 2479, 2395 and 2338 (control case). gVII, genu of the facial nerve; SO, superior olive; spV, spinal trigeminal nucleus; RB, restiform body. Bar represents 100μm.
[\textsuperscript{3}H]-leucine injections in dorsal pons and medulla
Including supragenual PPH:

Not including supragenual PPH:

Figure 8. Drawings of the location of the [\textsuperscript{3}H]-leucine injection sites in the 6 cases used in the anterograde tracing study. Approximate levels according to Berman’s atlas (1968). BC, brachium conjunctivum; CN, cochlear nuclei; IC, inferior colliculus; MVN, medial vestibular nucleus; NTS, nucleus of the solitary tract; P, pyramidal tract; SA, stria acustica; SO, superior olive; mV, motor trigeminal nucleus; pV, principal trigeminal nucleus; spV, spinal trigeminal nucleus; VII, facial nucleus; gVII, genu of the facial nerve; nVII, facial nerve.
In all six cases, but to a limited extent in case 1452, labeled fibers ascended in the area of the contralateral medial longitudinal fasciculus (MLF). In case 1061 (fig. 10), with the caudal PPH injection, some labeled fibers were found in the trochlear nucleus (nucleus IV), and many in the oculomotor nucleus (nucleus III) with the emphasis on its lateral part containing motoneurons innervating the medial rectus muscle (Tarlov and Tarlov, 1971; Gacek, 1974; Akagi, 1978). These projections to nuclei III and IV were bilateral, but with an ipsilateral preponderance (table 2). In cases with injections extending into the abducens nucleus (all cases except 1061 and 1452), similar projections to nucleus III were found, but with a contralateral preponderance (fig 11 and table 2).
PPH projections to dorsolateral PAG

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Table 2. Extent of injection sites in the dorsal medulla and pons with regard to the involvement of caudal PPH, supragenual PPH, abducens nucleus and LDT (left) and the relative density of anterograde labeling in dorsal mesencephalon (right) after [3H]-leucine or WGA-HRP injections.

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the injection did not extend into nucleus VI, but into the caudal periventricular gray bilaterally (case 1452), no labeled fibers were found in nuclei III (table 2) and IV, but many in the ventromedial and ventrolateral PAG regions bordering the aqueduct, bilaterally.

In all cases, some labeled fibers were also found in the area medially adjoining the parabigeminal nucleus, mainly ipsilaterally, and in the interpeduncular nuclei bilaterally.

In summary, the supragenual part of the PPH gave rise to a distinct projection to the dorsolateral PAG, and, albeit to a lesser extent, to the adjoining tectal parts. This distribution pattern in the PAGdl did not extend centrally to reach the aqueduct and also a small region on the dorsolateral border of the PAG (PAGdldl) was always devoid of labeled fibers. Neurons in the caudal PPH sent fibers to nuclei III and IV mainly ipsilaterally, and interneurons in nucleus VI to nucleus III mainly contralaterally.

In contrast, the supragenual part of the PPH did not project to nuclei III and IV. No projections to PAGdl were found when the injection sites did not involve the supragenual PPH, as in case 1061.

WGA-HRP injection sites in PPH

In addition to the [3H]-leucine cases, in five cases injections of WGA-HRP were made in dorsal medulla and pons (figs. 14 and 15). Three of these injections involved the region of the caudal and intermediate PPH (cases 2536, 2538 and 2541), (parts of) the
Figure 11. Darkfield photomicrographs of the periaqueductal gray and adjoining deep tectal layers after an injection of [3H]-leucine in the case 1498, with the center of the injection in the supragenual nucleus prepositus hypoglossi. Note the projection to the contralateral PAGdl. IC, inferior colliculus; MLF, medial longitudinal fascicle; nD, nucleus of Darkschewitsch; SC, superior colliculus. Bar represents 2mm.
Figure 12. Magnifications of photomicrographs B and D in figure 9, showing the labeling in the dorsolateral periaqueductal gray (PAGdl) after an injection of [3H]-leucine in the case 1498. Note that the dorsolateral part of the dorsolateral periaqueductal gray (PAGddl) is devoid of labeling. Aq, aqueduct; mesV, mesencephalic trigeminal nucleus; SC, superior colliculus. Bar represents 500μm.
medial vestibular nucleus and ventrally adjoining tegmental field. In case 2536 the injection also involved the hypoglossal nucleus.
In the remaining two cases (2539 and 2543) the injection sites involved the supragenual PPH, nucleus VI, and the ventrally adjoining tegmentum. In one of these cases (2539) the injection site extended also into the caudal PPH, the nucleus of the solitary tract and the dorsal vagal nucleus.

Labeled fibers in the midbrain
In cases 2536, 2538 and 2541, in which the supragenual part of the PPH was not or only to a very limited extent involved in the injection site, no (cases 2536 and 2541) or very few (case 2538) labeled fibers were seen in PAGdl, except in PAGdldl (fig 16, left and table 2). In the same three cases many labeled fibers were also seen in the ventrolateral and lateral parts of the lateral PAG bilaterally and in the intermediate and deep tectal layers, predominantly on the contralateral side (table 2). Only when the injections involved the supragenual PPH (cases 2539 and 2543), labeled fibers were observed in PAGdl (fig 16, right and table 2). Similar to the cases with [3H]-leucine injections, PAGdldl was devoid of labeling when the caudal PPH was not injected (case 2543). Accordingly, in case 2539, with an injection including both caudal and supragenual PPH, both PAGdl and PAGdldl were labeled (table 2).
WGA-HRP injections in the dorsal pons and medulla

Including supragenual PPH:

Figure 14. Drawings of the location of the WGA-HRP injection sites in the five cases used in the anterograde tracing study. The cores of the injection sites are indicated in dark gray. Rostrocaudal levels according to Berman’s atlas (1968). Abbreviations as in figure 8.
DISCUSSION

NADPH-d staining in PAGdl

Staining for NADPH-d is often used to define PAGdl, because this enzyme can be found in PAGdl, and not in the adjacent dorsomedial and lateral columns. Our results in cat show that NADPH-d staining is indeed confined to PAGdl, but is certainly not evenly distributed throughout PAGdl. In fact, two clusters of neurons, one in the peripheral and one in the central part, are located in PAGdl. This seems also the case in rat (see Fig. 3 in Bandler and Shipley, 1994). Only after very long
incubation times (6 hrs at 37°, see Carrive and Paxinos, 1994) reaction product can be found throughout PAGdl, but the emphasis remains on the peripheral and central cell clusters. It can, therefore, be disputed that NADPH-d staining should be seen as a definitive marker for PAGdl.

It is important to emphasize that within the confines of the PAG NADPH-d can also be found in areas other than PAGdl, such as the supraoculomotor cap, located dorsally from the supraoculomotor nucleus (fig. 1 present paper; Carrive and Paxinos, 1994) and in the intercalated PAG, lateral to the aqueduct (Carrive and Morgan, 2004).

Projections from PPH to dorsal midbrain

In the present study, for the first time, we have demonstrated projections from the PPH to PAGdl, with the caudal part of the PPH projecting to PAGdldl, and the rostral or supragenual PPH projecting to the PAGdl excluding PAGdldl (fig. 17). In addition, all parts of PPH project to the PAG bordering the aqueduct ventrolaterally, which explains why so many labeled neurons are present in PPH when the injections involved the ventral part of PAG. This part of PAG seems to correspond with the supraoculomotor cap as described by Carrive and Paxinos (1994) in a variety of mammals including cat and human can be stained for NADPH-d, just like PAGdl and PAGdldl (Carrive and Paxinos, 1994 and fig. 1).

The supragenual PPH sends limited projections, mainly contralaterally, to the intermediate and deep layers of the superior colliculus, which explains why labeled neurons were found in this part of the supragenual PPH after a control injection in the deep collicular layers (case 2338). The more caudal PPH sends much stronger projections to the superior colliculus, which is in accordance with McCrea and Baker (1985) and Corvisier and Hardy (1997). Our finding that the caudal and intermediate parts of the PPH, but not the supragenual PPH, project to nucleus IV and to the medial rectus motoneuronal cell group in nucleus III, also corresponds with McCrea (1988).

Two parts of the dorsolateral PAG

The differential projections to PAGdl from the caudal and supragenual PPH suggest that the dorsolateral column of the PAG consists of two different parts. These subdivisions PAGdl have also been shown in earlier studies. Small injections in substantia nigra pars reticulata (SNr) and pars lateralis (SNI) in cat resulted in labeled fibers in PAGdldl and not PAGdl (Harting et al., 1988; Harting and Van Lieshout, 1991). However, injections that were located at slightly different locations in SNr resulted in labeled PAGdl and not PAGdldl (Harting et al., 1988). Projections in cat from the nucleus of the brachium of the inferior colliculus and the parabigeminal nucleus terminate mainly in PAGdldl (Graybiel, 1978; Kudo et al., 1984), while projections from the caudal pedunculopontine nucleus (Harting and Van Lieshout, 1991) and the precommissural nucleus in rat (see fig. 6 in Canteras and Goto, 1999) seem to project to PAGdl, but not to PAGdldl. The distribution patterns of particular neurochemicals also point to the existence of two subdivisions. Within the feline PAG, acetylcholinesterase (McHaffie et al., 1991; Graybiel and Illing, 1994), GABA transporter-1 (GAT-1) immunoreactivity (Barbaresi et al., 1998; Barbaresi et al., 2001) and kainic acid receptor binding sides (Gundlach,
Figure 17. Schematic representation of the projections from the rostral or supragenual and of the caudal PPH to the dorsal midbrain. Strongest projections are in dark gray, weaker projections in lighter gray. IC, inferior colliculus; MLF, medial longitudinal fascicle; PAGd, dorsolateral PAG; PAGdidl, dorsolateral part of the dorsolateral PAG; III, oculomotor nucleus; IV, trochlear nucleus.
PPH projections to dorsolateral PAG

1991), and in rat neuropeptide Y (Yamazoe et al., 1985) have been demonstrated to be particularly dense in PAGdldl. In contrast, when in rat the midbrain is stained for dopaminergic fibers (Van Dijken, 2000), particularly PAGdldl shows very little staining compared to the rest of the PAG.

PAGdldl is adjacent to the deep layers of the superior colliculus and they share many afferent sources, such as the caudal PPH (this study), SNr and SNl (Harting et al., 1988; Harting and Van Lieshout, 1991), and prefrontal cortex area 10 (An et al., 1998). They contain many similar neurochemicals, such as NADPH-d (Mizukawa et al., 1989; Herbert and Saper, 1992; Carrive and Paxinos, 1994; Floyd et al., 2000), acetylcholinesterase (McHaffie et al., 1991; Graybiel and Illing, 1994), and cholecystokinin (Liu et al., 1994). This brings up the question of whether PAGdldl should actually be seen as a region continuous with, and functionally part of, the stratum griseum profundum and intermediate layers of the superior colliculus. On the other hand, projections from the prefrontal cortical areas 25 and 32 in monkey project to the dorsolateral PAG including PAGdldl, but not to the superior colliculus (An et al., 1998). Furthermore, fibers containing neuropeptide Y are abundant in PAGdldl in rat, but very sparse in the superior colliculus (Yamazoe et al., 1985). We conclude, therefore, that PAGdldl is distinct from both PAGdl and the deep superior colliculus.

Functional implications

Physiologically it has been shown that the caudal and intermediate PPH play a role in the control of eye movements, especially in controlling gaze holding and saccades as shown in physiological studies (Delgado-Garcia et al., 1989; Cheron et al., 1992; Hardy and Corvisier, 1996; Moreno-Lopez et al., 1996). These parts of the PPH, therefore, maintain numerous connections with brain areas involved in the oculomotor system (for review, see McCrea, 1988). Regarding the efferents from the supragenual part of the PPH, anatomical tracing studies in rat (Blanks et al., 1983), cat (Kotchabhakdi et al., 1978; Sato et al., 1983) and monkey (Brodal and Brodal, 1983; Langer et al., 1985) have shown that they terminate in the flocculus and perhaps vermis of the cerebellum. Present results show that the supragenual PPH also has, albeit relatively weak, projections to the superior colliculus. Afferents of the nucleus supragenualis originate in the rostral paramedian reticular formation in cat (Gerrits and Voogd, 1986). The fact that the areas with which supragenual PPH maintains connections are all involved in oculomotor control, points to a role of the supragenual PPH in the oculomotor system. On the other hand, defects in both vertical and horizontal gaze holding occur after injections of ketamine into the caudal supragenual PPH, but not after injections into the rostral part of this subnucleus (Cheron et al., 1992). Furthermore, the supragenual PPH, in contrast to the caudal and intermediate parts of the PPH, does not project to the trochlear and oculomotor nuclei (present study). These findings, together with the different projection patterns in PAGdl of the two parts of the PPH, suggest that the supragenual PPH plays another role in oculomotor control than the caudal and intermediate PPH, or perhaps might have a completely different function.

The dorsolateral column of the PAG is usually not associated with oculomotor
functions. However, a physiological study in monkeys has shown that PAGdl plays a role in saccadic eye movements. Electrophysiological recordings of cells located throughout PAGdl revealed the presence of cells in this column that had tonic discharge during steady fixation of the eyes, but paused just before the onset of and during spontaneous saccades. In contrast, cells in the overlying layers of the superior colliculus showed bursts preceding spontaneous saccades. The responses of the cells in PAGdl were related exclusively to saccades, because discharge rates were not altered by visual inputs, such as lights on and off, or in relation to eye position or slow eye movements (Kase et al., 1986).

Connections of PAGdl have been reported with other oculomotor related areas, such as parabigeminal nucleus (Graybiel, 1978; Baleydier and Magnin, 1979) and the frontal eye fields (Stanton et al., 1988; Leichnetz and Gonzalo-Ruiz, 1996), and with substantia nigra pars reticulata (Harting and Van Lieshout, 1991). Remarkably, projections from the parabigeminal nucleus, the frontal eye fields and substantia nigra pars reticulata apparently terminate in PAGdldl only, and not in PAGdl. This finding, together with the fact that not all parts of the supragenual PPH seem to be involved in gaze holding (see above), might suggest that only PAGdldl is oculomotor related and the rest of the dorsolateral column is not. On the other hand, saccade related neurons and cells projecting to the parabigeminal nucleus were observed throughout the dorsolateral PAG (Kase et al., 1986; Baleydier and Magnin, 1979).

The function of input from oculomotor related areas to PAGdl, which is usually associated with aversive behavior and defensive rage (for review, see Keay and Bandler, 2001; Vianna and Brandao, 2003), remains to be elucidated.