Chapter 6

The Segmental and Laminar Organization of the Spinal Neurons projecting to the Periaqueductal Gray (PAG) in the Cat suggests the Existence of at least Five Separate Spino-PAG Systems

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Abstract
The present study describes the spinal cord projection to the periaqueductal gray (PAG) in the cat, taking into account different regions of the PAG and all spinal segments. The retrograde wheat germ agglutinin-horseradish peroxidase results show that injecting different parts of the PAG leads to different laminar and segmental distributions of labeled spinal neurons. Evaluation of these results suggests that the spino-PAG projection is not one general ascending pathway, but consists of at least five anatomically, and probably functionally, separate spino-PAG systems. 

System I originates in laminae I and V throughout the length of the cord, terminates in all parts of the PAG, and is probably involved in nociception. 

System II begins in the ventrolateral part of laminae VI-VII of the C1-C4 spinal cord and terminates in the ventrolateral and lateral parts of the rostrocaudal PAG, and in the deep tectum. 

System III originates in lamina X of the thoracic and upper lumbar cord, and terminates in the PAG as well as in the deep tectum. 

System IV originates in the medial part of laminae VI-VII of the lumbosacral cord and projects predominantly to the lateral and ventrolateral caudal PAG. It might play a role in conveying tactile stimuli to the PAG during mating behavior. 

System V begins in the lateral part of lamina I of L6-S2 and in laminae V-VII and X of S1-S3. It terminates mainly in the central portion of the lateral and ventrolateral caudal PAG, and probably relays information concerning micturition and mating behavior.

Introduction
The mesencephalic periaqueductal gray (PAG) plays an integrative role in emotional responses necessary for basic survival. Stimulation in the PAG of the freely moving rat and cat elicits defense behaviors, such as fight, threat display, flight and immobility (Bandler and Depaulis, 1991; Carrive, 1993). These behaviors include movements, such as running, jumping, arching of the back, turning of the head, vocalization, pupil dilatation, ear positioning and piloerection. Stimulation in the PAG can also elicit cardiovascular changes (Carrive, 1991, Bandler and Keay, 1996, Lovick, 1996), micturition (Blok and Holstege, 1996), mating behavior (Sakuma and Pfaff, 1979) and analgesia (Basbaum and Fields, 1984, Besson and Chaouch, 1987, Besson et al. 1991). Other studies (Lonstein and Stern, 1997; Lonstein et al, 1998; Lonstein and Stern, 1998) in which different parts of the PAG were lesioned, showed that the PAG is also involved in maternal behavior and maternal aggression.

The question is how the PAG organizes these complex behaviors. Tracing studies revealed several of the efferent pathways that the PAG uses to generate its motor activities and analgesic effects (see Holstege, 1997 for review). Other studies have demonstrated that many afferent pathways to the PAG originate from various regions of the limbic system (Holstege, 1991; Shipley et al., 1991), but also from the lower brainstem and spinal cord (Mehler, 1969; Menetrey, 1982; Mantyh, 1982; Wiberg and Blomqvist, 1986; Bandler and Tork, 1987; Yezierski and Mendez, 1991;
Earlier tracing studies on the projections of the spinal cord to the PAG (rat: Keay et al., 1997, cat: Wiberg and Blomqvist, 1984; Keay and Bandler, 1992; Wiberg et al., 1987; Yeziorski and Mendez, 1991) showed that the spino-PAG neurons are located bilaterally in the cervical cord and contralaterally in the sacral cord, predominantly in lamina I, laminae IV-V, and laminae VI-VIII, and also contralaterally in the cervical and lumbar enlargements. In these latter parts many PAG projecting neurons were seen in lamina I. The spino-PAG neurons were found to terminate predominantly in the lateral and ventrolateral, and to a limited extent in the dorsal PAG (Björkeland and Boivie, 1984). Recently, more precise studies on the projections from the upper cervical (Keay and Bandler, 1992) and lumbosacral cord (VanderHorst et al., 1996) to the PAG in the cat demonstrated the existence of specific groups of neurons in the spinal cord projecting to different areas of the PAG. With respect to the C1 to C3 segments, Keay and Bandler (1992) reported that a majority of lamina I neurons projected to the lateral PAG and a majority of laminae VII-VIII neurons to the ventrolateral PAG. With respect to the L3-Coc3 segments a study from our group (VanderHorst et al., 1996) showed the existence of at least two different groups of neurons in the lumbosacral cord projecting to the PAG. One group of neurons extending from the lateral part of lamina I of L6-S2 into laminae V-VII of S2 projects to the central part of the lateral caudal PAG, and another group, located in the medial part of laminae VII and VIII of the L5-S1 segments, projects to the lateral part of the lateral caudal PAG and adjacent tegmentum. These findings argue against the idea of the existence of only one general spino-PAG projection system, with only one function -the relay of nociceptive information- and they raise the question of how the spino-PAG system is really organized and what other functions, besides the control of nociception, are involved. 

The present paper presents a detailed map of the complete spino-PAG projections in the cat, involving all spinal segments. The results suggest that the spino-PAG projection is not one general ascending pathway, but consists of at least five anatomically separate spino-PAG systems, probably conveying different types of information.

**Materials and Methods**

**Surgical procedures**

A total of 14 adult cats was used. The surgical procedures, pre- and postoperative care, as well as the handling and housing of the animals followed protocols approved by the Faculty of Medicine of the University of Groningen.

For surgery the animals were initially anesthetized with intramuscular ketamin (Nimatek, 0.1 ml/kg) and xylazine (Sedamun, 0.1 ml/kg), after which they were kept anesthetized either by ventilation with a mixture of O$_2$, N$_2$O and halothane or by intravenous 6% pentobarbital sodium (= 0.2 ml per hour). During surgery, ECG and body temperature were monitored. Following a survival time of 3 days the animals were initially anesthetized with intramuscular ketamin (Nimatek, 0.1 ml/kg) and xylazine (Sedamun, 0.1 ml/kg), followed by an overdose of intraperitoneal 6% pentobarbital sodium. The cats were perfused transcardially with two liters of 0.9% saline at 37°C, immediately followed by two liters of 0.1M phosphate buffer, containing 4% sucrose, 1% paraformaldehyde and 2% glutaraldehyde.

**Histological procedures**

In 11 female cats 20-100 nl 2.5% wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP) in saline was injected into the PAG either with a Hamilton syringe, or with a glass micro pipette using a pneumatic picopump (World Precision Instruments PV830). In one control case an injection was made in the tectal area lateral to the PAG. In two other control cases the injections were
Five spino-PAG systems

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Fig. 1. Photographs and drawings showing the injections sites of four cases. On the left: darkfield photographs and in the middle: brightfield photographs of the injection areas (scale bar, 1000 µm). On the right: drawings of the injection sites in gray. The core of the injections is indicated in black.

placed into the cerebrospinal fluid of the aqueduct. Except for case 2316, all injections were made stereotaxically, using Berman’s atlas (1968) and approaching the PAG dorsally through the superior colliculus, dorsolaterally through the inferior colliculus, or caudally through the cerebellum and fourth ventricle (case 2471). In case 2316 the cortical areas overlying the PAG were removed and the injection was made under visual guidance. After perfusion the brains and spinal cords were removed, post-fixed for two hours and stored overnight in 20% sucrose in phosphate buffer at 4°C. Subsequently, the spinal cords were cut into 33 separate segments (C1-Coc2). Each segment was cut into 40 µm frozen transverse sections of which every fourth (cases 2300, 2316, 2338, 2367, 2385, 2390, 2395, 2401 and 2471) or every fifth (cases 2155, 2159 and 2182) section was incubated according to the tetramethyl benzidine method, dehydrated, and coverslipped. The brainstem area, containing the injection site, was cut in 40 µm sections and every fourth section was processed with diaminobenzidine (DAB). The injection sites were plotted with the aid of a drawing tube connected to a Zeiss brightfield stereomicro-
scope, and were described in terms of dorso-medial, dorsolateral, lateral and ventrolateral parts of the rostral, intermediate or caudal PAG.

**Quantification of retrogradely labeled neurons**

To enable counting the spino-PAG neurons in the various spinal segments, in each case the retrogradely labeled neurons in all processed transverse sections of the C1-Coc2 spinal cord were plotted in drawings, using a drawing tube connected to a Zeiss Axioskop with darkfield polarized illumination, a digitizer, and a Macintosh computer. From these drawings the numbers of ipsilaterally and contralaterally located PAG projecting neurons per segment were counted. It should be emphasized that it was not the aim of this study to determine the absolute number of spino-PAG projecting neurons.

In seven of the PAG and/or tectum injected cases one out of every four sections was processed, in three cases one out of five. In order to compare the numbers from these two groups the 1:5 processed cases were corrected into 1:4 cases by multiplying the counted numbers by 1.25. Such a correction is allowed because in both groups an unbiased sample was taken and double counting of cells could not occur in a 1:5 series of 40µm sections. The corrected numbers were used in all further analysis.

In order to describe the laminar location of the spinal neurons projecting to the PAG, in all drawings the laminae of Rexed (1954) were depicted as well as a line dividing laminae VI and VII into a medial and lateral part. This line was set at half the distance between the lateral border of lamina X and the lateral border of the gray matter, dorsoventrally at the level of the central canal (Fig. 3). The labeled neurons were counted in eight different areas, i.e.: lamina I, laminae II-IV, lamina V, the medial part of laminae VI-VII, the lateral part of laminae VI-VII, including the lateral motoneuronal cell groups, lamina VIII with the medial motoneuronal cell groups, lamina X, and the white matter. Results of these countings per segment are presented in histograms. To enable reliable comparisons between the different histograms, the magni-

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five spino-PAG systems

Three of the y-scale of all histograms is kept standard, except for the histograms showing the total numbers per segment and those showing the numbers of neurons per segment in lamina X. The y-scale of the histograms showing the total numbers of neurons per segment, due to the high numbers per segment, is set at half the standard size. The y-scale of the histograms showing the numbers of neurons in lamina X, due to the small numbers of labeled neurons per segment, is set at three times the standard size.

Results

Injection sites

Fig. 1 contains photomicrographs of four of the injection sites and the corresponding schematic drawings. In the drawings the core of the injections, as observed with brightfield illumination, is shown in black. Fig. 3 contains schematic representations of all injection sites in the PAG and adjacent deep tectal areas. In cases 2367 and 2159 the large injections involved the rostral PAG and in cases 2385 and 2155 the intermediate and caudal PAG. The other, smaller, injections were placed in the intermediate and/or caudal PAG, involving the dorsomedial and/or dorsolateral (cases 2316 and 2395) and the lateral and/or ventrolateral parts (cases 2300, 2390, 2401, 2471 and 2182). In control case 2338 the injection involved the deep layers of the superior colliculus at the level of the rostral and intermediate PAG, and extended to only a very limited extent into the lateral part of the PAG (Fig. 1).

In cases 2270 and 2293 the WGA-HRP was injected in the cerebrospinal fluid of the aqueduct. These cases showed labeling of the ependymal layer surrounding the aqueduct as well as of the ependymal layer around the central canal of the spinal cord. No labeled neurons were found in these two cases, neither in the brainstem nor in the spinal cord. In all other cases, except for case 2471, labeling of the ependyma was not observed. In case 2471, in which the injection was made approaching the PAG caudally through the cerebellum and fourth ventricle, a small amount of tracer leaked into the aqueduct, resulting in labeling of the ependyma around the central canal throughout the length of the spinal cord.

Labeled neurons in the lateral cervical nucleus and dorsal column nuclei

In the upper cervical spinal cord labeled neurons were found in the cuneate and gracile nuclei and in the lateral cervical nucleus. These neurons are not included in this study.

Total number of labeled spino-PAG neurons

In all cases many labeled neurons were present throughout the length of the spinal cord (Figs. 2-3). In cases 2385 and 2155, with large injections involving the intermediate and caudal PAG and a small part of the tectum, but not the rostral and most caudal PAG, a total number of 5268 and 7468 labeled neurons respectively were found. Taking into account that these numbers are from a series of 1:4 transverse sections, but also considering that multiplication by four would lead to an overestimation (Coggeshall and Lekan, 1996), a conservative estimation is that there are at least 15,000 spino-PAG neurons.

The number of labeled neurons varied considerably from case to case. In case 2395, with an injection in the dorsolateral PAG, 539 labeled cells were counted, in contrast to the 7468 in case 2155, with a large injection involving the intermediate and caudal PAG (Table 1). Obviously, differences in numbers of neurons per case largely reflect the differences in injection size. On the other hand, the very small injection in case 2471 did not result in the lowest numbers of labeled neurons, and the slightly larger, but still limited injections in cases 2300, 2316, 2390, 2395, and 2401 showed large variations in the numbers of neurons, from 539 neurons in case 2395 to 1924 neurons in cases 2300. This demonstrates that certain regions of the PAG receive more spinal afferents than others. For
Fig. 2. left and right: Schematic drawings of the WGA-HRP injection sites in the different parts of the PAG. The AP coordinates are according to the atlas of Berman (1968). The black areas are considered the core of the injection sites. The histograms show the total numbers of retrogradely labeled neurons.
Five spino-PAG systems

Per segment, in a series of one out of four 40 µm transverse sections. The labeled neurons in the lateral cervical nucleus and in the dorsal column nuclei are not included.
Table 2A. Numbers of ipsilaterally located labeled neurons (counted in 1:4 series) per subarea throughout the cord, after WGA-HRP injections in different parts of the PAG and adjacent deep tectal layers. The percentages relate to the total of the ipsilaterally located labeled neurons throughout the length of the spinal cord. *wm*, white matter.

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<th>VI-VII med</th>
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Table 2B. Numbers of contralaterally located labeled neurons (counted in 1:4 series) per subarea throughout the cord, after WGA-HRP injections in different parts of the PAG and adjacent deep tectal layers. The percentages relate to the total of the contralaterally located labeled neurons throughout the length of the spinal cord. \textit{wm}, white matter.

\begin{table}[h]
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\begin{tabular}{cccccccccccc}
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\textbf{case} & \textbf{total} & \textbf{I} & \textbf{II-IV} & \textbf{V} & \multicolumn{2}{c}{\textbf{VI-VII}} & \textbf{VIII} & \textbf{X} & \textbf{wm} \\
& & & & & \textbf{lat} & \textbf{med} & & & \\
\hline
2367 & 1466 & 222 & 15.1\% & 8 & 0.5\% & 406 & 27.7\% & 231 & 15.8\% & 313 & 21.4\% & 255 & 17.4\% & 25 & 1.7\% & 6 & 0.4\% \\
2159 & 1468 & 213 & 14.5\% & 8 & 0.5\% & 410 & 27.9\% & 340 & 23.2\% & 404 & 27.5\% & 53 & 3.6\% & 35 & 2.4\% & 5 & 0.3\% \\
2385 & 3806 & 1076 & 28.3\% & 60 & 1.6\% & 1070 & 28.1\% & 623 & 16.4\% & 693 & 18.2\% & 214 & 5.6\% & 46 & 1.2\% & 24 & 0.6\% \\
2155 & 5070 & 1323 & 26.1\% & 104 & 2.1\% & 1168 & 23.0\% & 811 & 16.0\% & 1050 & 20.7\% & 374 & 7.4\% & 194 & 3.8\% & 46 & 0.9\% \\
2316 & 340 & 118 & 34.7\% & 0 & 0.0\% & 157 & 46.2\% & 28 & 8.2\% & 31 & 9.1\% & 3 & 0.9\% & 2 & 0.6\% & 1 & 0.3\% \\
2395 & 417 & 46 & 11.0\% & 2 & 0.5\% & 228 & 54.7\% & 35 & 8.4\% & 28 & 6.7\% & 67 & 16.1\% & 10 & 2.4\% & 1 & 0.2\% \\
2300 & 1402 & 364 & 26.0\% & 68 & 4.9\% & 463 & 33.0\% & 187 & 13.3\% & 243 & 17.3\% & 32 & 2.3\% & 34 & 2.4\% & 11 & 0.8\% \\
2390 & 1345 & 324 & 24.1\% & 11 & 0.8\% & 498 & 37.0\% & 181 & 13.5\% & 241 & 17.9\% & 57 & 4.2\% & 21 & 1.6\% & 12 & 0.9\% \\
2401 & 780 & 134 & 17.2\% & 5 & 0.6\% & 333 & 42.7\% & 117 & 15.0\% & 104 & 13.3\% & 64 & 8.2\% & 10 & 1.3\% & 13 & 1.7\% \\
2471 & 589 & 234 & 39.7\% & 0 & 0.0\% & 79 & 13.4\% & 117 & 19.9\% & 126 & 21.4\% & 27 & 4.6\% & 6 & 1.0\% & 0 & 0.0\% \\
2182 & 2006 & 810 & 40.4\% & 50 & 2.5\% & 495 & 24.7\% & 175 & 8.7\% & 306 & 15.3\% & 100 & 5.0\% & 44 & 2.2\% & 26 & 1.3\% \\
2338 & 987 & 32 & 3.2\% & 8 & 0.8\% & 411 & 41.6\% & 246 & 24.9\% & 184 & 18.6\% & 82 & 8.3\% & 21 & 2.1\% & 3 & 0.3\% \\
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\end{tabular}
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Fig. 3. Schematic drawings of the labeled neurons in the C1-Coc2 spinal cord after a WGA-HRP injection in the lateral intermediate and caudal PAG (case 2390). Each drawing represents twelve 40 µm thick sections. *wm*, white matter; *cun*, cuneate nucleus; *grac*, gracile nucleus.
example, many more spinal neurons projected to the lateral and ventrolateral PAG (cases 2300, 2390, 2401 and 2471) than to the dorsomedial and dorsolateral PAG (cases 2316 and 2395). The central part of the ventrolateral and lateral PAG (cases 2300 and 2471) received the strongest projections. Although the spino-PAG projections were bilateral, about 70% of the PAG projecting spinal neurons was located contralaterally (Table 1). In the control case with an injection in the deep tectal layers (case 2338) 60% of the labeled neurons was found on the contralateral side.

**Total number of labeled neurons per lamina**

Different injection sites resulted in different numbers of labeled neurons per lamina throughout the cord. The largest numbers of labeled neurons were found contralaterally in laminae I, V and lateral VI-VII, and ipsilaterally in lamina V and the lateral part of laminae VI-VII (Table 2). Smaller numbers were found contralaterally in laminae VIII and X and ipsilaterally in lamina I, the medial part of laminae VI-VII and in laminae VIII and X. Relatively few labeled neurons were present in laminae II-IV and no labeled neurons were present in the motoneuronal cell groups. A few labeled neurons were located in the white matter lateral to the intermediate zone and ventral horn, mainly contralaterally. In case 2338 with an injection in the deep tectal layers most of the labeled neurons were found in lamina V contralaterally, and in laminae VI-VII ipsilaterally. In contrast to most PAG injected cases, this tectum injected case showed almost no labeled cells in lamina I.

**Distribution per segment**

**General overview**

Fig. 2 shows the total number of labeled neurons per segment. Large segmental differences were found in the numbers of spino-PAG projecting neurons, even between adjacent segments. In most cases the spinal afferents came mainly from the upper cervical (40-60%) and lumbosacral cord (10-30%). In cases 2316 and 2471 the majority of labeled neurons was found in the lumbosacral cord. A moderate number of afferents originated from the thoracic cord, mainly from the upper thoracic segments, and a few from the coccygeal segments. With the exception of C1, most labeled neurons were present contralaterally. In C1, a large number of ipsilaterally located neurons was found, sometimes even exceeding the number of contralateral cells at this level (cases 2159 and 2401).

**Lamina I**

Throughout the cord approximately 1600 and 1200 labeled lamina I neurons were found in cases 2155 and 2385 respectively, with large injections in the caudal and intermediate PAG (Table 2). Only 230 labeled lamina I neurons were found in cases with large injections in the rostral PAG (2367 and 2159), and only 54 after the injection in the deep tectal layers (case 2338).

With the exception of the sacral cord, all lamina I-PAG projecting neurons were located in the dorsal and dorsolateral parts of lamina I, and not in its medial or ventrolateral parts. In the sacral cord labeled lamina I neurons were found in the dorsal and dorsolateral parts, but also more ventrolaterally (Figs. 3 and 9). With the exception of the sacral cord, the contralateral lamina I-PAG projection was five times stronger than the ipsilateral one.

Fig. 4 shows the numbers of lamina I neurons per segment in each case. The results indicate that all spinal segments contain lamina I neurons projecting to the PAG, and that they are most numerous in the cervical and lumbosacral enlargements. Another finding is that labeled lamina I neurons were present in both the rostral and caudal PAG injected cases (cases 2367, 2159, 2385 and 2155), indicating that the lamina I-PAG projections target the entire rostro-caudal extent of the PAG. However, independent of the spinal level, the lamina I projection was always...
Fig. 4. Histograms showing the segmental distribution of the labeled neurons in lamina I in the C1-Coc2 spinal cord. The numbers represent the labeled neurons in a series of 1:4 transverse sections.
Fig. 5. Histograms showing the segmental organization of the labeled neurons in lamina V in the C1-Coc2 spinal cord. The numbers represent the labeled neurons in a series of 1:4 transverse sections.
stronger to the intermediate and caudal PAG than to its rostral part. In other words, the caudal PAG injected case 2385, compared to the rostral PAG injected case 2367, not only showed more labeled lamina I neurons in the lumbosacral cord, but also in the cervical cord. Apparently, all lamina I neurons have a similar projection pattern in the PAG, which is independent of their segmental location. The results also revealed that the ventrolateral and lateral parts of the intermediate PAG, and especially the medial area adjacent to the aqueduct, received strong afferent projections from the lateral part of lamina I of the L6-S2 segments (cases 2155, 2385, 2300, 2390, 2471 and 2182). These neurons are part of the earlier defined specific lumbosacral-PAG projection, which also involves neurons in the lateral part of laminae V, VI and VII (see VanderHorst et al., 1996; and system V in the discussion).

Laminae II, III and IV
Only very few labeled neurons were found in laminae II, III and IV (Table 2). More than half of these labeled laminae II-IV neurons was located in the C1 segment.

Lamina V
Within lamina V, most PAG projecting neurons were located laterally (Figs. 3 and 9), and only in the C1-C2 and sacral segments also medially (Fig. 3). The contralateral lamina V-PAG projection was about twice as strong as the ipsilateral one (Fig. 5). The rostrocaudal distribution of the lamina V-PAG neurons resembled that of the lamina I neurons, except that in the upper cervical cord, and especially in the C1 segment, many more lamina V-PAG neurons were found (Fig. 5). These higher numbers of labeled lamina V cells in the upper cervical cord were not present in cases 2316 and 2471. Another difference between the distributions of labeled neurons in lamina I and V was in which lumbosacral segments the large numbers of labeled neurons were found. They were not found in the L6-S2 segments, as in lamina I, but in the S1-S3 segments. However, both groups of cells belong to the same distinct lumbosacral cell group projecting to the PAG (see system V in the discussion). The deep tectal layers received much stronger projections from lamina V than from lamina I, but mainly from the upper cervical cord (control case 2338).

Lateral part of laminae VI and VII
Only a limited number of labeled neurons were found in the lateral part of laminae VI and VII, with the exception of the cervical and sacral cord. In the upper cervical cord, and especially in C1, in all cases, except 2316, 2395 and 2471, large numbers of labeled neurons were found in this area (Fig. 6). The great majority was located in the ventrolateral portion of lamina VII (Figs. 3 and 9). In contrast to all other spino-PAG projections, this upper cervical laminae VI-VII projection to the PAG and deep tectal layers was stronger ipsilaterally than contralaterally. Another group of labeled laminae VI-VII neurons was found laterally in laminae VI-VII in the sacral segments, but only in the cases with an injection involving the lateral or ventrolateral intermediate and caudal PAG (cases 2385, 2155, 2300, 2390, 2401, 2471 and 2182). This group of neurons also takes part in the earlier mentioned lumbosacral cell group (see system V of the discussion).

Medial part of laminae VI and VII
Throughout the length of the spinal cord three separate populations of labeled neurons were observed in the medial part of laminae VI-VII (Fig. 7). The first population was located in the C1-C2 segments, between the cuneate nucleus and the medial border of lamina V bilaterally, with a contralateral preponderance. The second population consisted of neurons scattered throughout the medial part of laminae VI and VII in the cervical and upper thoracic segments, mainly contralaterally. These two populations were present in all
cases in which the injections involved the tec-
tum (cases 2155, 2159, 2182, 2338, 2367 and
2385), but were almost absent when the tec-
tum was not (case 2471), or only slightly in-
volved in the injection site (cases 2300, 2316,
2390, 2395, and 2401).

The third population was located mainly
contralaterally in the L5-S3 segments (Figs.
3 and 9). This group was most prominent in
the cases with injections involving the lateral
and ventrolateral parts of the intermediate and
caudal PAG, and also takes part in the dis-
tinct lumbosacral-PAG projection (see system
V of the discussion).

**Lamina VIII**
In all cases most labeled lamina VIII neurons
were observed in the cervical, and a few in
the lumbosacral cord (Fig. 8). Within lamina
VIII most labeled neurons were located
dorsolaterally, some dorsomedially and only
a few in other parts (Fig. 3).

**Lamina X**
In all cases labeled neurons were found in
lamina X, mainly contralaterally. In contrast
to all other groups of retrogradely labeled
neurons, most of the labeled lamina X neu-
rons were located in the thoracic and upper
lumbar cord, and virtually none in the cervi-
cal cord (Fig. 9). Especially in cases 2155 and
2300 relatively many labeled neurons were
also found in lamina X of the sacral cord, but
the impression was gained that also these neu-
rons belonged to the distinct cell group lo-
cated in laminae I and VI-VIII of the lum-
bosacral cord.

**Discussion**

**Technical considerations**
One major concern in tracing studies is the
possibility of uptake of tracer by fibers of
passage. In the present study the retrograde
tracer used is WGA-HRP, of which is known
that the problem of uptake of tracer by fibers
of passage is very small compared to many
other tracers. Furthermore, a comparison be-
tween the results from different cases revealed
that uptake of tracer did not cause a major
problem. In case 2300 a relatively small in-
jection of WGA-HRP was made in the cen-
tral part of the lateral PAG, involving only a
small part of the laterally adjacent tegmen-
tum. This injection resulted in many labeled
neurons in the upper cervical and lumbosac-
cral cord. In cases 2390 and 2401 WGA-HRP
injections were placed more laterally in the
lateral PAG, and in case 2338 the injection
almost exclusively involved the deep tectal
area laterally adjoining the lateral PAG. The
spinal afferent fibers always enter the PAG
ventrolaterally and laterally (Mehler, 1969;
Björkeland and Boivie, 1984; Yezierski,
1988). An injection in the lateral PAG and/or
the laterally adjacent deep tectal area, involves
the fibers terminating in the lateral PAG, as
well as the fibers passing to the medial part
of the lateral PAG. On the other hand, an in-
jection in the medial part of the lateral PAG
will exclusively involve the medially located
terminals. If a substantial uptake of WGA-
HRP occurs by fibers of passage, it would
result in more labeled spinal neurons in the
laterally injected cases 2390, 2401, and 2338
than in the medially injected case 2300. The
results, however, show exactly the opposite.
Thus, although one can never rule out the
possibility of some uptake of tracer by fibers
of passage, the results demonstrate that in the
present study this has not been a major
problem.

**Comparison with earlier spino-PAG studies**
In the present study as many as 5000 to 7500
labeled neurons were found in cases with large
injections in the intermediate and caudal PAG,
counted in a 1:4 series of sections. Such a
large number of spino-PAG neurons has never
been reported before, but in none of the ear-
lier studies on the spino-PAG projections (rat:
Keay et al, 1997, Yezierski and Mendez, 1991;
cat: Wiberg and Blomqvist, 1984; Keay and
Bandler, 1992; monkey: Wiberg et al., 1987;
Zhang et al, 1990) were injections made in-
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lateral part of lamina VI/VII

2367

2159

2385

2155

2316

2395

2300

2390
Fig. 6. left and right: Histograms showing the segmental organization of the labeled neurons in the lateral part of laminae VI-VII in the C1-Coc2 spinal cord. The numbers represent the labeled neurons in a series of 1:4 transverse sections.
Fig. 7. Histograms showing the segmental organization of the labeled neurons in the medial part of laminae VI-VII in the C1-Coc2 spinal cord. The numbers represent the labeled neurons in a series of 1:4 transverse sections.
Fig. 8. Histograms showing the segmental organization of the labeled neurons in lamina VIII in the C1-Coc2 spinal cord. The numbers represent the labeled neurons in a series of 1:4 transverse sections.
Fig. 9. Histograms showing the segmental organization of the labeled neurons in lamina X in the C1-Coc2 spinal cord. The numbers represent the labeled neurons in a series of 1:4 transverse sections. Note that the y-scaling is three times larger than that of the histograms shown in figures 3-8.
volving such a large rostrocaudal part of the PAG. It should be emphasized, however, that a precise comparison between the numbers of spino-PAG neurons (totals, numbers per laminae, or numbers per segment) observed in the present study, and the numbers presented in other studies, is difficult. There are three reasons that the numbers in all earlier studies are much less precise. First, in several of these studies these numbers are the result of large injections primarily targeting the intercollicular area and not the PAG (Wiberg and Blomqvist, 1984; Wiberg et al, 1987; Yezierski and Mendez, 1991). Second, often the numbers of labeled neurons were derived from blocks consisting of several segments, and were “averaged”. For instance, the total number of neurons was taken from 25 sections out of a block containing the C1-C4 segments (Yezierski and Mendez, 1991). Third, some of the earlier studies give numbers that result from averaging the numbers obtained from several cases with different injection sites, even when there were large differences between the numbers of neurons per case (Keay et al., 1997).

The segmental and laminar distribution of the spino-PAG neurons observed in the present study are generally in good agreement with those found in earlier studies. Similar to the earlier studies, the present results show many labeled neurons bilaterally in the cervical cord, contralaterally in the sacral cord, and in lamina I of the cervical and lumbar enlargements. However, the present results do not show a different input from C1-C3 to the ventrolateral PAG compared to the lateral PAG, as reported by Keay and Bandler (1992) in the cat and by Keay et al. (1997) in the rat. Instead, it was found that the upper cervical projection to the central part of both the lateral and ventrolateral PAG was much stronger than to the more lateral parts of these same regions. Differences in the spinal projections to the medial versus the lateral PAG were also reported by Mantyh (1982) in the primate, but this study did not report precisely which segments were involved, which prohibits a reliable comparison with the present results.

The same is true in respect to comparing the segmental and laminar distributions of the spino-PAG neurons observed in the present study and those in other earlier studies (Mehler, 1969; Hazlett et al., 1972; Beitz, 1982; Björkland and Boivie, 1984; Wiberg and Blomqvist, 1984; Meller and Dennis, 1986; Wiberg et al., 1987; Harmann et al., 1988; Lima and Coimbra, 1989; Zhang et al., 1990; Yezierski, 1991; Keay and Bandler, 1992; Bernard et al., 1995; Craig, 1995; VanderHorst et al., 1996; Keay et al., 1997), mainly because none of them has investigated the different parts of the PAG on one hand and all separate spinal segments on the other hand, as was done in the present study.

**The existence of five separate spino-PAG systems**

Comparing the different laminar and segmental distributions of the spino-PAG neurons after injections in different parts of the PAG, gives the impression that the spino-PAG projection is not one general ascending system, but consists of different groups of spinal neurons that project to different parts of the PAG. In all cases in all segments labeled lamina I and V neurons were found, which suggests the existence of one particular system originating in laminae I and V throughout the length of the spinal cord and projects to all parts of the entire rostrocaudal PAG, but stronger to the intermediate and caudal PAG. However, besides this rather diffuse system, at least four other specific systems can be distinguished on the basis of the laminar and segmental locations of their neurons. It has led to the hypothesis that there are at least five separate spino-PAG systems (Figs. 10 and 11):

**System I** (indicated in red in Fig. 11) is predominantly contralateral and originates in the dorsolateral part of lamina I and the lateral part of V (Fig. 10) throughout the length of the spinal cord. About 50% of all spino-PAG
neurons belong to this system. **System II** (indicated in gray in Fig. 11) originates exclusively from the upper cervical cord (C1-C3) and most strongly from C1. This system is bilateral, but predominantly ipsilateral, and originates from the lateral part of laminae VI-VII and the dorsolateral part of lamina VIII (Fig. 10). System II projects to the ventrolateral and lateral parts of the entire rostrocaudal PAG and to the adjacent deep tectal layers. Almost 20% of all spino-PAG neurons belong to this system. **System III** (indicated in green in Fig. 11) is predominantly contralateral, originates in lamina X of the thoracic and upper lumbar spinal cord (Fig 10) and projects to the PAG as well as to the adjacent deep tectal area. About 2% of all spino-PAG neurons belong to this system. **System IV** (indicated in yellow in Fig. 11) is predominantly contralateral and originates in the medial part of laminae VI-VII of the L5-S3 segments (Fig. 10). It terminates in the lateral and ventrolateral parts of the rostrocaudal PAG, but mainly in its intermediate and caudal region. About 10% of all spino-PAG neurons take part in this system. **System V** (indicated in blue in Fig. 11) has been described in detail in an earlier paper of our department (VanderHorst et al., 1996) as a system that is predominantly contralateral and originates in the lateral part of lamina I of the L6-S2 segments and the lateral parts of laminae V-VII and X at the S1-S3 levels (Fig. 10). The present results show that there are also some neurons located in lamina X of the sacral cord that may belong to this system. System V projects to the lateral, ventrolateral and dorsomedial parts of the intermediate and caudal PAG, but most strongly to its central portion (see cases 2300 and 2471). About 10% of all spino-PAG neurons belong to system V.

**Possible functions of the 5 systems**

Although physiological data are scarce, it is tempting to predict that each of the 5 systems subserves a different function. Considering the locations of the cells of origin of each of the different systems the following functional considerations can be made: **System I** contains laminae I and V neurons throughout the length of the cord that project to all parts of the PAG. Lamina I is known to receive afferent fibers from nociceptors and thermoreceptors from the skin as well as from muscles, joints and viscera. Lamina V receives afferent fibers from mechanoreceptors in the skin, joints and viscera (Willis and Coggeshall, 1991). Apparently, the entire rostro-caudal PAG, but mainly its lateral and ventrolateral parts, receive nociceptive, thermic, mechanical and/or visceral information from all parts of the body. **System II** contains neurons in the lateral part of laminae VI-VIII of the C1-3 segments which project to the lateral and ventrolateral PAG and to the deep tectum. Keay and Bandler (1992) and Keay et al. (1997) also found many laminae VI-VII neurons in these segments projecting to the PAG and suggested this projection to play a role in conveying information from the deep neck muscles to the ventrolateral PAG. The results of the present study, however, do not support this hypothesis, because the location of the large numbers of C1-C3 laminae VI-VII neurons projecting to the PAG does not coincide with the area where the deep neck muscle afferents terminate. The former terminate medially in the upper cervical intermediate zone (Hirai et al., 1984; Bakker et al., 1984; Abrahams et al., 1984; Nyberg and Blomqvist, 1984; Rose and Keirstead, 1986), while the PAG projecting neurons were found more laterally. The medial region also contained retrogradely labeled neurons when the injection involved the deep tectum, suggesting that the neck muscle information is not conveyed primarily to the PAG, but to the deep tectum. The lateral part of the intermediate zone, with the many PAG projecting neurons, receives many afferents from the ventrolateral and lat-
Fig. 10. Darkfield polarized photomicrographs showing labeled neurons of each of the five spino-PAG systems, after WGA-HRP injections in the PAG (case 2385). Abbreviation: CC: central canal.
Fig. 11. Schematic representation of the five spino-PAG systems. For each system the location of the neurons is indicated by color and their termination area is described on top. *dm*, dorsomedial; *dl*, dorsolateral; *l*, lateral; *vl*, ventrolateral. Areas between parentheses receive much fewer spinal projections than the other areas mentioned.
eral PAG and deep tectum (Mouton and Holstege, 1994). Furthermore, it has been shown that cells in this same region send many fibers to the motoneuronal cell groups of the cervical enlargement (Holstege, 1988). It is not yet determined whether these motoneuron projecting cells receive afferents from the PAG, but one might speculate that via this projection the PAG has access to fore-limb motoneurons. Such a projection might be important for eliciting the motor activities in for example the framework of aggressive behavior.

**System III** contains neurons in lamina X of the thoracic and upper lumbar spinal cord that project to the PAG as well as to the deep tectal area. Anatomical and physiological studies in cat and monkey (Light and Perl, 1979; Honda and Perl, 1985; Honda, 1985) showed that lamina X plays a role in nociception, as thin myelinated nociceptive primary afferents from both cutaneous, subcutaneous and visceral structures terminate in this area. However, as these studies were confined to the lower lumbar and sacral cord, it is unknown whether this is also true for the thoracic lamina X-PAG projecting neurons. On the other hand, a striking feature is that the lamina X cells are exclusively located at the level of the sympathetic preganglionic motoneurons. Perhaps the lamina X cells at this level relay some specific visceral afferent feedback information to the PAG concerning the total output activity of the sympathetic system.

**System IV** contains neurons in the medial part of laminae VI-VII of the L5-S3 segments that project to the lateral and ventrolateral parts of the contralateral PAG. The function of system IV is not known, but, unlike all other spino-PAG cells in the lumbosacral cord, several of its neurons contain estrogen receptors (VanderHorst et al., 1997). Possibly, they are involved in relaying information of tactile stimuli to the PAG, for example in the context of the receptive behavior induced by tactile stimuli of the flanks of an estrous female (Kow et al., 1979, 1980).

**System V** contains neurons in the lateral part of lamina I of the L6-S1 and laminae V-VII and X at the S1-S3 segments. In this area the afferents from the pelvic and pudendal nerves (Morgan et al., 1981) terminate. These afferents convey information from the bladder, perineum, vagina and cervix. In all likelihood, system V plays an important role in the relay of specific information from the pelvic organs to the PAG concerning micturition and mating behavior (Blok et al., 1995; VanderHorst et al., 1996).

**Conclusion**

The PAG is known to elicit analgesia, but also many motor functions related to survival and reproduction. To accomplish this it needs many afferents from rostrally as well as from caudally located structures. The present study shows that the PAG receives a strong, direct, projection from the spinal cord. Results showed the existence of at least 15,000 spinal cord neurons projecting to the PAG, located throughout the entire length of the cord. The different laminar and segmental distributions of labeled neurons suggest that the spino-PAG projection is not one general ascending system of which the main function is the relay of nociceptive information, but consists of at least five separate systems, each of which might have a separate function.