Chapter 9

C1-C3 spinal cord projections to periaqueductal gray and thalamus; a quantitative retrograde tracing study in cat

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ABSTRACT
By far the strongest spinal cord projections to periaqueductal gray (PAG) and thalamus originate from the upper three cervical segments, but their precise organization and function is not known. In the present study in cat tracer injections in PAG or in thalamus resulted in more than 2400 labeled cells, mainly contralaterally, in the first three cervical segments (C1-C3), in a 1:4 series of sections, excluding cells in the dorsal column and lateral cervical nuclei. These cells represent about 30% of all neurons in the entire spinal cord projecting to PAG, and about 45% of all spinothalamic neurons. About half of the C1-C3 - PAG and C1-C3 - thalamic neurons were clustered laterally in the ventral horn (C1-3vl), bilaterally, with a slight ipsilateral preponderance. The highest numbers of C1-3vl-PAG and C1-3vl-thalamic cells were found in C1, with the greatest density rostrocaudally in the middle part of C1. A concept is put forward that C1-3vl cells relay information from all levels of the cord to PAG and/or thalamus, although the processing of specific information from upper neck muscles and tendons or facet joints might also play a role.

INTRODUCTION
The spinothalamic tract is the best known projection from spinal cord to supraspinal levels. Especially the lamina I projections to thalamus are thoroughly studied, because these are clinically important for their role in relaying nociceptive information from body and extremities. Also the periaqueductal gray (PAG) is well known for its role in nociception and for its afferents from lamina I neurons throughout the length of the spinal cord. Recent quantitative tracer studies of our lab (Mouton et al., 2001; Klop et al., 2004b), however, have shown that no more than 30% of all spino-PAG and no more than 15% of all spino-thalamic neurons are located in lamina I. It means that other areas of the spinal cord contain large numbers of spino-PAG and spino-thalamic neurons, possibly relaying other information to PAG and thalamus than nociception. Earlier studies showed that the upper cervical cord is one of these other areas, as tracer injections in PAG of rat (Menetrey et al., 1982; Pechura and Liu, 1986; Yezierski and Mendez, 1991; Keay et al., 1997), cat (Wiberg and Blomqvist, 1984b; Keay and Bandler, 1992; Mouton and Holstege, 2000) and monkey (Wiberg et al., 1987; Zhang et al., 1990), as well as tracer injections in thalamus of rat (Giesler et al., 1979; Kevetter and Willis, 1983; Granum, 1986; Kemplay and Webster, 1986; Burstein et al., 1990b), cat (Carstens and Trevino, 1978a; Carstens and Trevino, 1978b; Comans and Snow, 1981) and monkey (Apkarian and Hodge, 1989a; Zhang et al., 1990; Willis et al.,
2001) resulted in large numbers of labeled cells in the upper three cervical segments (C1-C3). However, in no species precise quantitative data on both the spino-PAG and spino-thalamic neurons exists. In rat it has been estimated that 30 to 50% of all spino-PAG (Yezierski and Mendez, 1991; Keay et al., 1997) and 30 to 40% of all spino-thalamic neurons (Kemplay and Webster, 1986; Burstein et al., 1990b) are located in the C1-C3 segments. However, these are only estimates and are based on counts of about 60% of the spinal segments and not of all spinal segments (Burstein et al., 1990b; Keay et al., 1997), and/or the numbers of C1-C3 neurons also included labeled cells in the lateral cervical nucleus (LCN) and nucleus of the dorsolateral funiculus (Yezierski and Mendez, 1991; Keay et al., 1997), and/or just parts of PAG or thalamus are involved (Kemplay and Webster, 1986; Keay et al., 1997). Only in monkey precise data exist on the number of C1-C3 thalamic projecting neurons: counts of labeled neurons, after injections in the entire thalamus and studying all spinal segments, gave the conclusion that 35% of all spino-thalamic cells are located in C1-C3 (Apkarian and Hodge, 1989a). Precise data on the C1-C3 PAG projecting neurons in monkey, however, do not exist. In cat no precise quantitative data exists on both the C1-C3 PAG projections as well as the C1-C3 thalamus projections.

Within the gray matter of C1-C3 the PAG projecting neurons are located in the lateral part of the ventral horn (C1-3vl), bilaterally, in lamina I, mainly contralaterally, and in the lateral part of lamina V, also mainly contralaterally (Menetrey et al., 1982; Wiberg et al., 1987; Keay et al., 1997; Mouton and Holstege, 2000). The C1-C3 neurons that project to thalamus have a similar distribution pattern, except that an additional group of spino-thalamic neurons was found in lamina VI of C1 (Carstens and Trevino, 1978a; Giesler et al., 1981; Comans and Snow, 1981; Kevetter and Willis, 1983; Apkarian and Hodge, 1989a; Burstein et al., 1990b; Willis et al., 2001). Of all C1-C3 PAG and C1-C3 thalamus projecting cells the C1-3vl cells seem to form a considerable part (Carstens and Trevino, 1978b; Giesler et al., 1979; Comans and Snow, 1981; Kevetter and Willis, 1983; Wiberg et al., 1987; Burstein et al., 1990b; Keay and Bandler, 1992; Keay et al., 1997; Mouton and Holstege, 2000). However, no precise quantitative data exist on the numbers of cells belonging to this C1-3vl cell group projecting to PAG and C1-3vl cell group projecting to thalamus in any species.

To finally solve the questions which portion of all spino-PAG and which portion of all spino-thalamic cells are located in the upper cervical cord, and especially which portion is located in the lateral part of the ventral horn of this upper cervical cord, in the present study in cat we, for the first time, precisely determined the numbers of C1-C3 neurons projecting to PAG and C1-C3 neurons projecting to thalamus, as well as the numbers of C1-3vl-PAG and C1-3vl-thalamic neurons.

**MATERIALS AND METHODS**

**Surgical procedures**

Four female cats were used and the surgical procedures, pre- and postoperative care, handling and housing of the animals were in accordance with protocols approved by the Committee of Animal Experiments of the Faculty of Medicine of the University of Groningen. For surgery, the animals were initially anesthetized with intramuscular
C1-C3 projections to PAG and thalamus

<table>
<thead>
<tr>
<th>case</th>
<th>injection technique</th>
<th>total volume of WGA-HRP (µl)</th>
<th>number of injections</th>
<th>volume per injection (nl)</th>
<th>coordinates</th>
<th>survival time (days)</th>
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<td>200</td>
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<td></td>
<td></td>
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<td>LM 1.0 - 9.0</td>
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Table 1. Detailed information on the injection technique, number of injections, injected volumes and survival times per case. Coordinates according to the atlases of Berman (1986) and Berman and Jones (1982).
AP, anterior-posterior; DV, dorsal-ventral; LM, lateral-medial.

Ketamin (Nimatek, 0.1 ml/kg i.m.) and xylazine (Sedamun, 0.1 ml/kg i.m.), and subsequently ventilated with a mixture of \( O_2 \), \( N_2 O \) (1:2) and 1-2% halothane. During surgery, body temperature was monitored and maintained between 36.5 and 39°C using a heating pad, and basic physiological parameters were monitored through the use of a combined electrocardiogram (ECG), respiration, and \( CO_2 \) monitor. In two cases (2155 and 2385) multiple injections of 2.5% wheatgerm agglutinin-conjugated horseradish peroxidase (WGA-HRP) were made in the PAG, and in two other cases (2517 and 2529) in the thalamus (table 1). Hereafter a survival time of 3-4 days (table 1) was taken, as it has been proven that this is sufficient for the transport of WGA-HRP from thalamus or PAG to the lumbosacral cord (Wiberg and Blomqvist, 1984b; Yezierski, 1988; Mouton and Holstege, 2000; Klop et al., 2004a). Following survival time the animals were initially anesthetized with (Nimatek, 0.1 ml/kg i.m.) and xylazine (Sedamun, 0.1 ml/kg i.m.), followed by an overdose of 6% pentobarbital sodium (Nembutal; i.p.). They were perfused transcardially with 1.5-2 l of heparinized 0.9% saline at 37° C, immediately followed by 1.5 l of 0.1M phosphate buffer, containing 4% sucrose, 1% paraformaldehyde and 2% glutaraldehyde.

Histological procedures
After perfusion, the brains and spinal cords were removed, post-fixed for 2h and stored overnight in 25% sucrose in phosphate buffer at 4°C. The spinal cord was cut into 33 separate parts, from the first cervical to the second coccygeal segment (C1-
The rostral border of the C1 segment was set just rostral to the location at which the most rostral C1 dorsal rootlet enters the spinal cord, and the caudal border set just caudal to the location at which the most caudal dorsal rootlet of this segment enters the cord. Subsequently, the other more caudally located segments were each separated by cutting just caudal to the location at which the most caudal dorsal rootlet of the segment enters the cord. The brainstem and each spinal segment was cut into 40 µm frozen transverse sections of which every fourth (cases 2385, 2517 and 529) or every fifth (case 2155) section was incubated according to the tetramethylbenzidine method, dehydrated and coverslipped. From the area containing the injection site an extra series was processed with diamino-benzidine (DAB).

Quantification of labeled neurons
To determine the location and number of labeled neurons in the spinal cord, in each case all retrogradely labeled neurons in all processed sections of all spinal segments were plotted and counted. A neuron was considered labeled when its cells body as well as part of its axon or one or more of its dendrites was observed to contain tracer. In order to count the neurons located in laminae VI, VII and VIII of the C1-C3 segments, in all plottings the laminae of Rexed (1954) were depicted. An extra line, dividing laminae VI and VII into a medial and lateral part 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. 1). It must be emphasized that the labeled neurons in the lateral cervical nucleus (LCN) and dorsal column nuclei (DCN) were not included in any of the counts. To compare the numbers of case 2155, in which every fifth section instead of every fourth was processed, with the other cases, all counts of case 2155 were multiplied by 1.25. Such a correction is allowed because in all cases an unbiased sample was taken and double counting of cells could not occur in a 1:5 series of 40 µm sections. The corrected numbers were used in all additional analyses.

Figure 1. Schematic drawings of the gray matter of the three upper cervical segments, with the laminae of Rexed (1954) depicted. An extra line, dividing laminae VI and VII into a medial and lateral part 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. In gray the area in which the numbers of C1-3vl cells are counted.
As the present study did not attempt to come to a precise absolute total number of C1-C3 spinal neurons projecting to PAG or to thalamus, but aimed to give the percentages of C1-C3 and C1-3vl cells of the numbers of spino-PAG and spino-thalamus neurons in the entire cord, it was not necessary to use any specific method for cell countings.

The density of C1-3vl cells within the different rostrocaudal parts of the C1-C3 spinal cord was calculated by counting the total number of C1-3vl cells in 12 consecutive processed sections, starting from the rostral part of the segment, and dividing this number by 12. When at the caudal end of a segment only 6 to 11 sections were left, the number of labeled cells counted in these sections was divided by the number of sections. If 5 or less sections were left at the caudal end, no density was calculated.

RESULTS

PAG injectioned cases

The injections in the PAG injected cases (2155 and 2385) involved a large part of the PAG on one side (Figs. 2, 3). However, in both cases the most rostral part of the PAG escaped the injection, while in case 2385 the lateral part of the caudal PAG was also not included (Figs. 2, 3).

In the two PAG injected cases a total of 7468 and 5268 labeled neurons was counted in the spinal cord throughout its rostrocaudal extent, in a 1:4 series of sections, and excluding labeled cells in DCN and LCN (Table 2). Of these labeled neurons 32.5% was found in C1-C3, mainly contralaterally (Table 2). The cervical enlargement (C5-C8) contained 13.3% of these labeled spino-PAG neurons, averaged over both cases, and the lumbar enlargement (L5-S1) 14.5%.

In both PAG injected cases many of the C1-C3 labeled cells were located in the lateral part of laminae VI en VII and the dorsolateral part of lamina VIII, bilaterally (Figs. 4, 5), with in case 2385 a small ipsilateral preponderance, and in case 2155 about equal numbers of labeled C1-3vl cells on both sides (Table 3). Ipsilaterally, the

![Figure 2. Darkfield and brightfield photomicrographs showing sections with the WGA-HRP injection sites in the periaqueductual gray (left) and thalamus (right) after processing the sections with diaminobenzidine. Scale bar = 200 μm.](image)
Figure 3. Schematic drawings of the entire rostrocaudal extent of the WGA-HRP injection sites in periaqueductal gray or in thalamus. The core of each injection site is shown in dark gray. The rostrocaudal levels of mesencephalon and thalamus are given according to Berman’s atlases (Berman, 1968; Berman and Jones, 1982). P, posterior; A, anterior.

Table 2. Numbers of labeled cells in the upper cervical (C1, C2, C3) segments after large WGA-HRP injections in PAG or thalamus, in a 1:4 series of transverse sections, compared with the total number of labeled cells in the entire (C1-Coc2) spinal cord. Labeled cells in DCN and LCN were not included in any counts.
C1-C3 projections to PAG and thalamus

<table>
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<th>Total C1-3vl</th>
<th>C1vl</th>
<th>C2vl</th>
<th>C3vl</th>
<th>C1-2vl</th>
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<td>110</td>
<td>517</td>
<td>301</td>
<td>246</td>
<td>115</td>
<td>662</td>
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</table>

**Table 3.** The numbers of labeled cells in the lateral part of laminae VI-VII and in lamina VIII of the upper cervical segments C1-3vl after large WGA-HRP injections in PAG or thalamus, in a 1:4 series of transverse sections, compared with the total number of labeled cells in the entire (C1-Coc2) spinal cord. Labeled cells in DCN and LCN were not included in any counts.

C1-3vl-PAG projecting cells formed a relatively dense group, while contralaterally they were more scattered and extended more ventrally (Fig 5). The majority of the C1-3vl-PAG projecting cells was found in C1, with rostrocaudally the highest density in the middle part of this segment (Fig. 5). The C1-3vl-PAG projecting cells represent about 50% of all PAG projecting cells located in C1-C3, and 16% of all PAG projecting cells of the entire C1-Coc2 spinal cord (Tables 2, 3).

In addition to the retrogradely labeled neurons the upper cervical segments contained many anterogradely labeled fibers ipsilaterally in lamina V-VIII, and some

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**Figure 4.** Darkfield polarized photomicrographs showing labeled neurons in the lateral part of the ipsilateral (left) and contralateral (right) ventral horn of the first cervical segment (C1), after WGA-HRP injections in PAG or thalamus. Scale bar = 2000 μm.
Figure 5. Schematic drawings of labeled cells in the upper cervical segments (C1-C3) after WGA-HRP injections in PAG (case 2385) or thalamus (2517). Note that the drawings of the spinal cord contain labeled cells of 12 sections. The gray area indicates the area in which the number of labeled $C_{1-3vl}$ cells are counted. The histograms show the density of labeled $C_{1-3vl}$ cells ipsilaterally and contralaterally in the different rostrocaudal levels of the C1-C3 segments.

contralaterally. These anterogradely labeled fibers were not plotted in the present study.

*Thalamus injected cases*

Of the thalamus injected cases the injections site in case 2529 covered the complete thalamus, while in case 2517 the most medial thalamus escaped the injection site (Figs. 2, 3). In both cases many labeled neurons were found throughout the length of the spinal cord, located in laminae I, III-VIII and X. In addition, in case 2517, in contrast to case 2529, many very faintly labeled cells were found specifically in laminae III and IV. These cells were assumed to be labeled as the result of transsynaptic labeling via heavily labeled cells in LCN and DCN, due to a one day longer survival time. Therefore, labeled laminae III and IV cells in this case were not counted.

In the thalamus injected cases a total of 4817 and 6082 labeled cells was found.
throughout the length of the spinal cord (Table 2). Of these labeled cells 40 to 50% was found in C1-C3 (Table 2). Averaged over both cases, the cervical enlargement (C5-C8) contained 17.3% of these labeled spino-thalamic neurons, and the lumbar enlargement (L5-S1) 13.6%.

The C1-C3 thalamic projection shows a contralateral predominance (Table 2), which was more pronounced than found in the PAG injected cases. The reason for the thalamus injected cases to contain more C1-C3 contralaterally projecting neurons than the PAG injected cases, is a large cell group in lamina VI of C1 that projects to the contralateral thalamus but not to PAG (Fig. 5).

In both thalamus injected cases many labeled cells were located in the lateral part of laminae VI en VII and the dorsolateral part of lamina VIII of C1-C3 bilaterally (Figs. 4, 5), with a small ipsilateral preponderance (Table 3). Alike in the PAG injected cases, the labeled C1-3vl cells on the ipsilateral side formed a relatively dense group, while contralaterally they were more scattered and extended more ventrally (Fig. 5). In the thalamus injected case 2517 the total number of the C1-3vl cells was higher than in both PAG injected cases (Fig. 5, Table 3). Similar to the C1-3vl-PAG projecting cells, the majority of the C1-3vl-thalamus projecting cells was found in C1, with rostrocaudally the highest density in the middle part of this segment (Fig. 5). The C1-3vl-thalamic projecting cells comprise about 50% of all C1-C3 thalamic projecting cells, and about 20% to 30% of all C1-Coc2 spino-thalamic cells (Table 3).

**DISCUSSION**

**Technical considerations**

In the present study large tracer injections with WGA-HRP in PAG of two cats resulted in a total of 7468 (case 2155) and 5269 (case 2385) labeled cells throughout the length of the spinal cord, in a 1 out of 4 series of sections. The smaller total number of cells counted in case 2385 than in case 2155 can be explained by the fact that in case 2385 a smaller part of the PAG was involved in the injection site; in case 2385 the lateral part of the caudal PAG escaped the injection site, which lead to a diminished number of spino-PAG neurons in all laminae throughout the length of the cord (Mouton and Holstege, 2000). After large WGA-HRP injections in the thalamus of the cat 4817 and 6082 labeled neurons were found throughout the length of the spinal cord, in a 1 out of 4 series of sections. This difference is due to the fact that in case 2517 part of the medial thalamus was not involved in the injection site. Craig et al. (1989a) showed that the spinal enlargements contain more cells projecting to the lateral than to the medial thalamus. Therefore, missing a part of the medial thalamus will lead to a diminished total number of labeled spino-thalamic neurons.

In the PAG injected cases the retrogradely labeled neurons in the upper cervical segments were partly intermingled with anterogradely labeled fibers, especially ipsilaterally. These fibers are part of one of the descending pathways from PAG to spinal cord (Mouton and Holstege, 1994). It was never a problem to distinguish between labeled cells and labeled fibers.
Comparisons with earlier studies

The present results in cat for the first time demonstrate about 30% of all spino-PAG neurons and 40-50% of all spino-thalamic neurons are located in C1-C3. In a precise retrograde tracing study in monkey (Apkarian and Hodge, 1989a) it was found that 35% of all spino-thalamic cells was located in the C1-C3 segments. This is lower than the result presently found in cat. As no technical differences seem to be present between our study and that in monkey the discrepancy might be a species difference.

The present study in cat showed that 50% of all upper cervical neurons projecting to PAG and upper cervical neurons projecting to thalamus is located in the lateral parts of the ventral horn, bilaterally, with a slight ipsilateral preponderance. The ipsilaterally projecting cells formed a cluster in the lateral ventral horn and the contralaterally projecting cells were more widely distributed. Concerning the C1-3vl-thalamic projections, Granum (1986) has reported a similar distribution pattern in rat, i.e. the contralaterally projecting C1-3vl-thalamic neurons located deeper in the ventral horn than the ipsilateral ones, even deeper than found in the present study.

Our results show that rostrocaudally the highest number of C1-3vl-thalamic cells is in C1. This is in accordance with earlier results in cat of Comans and Snow (1981), but not of those of Carstens and Trevino (1978a), also in cat, who found the highest number of C1-3vl-thalamic cells in C2.

Comparison with the numbers of lamina I-PAG and lamina I-thalamic neurons

In earlier work of our lab (Mouton et al., 2001; Klop et al., 2004b), using the same materials and methods, the total numbers of lamina I neurons were counted throughout the length of the spinal cord, after large injections in PAG and thalamus. Comparing these numbers with the present results leads to the striking conclusion that the number of C1-C3 neurons projecting to PAG or thalamus, excluding cells in the lateral cervical nucleus and DCN, is respectively 1.5 and 3.5 times higher than the total number of lamina I neurons projecting to PAG or thalamus. For the C1-3vl neurons such comparisons are 0.7 and 2.0 respectively. Considering this, it is surprising that until now so little attentions was given to the upper cervical projection systems.

Functional considerations

Considering the many cells in the lateral ventral horn projecting to PAG or thalamus the intriguing question is what information is relayed by these C1-3vl cells. Although some studies paid attention to the upper cervical cord cells projecting to PAG or to thalamus, no definite function of these cells was revealed. According to the physiological study of Carstens and Trevino in cat (1978a) about 40% of the cells in the lateral ventral horn of C2 projecting to the ipsilateral thalamus is not responsive to any type of natural stimuli given, i.e. not to light brushing, touch, light tap, pressure, pinch, pin-prick, cooling, noxious heat, joint movements, sound, or visual stimuli. About 35% of the recorded cells in this region had large complex receptive fields and were responsive to several different stimuli, while the other cells had smaller receptive fields. In another physiological study in cat on the functional properties of C1-C3 mesencephalic cells, Yezierski and Broton (1991) found that none of the cells
in the lateral ventral horn responded to cutaneous mechanical, cutaneous thermal, joint, or deep muscle stimuli applied to face, neck, forelimbs, hind limbs, body or tail. These cells were also non-responsive to genital stimulation (Yezierski and Broton, 1991). Accordingly, in another study in rat involving the upper cervical segments (Keay et al., 2001), almost no Fos-like immunoreactive cells were found in the lateral ventral horn after deep or cutaneous noxious stimuli in the neck region. So it seems that the C1-C3vl cells, or at least part of them, do not to respond on stimuli applied to the neck-region, but have wide receptive fields. It might perhaps be that these cells, similar to DCN and LCN relay information from all levels of the cord to PAG and thalamus.

A second question that can be raised is whether the C1-C3vl-PAG and C1-C3vl-thalamic systems are mutually related. The striking similarity between the numbers and location of C1-C3vl-PAG and C1-C3vl-thalamic cells suggests that the same cells might project to both PAG and thalamus. However, Liu (1986) in rat and Zhang et al. (1990) in monkey report no double labeled cells in the lateral ventral horn after tracer injections in thalamus and in mesencephalon. It should be noted though, that in these studies the injections sites did not involve the entire PAG and thalamus. Kevetter and Willis (1983) found in rat C1-C3vl cells double labeled after injecting medial and lateral thalamus, indicating that these cells have multiple targets within the thalamus, but whether the same cells also project to PAG is not known.

CONCLUSION

In cat the C1-C3 segments together measure about 25 mm, while the average total length of the complete cervical to coccygeal spinal cord is 290 mm. Thus, the C1-C3 segments represent about 8.6% of the total length of the spinal cord. The present retrograde tracing study in cat, involving all spinal segments, for the first time demonstrates that within this 8.6% of the length of the spinal cord about 30% of all spino-PAG neurons and 40-50% of all spino-thalamic neurons are located. These are strikingly high percentages when comparing them with the 13% and 14% of all spino-PAG neurons or 17% and 13% of all spino-thalamic neurons located in the cervical or lumbar enlargements, respectively. It is also a high percentage realizing that the well-known lamina I projections to PAG or to thalamus comprise only 30% of all spino-PAG or 15% of all spinothalamic neurons.

Of the C1-C3 neurons projecting to PAG or thalamus about half are located grouped laterally in the ventral horn, i.e. laterally in laminae VI and VIII and dorsolaterally in lamina VIII. It might be that these cells relay information from all levels of the cord to PAG and/or thalamus, but it can also not be ruled out that they process specific information from the upper neck region.