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

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Chapter 8

In cat four times as many lamina I neurons project to the parabrachial nuclei and twice as many to the periaqueductal gray as to the thalamus

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ABSTRACT
The spinothalamic tract, and especially its fibers originating in lamina I, is the best known pathway for transmission of nociceptive information. On the other hand, different studies have suggested that more lamina I cells project to the parabrachial nuclei (PBN) and periaqueductal gray (PAG) than to the thalamus. The exact ratio of the number of lamina I projections to PBN, PAG and thalamus is not known, because comprehensive studies examining these three projections from all spinal segments, using the same tracers and counting methods, do not exist.

In the present study, the differences in number and distribution of retrogradely labeled lamina I cells in each segment of the cat spinal cord (C1-Coc2) were determined after large WGA-HRP injections in either parabrachial nuclei (PB), periaqueductal gray (PAG) or thalamus. We estimate that approximately 6000 lamina I cells project to PBN, 3000 to PAG and less than 1500 to the thalamus. Of the lamina I cells projecting to thalamus or PAG more than 80%, and of the lamina I-PBN cells approximately 60%, was located on the contralateral side. In all cases, most labeled lamina I cells were found in the upper two cervical segments and in the cervical and lumbar enlargements.

INTRODUCTION
Cells in lamina I, or marginal layer, of the spinal cord play an essential role in conveying information about noce and temperature to supraspinal structures. The great majority of these lamina I neurons receive Aδ and C fiber input and is nociception specific (Willis and Coggeshall 2004). The somatosensory thalamus is the best known supraspinal target of these lamina I neurons (Mott, 1892; Mott, 1895; Mehler, 1969; Trevino and Carstens, 1975; Carstens and Trevino, 1978b; Willis et al., 1979; Kemplay and Webster, 1986; Apkarian and Hodge, 1989a; Burstein et al., 1990b; Zhang et al., 1996; Zhang and Craig, 1997; Willis et al., 2001), but parabrachial nuclei (PBN) and periaqueductal gray (PAG) have also been shown to be important termination areas of lamina I projections (Trevino 1976; Menetrey et al., 1982; Wiberg and Blomqvist, 1984a; Panneton and Burton, 1985; Wiberg et al., 1987; Hyliden et al., 1989; Craig, 1995; Keay et al., 1997; Mouton and Holstege, 2000). Both PBN and PAG have also been associated with pain control (Basbaum and Fields, 1984; DeSalles et al., 1985; Besson and Chaouch, 1987; Besson et al. 1991). In PET studies in humans, both PAG and rostral pons, probably including PBN, have been shown to play an important role.
in both acute and chronic pain and in pain modulation (Hsieh et al., 1996; Petrovic et al., 1999; Kupers et al., 2000; Derbyshire et al., 2002; Tracey et al., 2002; Singer et al., 2004; Matharu et al., 2004). Furthermore, stimulation in PBN in patients with intractable pain has successfully been used for pain relief (Katayama et al., 1985; Young et al., 1992).

It has been suggested that more lamina I cells project to PBN (rat: Hylden et al., 1989) or to PAG (rat: Menetrey et al., 1982; cat: Mouton and Holstege, 1998; monkey: Trevino 1976) than to the thalamus, but it is not known what their ratio is, because these comparisons were based on studies of only few spinal segments, on data from the literature obtained with the use of different tracers and different counting methods, or on studies in which the injection sites did not cover the entire thalamus. In the present study in cat we have used the same method to determine the total number of lamina I-PBN, lamina I-PAG and lamina I-thalamus neurons.

MATERIALS AND METHODS

Experimental procedures

A total of 14 female cats was used with large injections into either PBN (cases 2500, 2592, 2602 and 2611), PAG (cases 2155, 2159, 2385, 2390 and 2424) or thalamus (cases 2517, 2519, 2529, 2545 and 2547). Six of these cases with the most successful injections are presented in this study. 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. All animals were initially anesthetized with intramuscular ketamine (Nimatek, 0.1 ml/kg) and xylazine (Sedamun, 0.1 ml/kg), and subsequently ventilated with a mixture of O₂, N₂O (1:2) and 1-2% halothane or isoflurane, while ECG and body temperature were monitored.

Injections of 2.5% wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP; Sigma, St. Louis, MO, USA) in saline were made into PBN (cases 2592 and 2611), PAG (cases 2155 and 2385) or thalamus (cases 2529 and 2547) under stereotaxic guidance using Berman’s (1968) atlas for PBN and PAG injections and the atlas of Berman and Jones (1982) for thalamus injections. A micropipette (PBN cases 2592 and 2611 and PAG case 2385) or a Hamilton syringe (PAG case 2155 and thalamus cases 2529 and 2547) was used to inject the tracer. In cases with thalamus or PAG injections a dorsal approach was used, while in cases with PBN injections a caudal approach under a 15° angle was used to avoid the osseous tentorium cerebelli, guiding the pipette through the cerebellum. Injections were aimed to cover all parts of the areas of interest and multiple injections were made in all regions. A total volume of approximately 0.15μl was injected in PBN and PAG, while 13.8μl (case 2529) and 4.3 μl (case 2547) was injected in the thalamus. Survival times were approximately three days.

For perfusion, all animals were initially anesthetized with intramuscular ketamine (Nimatek, 0.1ml/kg) and xylazine (Sedamun, 0.1ml/kg), followed by an overdose (6-10ml) of intraperitoneal 6% pentobarbital sodium. Subsequently, they were perfused transcardially with 2 liters of 0.9% saline, immediately followed by 2 liters of 0.1M phosphate buffer (pH 7.4), containing 4% sucrose, 1% paraformaldehyde.
and 2% glutaraldehyde, except in case 2592, in which the solution contained 1% glutaraldehyde and no sucrose.

After perfusion the brains and spinal cords were removed, post-fixed for two hours and stored overnight in 25% sucrose in phosphate buffer at 4°C. In order to define the extent of the injection sites, transverse 40µm frozen sections of the brainstem or thalamus were incubated with diaminobenzidine (DAB) in a 1:4 or 1:5 (case 2155) series of sections, and studied with brightfield illumination.

The spinal cord was divided into 33 segments (C1-Coc2) by placing a cut just caudal to each entering dorsal root. All spinal segments were cut into transverse serial 40µm frozen sections and every fourth or every fifth (case 2155) section was incubated according to the tetramethyl benzidine (TMB) method. In each case the retrogradely labeled lamina I neurons in all processed sections of all spinal segments were plotted and counted, using darkfield polarized illumination. To compare the numbers of labeled cells and of studied sections in case 2155 with those found in the other cases, numbers in case 2155 were multiplied with 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 40µm sections. The corrected numbers were used in all analyses shown in this paper.

In order to estimate the total number of lamina I cells projecting to PBN, PAG and thalamus based on the numbers found in a 1:4 series of sections, the correction factor of Abercrombie (1946) was used, because simply multiplying by four would lead to an overestimation (Coggeshall and Lekan, 1996). This correction factor is defined

**Figure 1.** Darkfield and brightfield photomicrographs showing the injection sites after diaminobenzidine (DAB) staining in the parabrachial nuclei (case 2611), periaqueductal gray (case 2385) and thalamus (case 2529). aq, cerebral aqueduct; BC, brachium conjunctivum; F, fornix; IC, internal capsule; LD, lateral dorsal nucleus; LGN, lateral geniculate nucleus; mesV, mesencephalic trigeminal tract; MT, mammillothalamic tract; OT, optic tract; PAG, periaqueductal gray; VBX, ventrobasal complex; VMP, principal ventral medial nucleus. Scale bar represents 1.5mm in all photographs.
as $M/(M+L)$, with $M$ being the thickness of the section (40μm) and $L$ the average rostrocaudal length of the neurons that were counted. Lamina I cells are known to have a specific rostrocaudal orientation, which cannot be measured in transverse sections. Therefore, the mean of the measurements of two cat studies was taken, which were 32μm (Galhardo and Lima, 1999) and 40 μm (Zhang et al., 1996). Using 36μm as the mean rostrocaudal length, the correction factor is $40/(40+36) = 0.526$. Photomicrographs in figure 3 were taken using a Leica digital camera and processed using Adobe Photoshop software.

RESULTS

Injection sites

Figures 1 and 2 show the injection sites in PBN, PAG and thalamus. Injections in PBN involved mainly the ventral and lateral parts in case 2592, and the dorsal, lateral and ventral parts in case 2611. In both cases the injection sites included the Kölliker-Fuse nucleus. Small leakage of the tracer into the cerebellum occurred along the needle track. Injection sites in the PAG involved the lateral and ventrolateral parts of the intermediate and caudal PAG and the adjacent tectal layers. In the thalamus the injection sites extended from the level of the anterior commissure to the level of the rostral midbrain. In case 2529 the entire left thalamus was injected. In case 2547 the injection site crossed the midline, but did not extend into the most lateral thalamus. In both cases the injection sites extended into the rostral mesencephalon caudally and, but to only a minor extent, into the hypothalamus ventrally. In case 2529 a large amount of tracer spread into the overlying cortical layers and internal capsule.

Retrogradely labeled neurons in the spinal cord

Labeled lamina I neurons were found in all cases (figs. 3 and 4). Table 1 gives the numbers of cells that were counted in lamina I after PBN, PAG and thalamus injections in an 1:4 series of sections. Results show that most labeled cells were found in lamina I after injections in the PBN (2595 and 3050 labeled neurons), and clearly less labeled cells were found after PAG injections (1615 and 1261 labeled cells). Even fewer labeled lamina I neurons were found in the two thalamus injected cases (628 and 701). In case 2547, the most lateral parts of the thalamus were not included in the injection site, which could have resulted in less labeled lamina I neurons. On the other hand, the spread of tracer into the rostral midbrain and over the midline (case 2547) might have caused extra lamina I cells to be labeled from this part of the brain.

Using the correction factor of Abercrombie (1946) leads to an estimation of an average of 5939 lamina I-PBN cells, 3026 lamina I-PAG cells and 1398 lamina I-thalamus cells. The ratio of the average numbers of labeled lamina I neurons from thalamus, PAG and PBN was $1 : 2.16 : 4.25$.

In all cases most labeled lamina I neurons were found on the contralateral side, but this contralateral preponderance was much stronger in the PAG and thalamus injected cases (81.9-85.3%) than in the PBN injected cases (approximately 60%). Histograms in figure 4 show that in the PBN injected cases the largest differences in ipsi- and contralaterally labeled lamina I neurons were present in the enlargements, while in
Lamina I projections to PBN, PAG and thalamus

Figure 2. Schematic drawings of the injection sites. The core of the injection is in dark gray and the halo in light gray. PAG, periaqueductal gray; PBN, parabrachial nuclei.

Table 1. Number of labeled lamina I cells found on the ipsi- and contralateral side in 1:4 series of sections per case. The number of sections studied in each case and the average number of labeled lamina I cells per section are given in the columns on the right. PAG; periaqueductal gray; PBN, parabrachial nuclei.

* All numbers in case 2155 have been recalculated to a 1:4 series of sections (see Methods).
Figure 3. Darkfield photomicrographs using polarized illumination of lamina I neurons in C8 segments in case 2611 with a parabrachial (PBN), case 2385 with a periaqueductal gray (PAG) and case 2529 with a thalamus injection. Scale bar represents 150μm.
most other segments the numbers labeled lamina I cells were almost equal on both sides. The ratio of the contralateral lamina I cells was 1 : 2.17 : 3.16, for thalamus, PAG and PBN, and of the ipsilateral lamina I cells 1 : 2.15 : 9.63.

The distribution of lamina I cells over the rostrocaudal extent of the cord is generally similar in all cases (fig. 4). Relatively many labeled lamina I cells were found in the upper cervical segments, although relatively few in PBN injected case 2592 and thalamus injected case 2529. Many labeled lamina I cells were also found in the enlargements. Only in parabrachial injected case 2611 relatively few labeled cells were found in lamina I of the segments of the lumbar enlargement.

Within lamina I, the majority of retrogradely labeled neurons was found on the dorsal cap of the dorsal horn in all cases, irrespective of the injection site in either PBN, PAG or thalamus. A smaller number of labeled neurons was found medially and laterally to the dorsal cap along the lateral edge of the dorsal horn.

Figure 4. Histograms showing the numbers from 1:4 series of sections and estimated numbers per case of labeled lamina I cells per segment on the ipsi- and contralateral side. All numbers in case 2155 have been recalculated to a 1:4 series of sections (see Methods).
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DISCUSSION

In the present study, for the first time, the numbers of lamina I cells from all segments of the spinal cord projecting to PBN, PAG and thalamus have been compared using the same tracer, the same histological procedures and the same counting techniques. The present results showed that the number of lamina I-PBN cells was approximately four times, and that of lamina I-PAG cells approximately two times, higher than the number of lamina I-thalamus cells (fig. 5). The total estimated number of lamina I cells projecting to PBN was 6000, to PAG 3000 and to thalamus less than 1500.

Technical and methodological considerations

For this study we have chosen WGA-HRP as our tracer. WGA-HRP, in contrast to HRP without a conjugate, produces the same results concerning the number of lamina I neurons after injections in brainstem and thalamus compared to fluorescent tracers and cholera toxin subunit b (Craig et al., 1989a; Craig et al., 1989b; Zhang et al., 1996; present study). Moreover, WGA-HRP, is a better tracer with regard to the retrograde labeling of cells in other parts of the spinal gray matter (Craig et al., 1989b), which is important, because the results from the retrogradely labeled neurons in the other laminae have been used (Klop et al., 2004a; Klop et al., 2004b) and will be used for other studies in the future. Another advantage of WGA-HRP is that it spreads relatively far from the center of the injection site, making it possible to cover large parts of a particular brain structure.

The quality of the retrograde labeling in the present study was similar in all cases and at all spinal cord levels. In an earlier study (Klop et al., 2004a; Klop et al., 2004b) we described that longer survival times for the thalamus injected cases did not result in more labeled (lamina I) neurons in the spinal cord. Although the injections in the thalamus were much larger than in PBN or PAG, in the thalamus injected cases a much smaller number of labeled lamina I cells was found. In the other parts of the spinal gray matter, however, numerous labeled cells were found in these cases (Klop et al., 2004a; Klop et al., 2004b).

Although we did not succeed to fill the structures of interest entirely with the tracer, the greater parts of PBN, PAG or thalamus were injected in the present study. Our most accurate injections (case 2611 for PBN, case 2155 for PAG and case 2529 for thalamus) covered those areas that have been described in anterograde tracing studies to receive lamina I projections (Craig, 1995; Craig, 2003b). However, although the greater part of these structures were injected in these cases, our injections might have missed some small areas to which lamina I neurons send fibers. It means that the numbers given in the present study might be lower than the actual number of lamina I neurons.

On the other hand, the injections also spread to neighboring structures, which might have led to overestimations. For example, in the PBN injected cases small tracer leakage in the cerebellum was found. This, however, does not pose a problem, since it has been shown in cat that only very few lamina I neurons project to the cerebellum (Matsushita et al., 1979; Matsushita and Hosoya, 1982; Grant et al., 1982). In our PAG injected cases, parts of the adjoining tectum and tegmentum were also involved in
the injection sites, but projections from lamina I to these structures are also limited (Craig, 1995; Mouton and Holstege, 2000). Concerning the thalamus injected cases, the numbers of retrogradely labeled lamina I cells found in case 2529 are probably most accurate, because in this case all parts of the thalamus were injected and only limited spread was seen to non-thalamic structures. Although the injection site also extended into the hypothalamus, this probably did not contribute much to the number of labeled lamina I neurons, since in cat, unlike in rat, almost no lamina I neurons project to the hypothalamus (Katter et al., 1991). In case 2547 the most lateral thalamus was not included in the injection, which might have caused less labeled lamina I neurons, although the figures in the study of Craig (2003b) show that the densest lamina I projections from the enlargements are not to this part of the thalamus. On the other hand, the injection in this case extended over the midline, probably causing extra labeled lamina I neurons from the submedial nucleus (Craig, 2003b).
In the PBN injected cases, the possibility exists of uptake by damaged fibers en route to the thalamus, although most fibers of the spinothalamic tract are located more ventrally. In the unlikely event that all lamina I-thalamic neurons were labeled via their fibers of passage in cases with PBN injections, even then the lamina I-PBN projection would still be three times stronger than the lamina I-thalamus projection.

**Comparison with earlier studies**

The present findings correspond well with suggestions of other authors that the projections from lamina I to the thalamus, compared to PAG and PBN, are limited. In cat, Mouton and Holstege (1998) estimated that three times as many lamina I neurons project to the PAG as to the thalamus. Their estimation was based on the number of lamina I cells after WGA-HRP injections in the PAG, compared with data from the study of Zhang et al. (1996) in which the feline thalamus was injected with cholera toxin-subunit b (CT-b). However, the number of retrogradely labeled lamina I cells after thalamic injections estimated in the present study (1500) exceeds the number of lamina I cells that was found in the study of Zhang et al. (1996), in which 1360 lamina I cells were found in horizontal sections in a 1:1 series of sections in the C3-Coc4 spinal cord, but in which no correction was used for double counted neurons, because relative proportions and not absolute numbers were their primary concern. This explains why Mouton and Holstege (1998), based on these data, came to an overestimation of three, instead of two, times more lamina I neurons projecting to the PAG than to the thalamus. The present estimation, based on comparisons using the same neural tracer and the same counting methods, is more accurate. In rat, cat and monkey, anterograde tracing studies (Craig, 1995; Bernard et al., 1995) have shown denser projections to PBN than to PAG. A retrograde tracing study of Trevino (1976) in monkey has shown more labeled lamina I neurons in selected segments after an injection covering a large part of the dorsal mesencephalon, including PAG, than after a large injection in the lateral parts of the thalamus. We are not aware of retrograde tracing studies revealing the total number of lamina I-PAG or lamina I-PBN projecting cells for the entire spinal cord in this species.

In the present study, higher percentages of ipsilaterally labeled lamina I neurons were found in the PBN injected cases than in the PAG and thalamus injected cases. This observation corresponds with retrograde tracing results found in earlier studies in cat (Panneton and Burton, 1985; Hylden et al., 1989) and with results from an anterograde study by Craig (1995) in cat and monkey. These studies show that projections from lamina I cells in the cervical and lumbar enlargements to PBN have a much larger ipsilateral component than projections to PAG and thalamus.

**Functional considerations**

Are all lamina I neurons projecting to PBN, PAG and thalamus nociceptive specific? Physiological studies in rat and cat have shown that the great majority of neurons in the superficial dorsal horn projecting to PBN and PAG is nociceptive specific (Hylden et al., 1986; Light et al., 1993; Bester et al., 2000; Todd et al., 2002), but it is important to realize that they also have functions not directly related to pain. ‘Nociceptive-
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I neurons only provide a limited part of all the spinal projections to the PAG (Mouton et al., 2001).

Although large differences exist in the total number and distribution of lamina I projections to PBN, PAG or thalamus, certain lamina I neurons might send their axons to two or all three structures. Different studies with different areas of interest, using different methods and in different species gave different results. Double labeling studies in rat and monkey have shown that indeed some lamina I cells project to both PAG and VPL of the thalamus, but the figures in the papers of Liu (1986) and Zhang et al. (1990) suggest that they represent only a small portion of all labeled lamina I cells. However, injection sites in these studies were small and, therefore, might lead to an underestimation of the number of these cells. Injections in PBN and VPL in rats (Hylden et al., 1989) have shown that 85% of lamina I-thalamus projecting cells in the enlargements have collaterals to the PBN. The reverse was not true, i.e. the majority of lamina I-PBN neurons were only single-labeled (Hylden et al., 1989). Spike et al. (2003) reported that 80% of the lamina I cells that projected to the caudal ventrolateral medulla also projected to the PBN and vice versa, and 90% of the lamina I cells labeled from PAG was also labeled from either medulla or PBN. On the other hand, lamina I-PBN and lamina I-PAG projection neurons in rats show different firing patterns in vitro, although half of the spino-PAG neurons showed the same firing pattern as the spino-PBN neurons (Ruscheweyh et al., 2003). A fluorescent labeling study in cat showed no double labeled cells in lamina I of the medullary dorsal horn after injections into PBN and the medial thalamus (Panneton and Burton, 1985). Moreover, in cat separate populations of lamina I cells appear to project to caudal medulla and thalamus (Andrew et al., 2003), or to medial and lateral thalamus (Craig et al., 1989a). It is tempting to conclude that, although in rat lamina I cells project to more than one structure, in cat different populations of lamina I neurons project to different supraspinal structures, possibly relaying different types of information.

In conclusion, the present findings, together with those from earlier studies, indicate that a much larger number of lamina I cells project to PBN and PAG than to the thalamus. Also in humans PBN and PAG have been associated with pain perception and pain control (Hsieh et al., 1996; Petrovic et al., 1999; Kupers et al., 2000; Derbyshire et al., 2002; Tracey et al., 2002; Singer et al., 2004; Matharu et al., 2004). However, in textbooks used in medical training a predominant role is still being attributed to the lamina I-thalamic projection concerning pain. The strong lamina I projections to the brainstem deserve more attention.
Lamina I projections to PBN, PAG and thalamus

Specific lamina I neurons have been reported to respond to changes in the tissue’s metabolic states not necessarily perceived as pain, and are probably important in homeostasis (for review see Craig, 2003a; Willis and Coggeshall, 2004). Furthermore, cells identified as wide dynamic range neurons or heat-pincha-cold cells have been found to project from lamina I to PBN, PAG and thalamus, as well as cells selectively responsive to innocuous cooling (Hodge and Apkarian, 1990 for review; Light et al., 1993).

One study in monkey showed that the morphological characteristics of lamina I cells correlate with their specific function (Han et al., 1998). Other studies in rat and cat, however, have shown that this is not the case in these species (Hylden et al., 1986; Todd et al., 2002) and that the presence of the neurokinin 1 receptor in lamina I neurons seems to be a better indicator of their function than their morphology (Todd et al., 2002). Also, in rat no consistent morphological differences exist between cells that project to PBN, PAG or caudal ventrolateral medulla (Spike et al., 2003). In our study, the morphology of labeled lamina I cells was not studied, because lamina I cells have a specific rostrocaudal orientation which can only be fully appreciated in horizontal and not in transverse sections (Zhang et al., 1996; Han et al., 1998; Galhardo and Lima, 1999).

Lamina I cells are not the only neurons involved in conveying information about noxa. Neurons in deeper laminae have been shown to convey noxious information to supraspinal structures. Wide dynamic range neurons located in the deep laminae of the dorsal horn have been shown to project to PBN, PAG and thalamus (Yezienski and Schwartz, 1986; Zhang et al., 1991; Bourgeais et al., 2001).

Anyway, the presence of extensive projections of lamina I neurons to the structures of the brainstem as compared to the thalamus found in the present and earlier studies, is in good agreement with the readopted view of pain as a homeostatic emotion, similar to temperature, hunger and thirst (Perl, 1998; Gauriau and Bernard, 2002; Craig, 2003a).

The majority of the PBN neurons in regions receiving lamina I input projects to either amygdala or hypothalamus and are nociceptive specific. These projections probably play a role in the emotional and autonomic aspects of pain (Bernard and Besson, 1990; Bester et al., 1995; Bernard et al., 1996). Both PBN and PAG are known to play a role in nociception control. Stimulation in PBN and PAG has been shown to produce analgesia in animals and humans (DeSalles et al., 1985; Katayama et al., 1985; Besson et al. 1991). In animals this analgesia has been shown to be accomplished through projections to the spinaly projecting raphe nuclei (Basbaum and Fields, 1984; Katayama et al., 1986; Besson and Chaouch, 1987; Vanegas and Schaible, 2004). The analgesia elicited in PAG and PBN should be seen as part of a wide array of survival mechanisms. Electrical or chemical stimulation of the PAG can elicit fight, flight or freezing accompanied by non-opioid mediated analgesia, as well as the appropriate autonomic responses, such as hypertension and tachycardia and vasodilation in specific parts of the body recruited for these behaviors (for review, see Keay and Bandler, 2001). The defensive response from the PAG might, among many others, be provoked by noxious stimuli. It is however, important to realize that lamina