Chapter 2

Afferent projections to the pontine micturition center in the cat

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Journal of Comparative Neurology; 494: 36-53 (2006)

Abstract
The pontine micturition center (PMC) or Barrington’s nucleus controls micturition by way of its descending projections to the sacral spinal cord. However, little is known about the afferents to the PMC that control its function and may be responsible for dysfunction in patients with urge-incontinence and overactive bladder. In five female cats wheatgerm agglutinin conjugated horseradish peroxidase (WGA-HRP) injections were made in the PMC and adjoining dorsolateral pontine tegmentum. Retrogradely labeled neurons were found in a large area including the medullary and pontine medial and lateral tegmental field, the dorsomedial, lateral and ventrolateral periaqueductal gray matter (PAG), posterior hypothalamus, medial preoptic area (MPO), bed nucleus of the stria terminalis, central nucleus of the amygdala, as well as the infralimbic, prelimbic and insular cortices. To verify whether these areas indeed project specifically to the PMC or perhaps only to adjacent structures in the pontine tegmentum, in 67 cats 3H-Leucine or WGA-HRP injections were made in each of these regions. Five cell groups appeared to have direct connections to the PMC, the ventromedial pontomedullary tegmental field, the ventrolateral and dorsomedial PAG, the MPO and the posterior hypothalamus. The possible functions of these projections are discussed. These results indicate that all other parts of the brain that influence micturition have no direct connection with

Introduction
Overactive bladder and urge incontinence are socially disabling conditions that affect tens of millions of people in the US and have an economic cost of over $30 billion (Hu et al., 2004). The spinal cord, bladder, and brainstem function normally in these conditions (Blaivas, 1982), suggesting that the defect may be in the inputs to the pontine micturition center (Holstege and Mouton, 2003). Normal micturition is a coordinated activation of bladder and relaxation of the external
urethral sphincter muscle (EUS). Coordination does not take place in the spinal cord, but in the PMC (Holstege et al., 1979; figure 1). Often the nucleus is defined as the neurons in the dorsolateral pontine tegmentum which are retrogradely labeled from the sacral spinal cord although it should be noted that part of the locus coeruleus is also labeled from the sacral cord. This nucleus is also known as Barrington’s nucleus (Barrington, 1925) or the M-region (Holstege et al., 1986). Electrical stimulation of the PMC results in coordinated micturition and bilateral lesions of the PMC result in long-term retention of urine (Holstege et al., 1986). Also in humans a lesion of the right-sided PMC results in urinary retention (Komiyama et al., 1998) and neuroimaging studies are in agreement with the idea that in cats and humans a similar organization of micturition exists (Blok et al., 1997b; Blok et al., 1998a; Nour et al., 2000).

The PMC coordinates micturition by way of a specific pattern of projections to the sacral segments of the spinal cord. First of all, PMC cells have a direct excitatory effect on parasympathetic preganglionic motoneurons in the sacral
intermediolateral cell column which innervate the detrusor muscle of the bladder (Morgan et al., 1979; Blok and Holstege, 1997). The same PMC cells also project to neurons located in the intermediomedial cell column (IMM) of the sacral spinal cord (Holstege et al., 1986) where they excite γ-aminobutyric acid (GABA) and glycine, gering inhibitory interneurons (Blok et al., 1997a; Sie et al., 2001). Electrical stimulation in IMM results in sphincter relaxation (Blok et al., 1998b), because these inhibitory interneurons project to EUS somatic motoneurons (Nadelhaft and Vera, 1996) which are located in the ventrolateral part of the nucleus of Onuf (Sato et al., 1978). Thus the PMC elicits simultaneously bladder contraction and EUS relaxation through descending excitatory projections to the parasympathetic preganglionic bladder motoneurons and inhibitory interneurons in the IMM.

In cat the only two regions known to maintain direct projections to the PMC are the periaqueductal gray matter (PAG) (Blok and Holstege, 1994) and the medial preoptic nucleus of the hypothalamus (Holstege, 1987), of which the PAG-PMC projection is thought to take part in the micturition reflex. Afferent information from the bladder enters the sacral dorsal horn where it is relayed to the central parts of the PAG (Blok et al., 1995; VanderHorst et al., 1996; Mouton and Holstege, 2000). Other cells in the PAG project directly to the PMC, which, as indicated above, controls micturition. This PAG-PMC projection explains why stimulation in the PAG causes micturition (Kabat et al., 1936). In humans the PAG seems to be involved in micturition in a similar way: lesions in the PAG can cause urinary retention (Yaguchi et al., 2004) and the PAG is active during micturition (Blok et al., 1997b; Blok et al., 1998a). The nature of the projection from MPO to PMC is not known, although according to Gjone (1966), stimulation in MPO in cat elicits bladder contractions.
In rat Valentino et al. (1994) also showed PMC afferents from MPO, posterior hypothalamus, ventrolateral and dorsomedial PAG and Kölliker-Fuse nucleus. However, a systematic study describing all afferents to PMC does not exist and the present study is the first to show all afferents of the PMC based on retrograde and anterograde tracing techniques.

**Methods**

**Surgical procedures**

A total of 72 adult cats was used. For the retrograde and anterograde experiments using wheatgerm agglutinin - horseradish peroxidase (WGA-HRP) the surgical procedures, pre- and postoperative care, as well as the handling and housing of the animals followed protocols approved by the Ethical Committee of the Faculty of Medicine of the University of Groningen. The animals were initially anesthetized with intramuscular ketamine (Nimatek, 0.1 ml/kg) and xylazine (Sedamun, 0.1 ml/kg), and subsequently ventilated with a mixture of O_2, N_2O (1:2) and 1-2% halothane. During surgery, body temperature was monitored and maintained between 36.5°C and 39°C using a heating pad. Physiological parameters were monitored through the use of an electrocardiogram (ECG), respiration and CO_2 monitor. In the five retrograde tracing cases (2532, 2546, 2548, 2549 and 2562), injections of approximately 50 nl of 2,5% WGA-HRP were made in the PMC. These injections were made stereotactically with a glass micropipette with a pneumatic picopump (World Precision Instruments PV 830) using coordinates from Berman’s atlas (Berman, 1968; Berman and Jones, 1982). The PMC was approached through the cerebellum.

For the anterograde tracing cases using WGA-HRP a total of six animals was used. In three cases approximately 700 nl WGA-HRP was injected either in the dorso- and ventromedial prefrontal cortex (case 2556), the prelimbic cortex (case 2551) and the infralimbic cortex (case 2584). In two cases approximately 500 nl WGA-HRP was injected in the insular cortex (cases 2569 and 2603). In one case (2601) approximately 200 nl WGA-HRP was injected in the hypothalamus at the level of the optic chiasm. After a survival time of three days the animals were initially anesthetized with intramuscular ketamine (Nimatek, 0.1ml/kg) and xylazine (Sedamun, 0.1 ml/kg), followed by an overdose (6-10 ml) of intraperitoneal 6% pentobarbital sodium. Subsequently, they were perfused transcardially with 2 liters of 0.9% saline at 37°C, immediately followed by 2 liters of 0.1M phosphate buffer (pH 7.4), containing 4% sucrose, 1% paraformaldehyde and 2% glutaraldehyde. After perfusion the brain and spinal cord was removed, post-fixed in the same fixative for two hours and stored overnight in 20% sucrose in phosphate buffer at...
4°C. The next day forebrain, brainstem and all spinal segments were transversely cut and frozen using an isopentane bath (-55°C).

For the anterograde tracing experiments using the autoradiographic technique, a total of sixty-one cats was used. The injections were made stereotactically using a Hamilton microsyringe fitted with a 22-gauge needle. In all cases 0.25-0.5µl containing 50µCi \[^{3}H\]-leucine (specific activity > 100 Ci/mmol) was injected over a period of 5 minutes, after which the needle was left in place for an additional 30 minutes to minimize the spread along the needle track. \[^{3}H\]-leucine injections were made either in the amygdala (case 1636), the bed nucleus of the stria terminalis (case 1520), the hypothalamus (cases 1522, 1654 and 1710), the PAG (cases 1434 and 1486), the lateral pons (case 863), the medullary lateral tegmental field and medial spinal trigeminal nucleus (cases 657, 1281), the pontine medial tegmental field (case 1549), the caudal pontine and medullary dorsomedial tegmental field (cases 1185), the caudal pontine and medullary ventromedial tegmental field (cases 1174, 506) and the nucleus of the solitary tract (NTS; case 1111). All other injections were placed in the brainstem tegmental field.

After a survival period of six weeks the animals were deeply anesthetized with 6-10ml of 6% intraperitoneal pentobarbital and perfused with saline followed by 4% paraformaldehyde. Subsequently, brain and spinal cord were removed and postfixed in 4% paraformaldehyde for at least one week.

Retrograde tracing

Histological procedures
Serial 40µm frozen transversal sections of forebrain, brainstem and sacral spinal cord were cut using a cryostat. Every fourth section was incubated according to the tetramethyl benzidine (TMB) method (Mesulam, 1978; Gibson et al., 1984). All sections were mounted on chromalum-gelatine coated slides, dried, dehydrated and coverslipped with Permount mounting medium. In order to define the extent of the injection site, of the brain area containing the injection an extra series of sections was incubated with dianaminobenzidine (DAB). The injection sites were plotted using a drawing tube connected to a Zeiss brightfield stereomicroscope. Digital photomicrographs of the sections containing the core of the injection were made with a Zeiss digital camera connected to a Zeiss stereomicroscope.

Visualization of anterogradely labeled fibers and terminals in the sacral spinal cord
After WGA-HRP injections in the PMC, the sacral IML and IMM were screened for the presence of anterogradely labeled fibers and terminals for additional evidence that the injection sites in the retrograde tracing studies involved the
PMC. Photomicrographs of the relevant sacral cord sections were taken using a Leica DC500 digital camera, connected to a Leica DM400B microscope with darkfield-polarized illumination, using Leica Qwin software.

**Visualization of retrogradely labeled neurons**
In order to determine the location of the retrogradely labeled neurons in the brain and brainstem every 32nd section was plotted using a Neurolucida System (MicroBrightField Inc., Colchester, USA) connected to a Zeiss Axioplan microscope with darkfield polarized illumination.

**Anterograde tracing studies**

**Histological procedures**
In the WGA-HRP cases, the same methods were used as described for the retrograde tracing procedures. In the autoradiographic cases forebrain and brainstem were cut into 25µm transverse frozen sections. One series of every tenth section was mounted, coated with Ilford G5 emulsion by dipping, and stored in the dark at 5°C for 3 months (Cowan et al., 1972; Holstege et al., 1979). Subsequently, the material was developed with Kodak D19 at 16°C, fixed and counterstained with cresyl violet. The injection area in all experiments was defined as that area in which the silver grains over the cell bodies were either as numerous as, or more numerous than, the surrounding neuropil (Holstege et al., 1977; Holstege et al., 1979).

**Visualization of anterogradely labeled fibers and terminals**
After WGA-HRP injections in regions in which retrogradely labeled fibers were found in the retrograde tracing experiments, the area of the PMC was screened for the presence of anterogradely labeled terminals. Photomicrographs of the pons at the level of the PMC were taken using a Leica DC500 digital camera, connected to a Leica DM400B microscope with darkfield-polarized illumination, using Leica Qwin software. In the autoradiography cases, silver grains representing anterogradely labeled fibers and nerve terminals in the PMC were studied using a Wild darkfield stereomicroscope. Darkfield photomicrographs were taken using a Zeiss Axiocam digital photo camera, attached to a Zeiss Axioplan stereomicroscope with darkfield illumination, using Axiovision software. All digital photomicrographs of both retrograde and anterograde tracing results were further processed using Adobe PhotoShop software.
Figure 4. Darkfield photomicrograph depicting anterograde labeling in the second sacral segment of the spinal cord after a PMC injection in case 2549. The WGA-HRP injection site is depicted in the small box. Note the descending fibers in the dorsolateral funiculus (black arrow), dense labeling in the intermediolateral (IML; single white arrow) and intermediomedial (IMM; double white arrows) cell groups. Scale bar = 500μm.

Results

Retrograde tracing studies
Injection sites
In all cases (2532, 2546, 2548, 2549 and 2562) WGA-HRP injections involved the left PMC (figures 2, 3) because in all these cases a distinct distribution pattern of anterogradely labeled fibers and terminals were observed bilaterally in the sacral IML and IMM (figure 4). In one case (2562) the anterograde labeling in the sacral IML and IMM was much less dense compared to the other PMC injected cases, probably because the injection site involved only the ventral part of the PMC. In case 2532 the injection site in the PMC extended into the locus coeruleus and the dorsal parts of the medial and lateral parabrachial nuclei, while in cases 2546 and 2549 the injections were located more medially and involved the PMC, the locus coeruleus and the medial parabrachial nuclei. The injections in case 2548 was smaller and only involved the PMC and the ventral part of the locus coeruleus. In cases 2532, 2546 and 2549 the injections also included the most lateral part of the periventricular gray matter.

Distribution of retrogradely labeled neurons
Despite the differences in injection sites, all PMC injected cases (figures 2, 3) showed a similar pattern of retrogradely labeled neurons in cortex, basal forebrain, hypothalamus, midbrain and brainstem. The distribution of retrogradely labeled neurons of case 2549 (figure 5) was chosen as the representative case and is described in the following paragraphs. Eventual differences with the distribution patterns in the other cases will be pointed out.
Figure 2. Schematic overview of WGA-HRP injections that included the PMC.
Cortex
A substantial number of retrogradely labeled neurons was found in the medial bank of the cortex, i.e. in the dorsomedial and ventromedial prefrontal cortices (figure 5A-D), the prelimbic cortex (area 32 of Rose and Woolsey (1948); figure 5E-G) and the infralimbic cortex (just ventral to the rostral extent of the corpus callosum; area 25 of (Rose and Woolsey, 1948); figure 5H). More laterally, labeled neurons were also found in the insular cortex (figure 5G-L) and in the more rostrally located anterior orbital gyrus (figure 5A-F) and some were located in the ventral bank of the cruciate sulcus (area 6A; figure 5C-H). In all cases the retrogradely labeled neurons in the cortex were found mainly on the side ipsilateral to the injection site.

Basal forebrain and hypothalamus
In the basal forebrain numerous retrogradely labeled neurons were located ipsilaterally in a band running from the lateral bed nucleus of the stria terminalis (figure 5L) through the substantia innominata into the central nucleus of the amygdala (figures 5K-O). The other subnuclei of the amygdala were devoid of labeled cells. On the contralateral side in these locations only very few retrogradely labeled cells were found.
The anterior hypothalamus and especially the medial preoptic area (MPO; figure
Figure 5. Schematic depiction of the distribution of retrogradely labeled neurons in brain and brainstem after an WGA-HRP injection in the PMC in case 2549. Note that labeled neurons immediately surrounding the injection site have not been plotted because there was too dense staining.
Figure 5. (continued)
Figure 5. (continued)
5L) and the nucleus of the anterior commissure (figure 5K) contained a large number of labeled neurons (figure 5J-L). A few retrogradely labeled neurons were observed in the ipsilateral paraventricular nucleus (figure 5M, N). Since in cases with smaller PMC injections (2548 and 2562) no labeled neurons were found in the paraventricular nucleus this area does not seem to contain neurons projecting to the PMC.

Caudally in the hypothalamus numerous labeled neurons were found in the posterior, dorsal and lateral hypothalamic areas around the fornix (figure 5N-P) at the level of, and just rostral to the mammillary bodies. This region is also known as the perifornical area of the posterior hypothalamus. The distribution of retrogradely labeled neurons in the hypothalamus was bilateral, but with an ipsilateral predominance.

**Mesencephalon**
Large clusters of labeled neurons were found in the PAG (figure 5Q-V). Especially in the ventrolateral and dorsomedial PAG and, but to a much lesser extent, in the dorsolateral PAG (figures 5R-T). A large number of labeled neurons was present in the most caudal PAG (figures 5V). Our finding that in the cases with small PMC injections (2548 and 2562, figure 2) very few or no labeled neurons were found in the dorsolateral and most caudal PAG indicates that these areas of the PAG do not project to the PMC. In addition, in all cases, scattered retrogradely labeled neurons were observed in the mesencephalic tegmentum lateral and ventrolateral to the PAG (figure 5Q-V). In cases 2532, 2549 and 2546, labeled neurons were found in the substantia nigra pars reticulata (figure 5Q-S), but in the cases with small PMC injections (2548 and 2562) only few labeled neurons were found in this region. Apparently, the neurons in the substantia nigra pars reticulata do not project to the PMC but to the more laterally located regions. All mesencephalic labeling was, similar to the hypothalamic labeling, bilateral but with a very strong ipsilateral predominance.

**Pons and medulla oblongata**
In the caudal brainstem, retrogradely labeled neurons were found bilaterally in the medial and lateral parabrachial nuclei, in the Kölliker-Fuse nuclei (figures 5V-Y) and throughout the rostrocaudal extent of the lateral tegmental field (figures 5U-II). In the medial tegmentum labeled neurons were most numerous in its ventral part ipsilaterally and in its dorsal part contralaterally (figures 5Z-EE). Only in those cases in which the injections extended into the medial parabrachial nuclei and the locus coeruleus (2532, 2546 and 2549), a cluster of labeled neurons was found in the nucleus of the solitary tract (NTS; figures 5DD-GG).
The fact that in the other cases such a cluster was not found suggests that the NTS is not a source of PMC afferents. In all cases retrogradely labeled neurons were found in the most medial part of the spinal trigeminal nucleus (figures 5AA-EE). In two cases, probably because of leakage of tracer in the cerebellum, (2532 and 2549) labeled neurons were observed in the contralateral inferior olive (figure 5EE-FF).

**Anterograde tracing studies**

**Cortex**

Since, according to the retrograde tracing results, the prefrontal, prelimbic, infralimbic and insular cortices might project to the PMC, in case 2556 an WGA-HRP injection was placed in the ventro/dorsomedial prefrontal cortex (figure 6A), in case 2551 in the prelimbic cortex (figure 6B) and in a case 2584 in the whole rostrocaudal extent of the infralimbic cortex (figure 6C). In two other cases (2569 and 2603) the injections were placed laterally in the cortex and involved the insular cortex (figure 6D) and the more rostrally located anterior orbital gyrus (figure 6E). In all five cases a similar distribution pattern was found in the dorsolateral pontine tegmentum with many labeled fibers in the medial parabrachial nuclei and, albeit to a lesser extent, in the locus coeruleus and subcoeruleus. Very few or no labeled fibers were observed within the PMC (figures 6A-E). In all PMC injected cases, labeled neurons were found in the ventral bank of the cruciate sulcus. The fact that the injection in case 2551, which included the ventral bank of the cruciate sulcus, did not result in anterograde labeling in the PMC (figure 6B) shows that this part of the cortex does not project to the PMC.

**Basal forebrain and hypothalamus**

After $[^3]H$-Leucine injections in the central nucleus of the amygdala (case 1636; figure 6F) and bed nucleus of the stria terminalis (case 1520; figure 6G) anterogradely labeled fibers were found in the locus coeruleus, nucleus subcoeruleus and medial and lateral parabrachial nuclei, but very few, if any, in the PMC (figures 6F-G). On the other hand, in case 1710 with a $[^3]H$-Leucine injection in the lateral part of the MPO and nucleus of the anterior commissure (figure 7A) or in case 1654 with a $[^3]H$-Leucine injection in the medial part of the MPO (figure 7B), a distinct projection of anterogradely labeled terminals was found to the PMC. A less distinct, but still clearly visible, projection was found in case 2601 after a WGA-HRP injection in the MPO (figure 7C). In another case (1522), with a $[^3]H$-Leucine injection in the dorsal and lateral areas of the posterior hypothalamus just dorsal to the fornix (figure 7D) a similar
distinct projection to PMC was found (figure 7D), although less strong than in the cases with injections in the anterior hypothalamus.

**Mesencephalon**

[³H]-Leucine injections in the dorsomedial (case 1486, figure 7E) and ventrolateral PAG (case 1434, figure 7F) resulted in anterogradely labeled terminals in the PMC (figures 7E-F), although much stronger in the case with the ventrolateral than with the dorsomedial injection. In none of the several cases with leucine injections in the mesencephalic tegmental field ventral and lateral to the PAG (figure 8A-G), specific anterograde labeling was found in the PMC. Apparently, the mesencephalic tegmental field does not project to the PMC.

**Pons and medulla oblongata**

The retrograde tracing showed a great many retrogradely labeled neurons in the lateral tegmentum of caudal pons and medulla. In order to verify whether these cells indeed project to the PMC or to nearby areas, in many cases leucine injections were made in the lateral tegmentum (figures 6H, I, L; 8H-R). In all these cases many fibers terminated in the parabrachial nuclei and locus coeruleus, but not in the PMC. Even in case 863 with a leucine injection in the lateral pontine tegmentum and Kölliker-Fuse nucleus, no anterograde labeling was found in the PMC (figure 6H). Also injections in the dorsal part of the medial medullary tegmentum did not result in labeled fibers in the PMC (case 1185, figure 6J). On the other hand, in all cases with injections in the ventromedial pontine and medullary tegmentum (figures 7G-I), a diffuse but clearly visible nonspecific pattern of labeled fibers was observed in the parabrachial nuclei, the adjoining pontine tegmentum and in the PMC. This probably indicates that the diffusely projecting level setting systems in the ventral medial tegmental field also include the PMC.

In all retrograde cases, labeled neurons were observed in the medial part of the spinal trigeminal nucleus but leucine injections in this part of the spinal trigeminal nucleus did not result in any anterograde labeling in the PMC (cases 657, 1281; figures 6I, L). In some of the PMC injected cases in the retrograde tracing study, labeled neurons were found in the NTS. However a leucine injection that involved the NTS did only result in anterograde labeling in the parabrachial nuclei but not in the PMC (case 1111; figure 6K). This shows that the NTS does not project to the PMC.
Figure 6 (A–J). Schematic depiction of an injection site (left) of tritiated leucine and WGA-HRP in cases in which no anterograde labeling was observed in the PMC. Darkfield photomicrograph (right) showing anterograde labeling in the dorsolateral pontine tegmentum in the same case. Note that no anterograde labeling is present in the PMC (indicated with dotted line). Scale bar = 500μm.
Figure 6 (G-L). (continued)
Figure 7 (A-I). Schematic depiction of tritiated leucine and WGA-HRP injection sites (left) in cases which anterograde labeling was observed in the PMC. Darkfield photomicrograph (right) showing anterograde labeled fibers in the dorsolateral pontine tegmentum in the same case. Note that anterograde labeling is present in the PMC (indicated with dotted line). Scale bar = 500μm.
Discussion

The results show that the PMC in cat only receives afferents from various parts of the preoptic area, the perifornical area of the posterior hypothalamus and the dorsomedial and ventrolateral PAG and from the pontomedullary ventromedial tegmental field (figure 9). The dorsomedial prefrontal cortex, the prelimbic cortex, the infralimbic cortex, the bed nucleus of the stria terminalis, the central nucleus of the amygdala, the lateral pontomedullary tegmental field or the NTS do not project directly to the PMC. In some of the cases where these structures were injected with an anterograde tracer, labeling was not completely absent from the PMC but was observed diffusely throughout dorsolateral pontine tegmentum. However this labeling was always weaker than the diffuse labeling from the ventromedial pontine and medullary tegmentum, which could always easily be observed especially when multiple sections were studied. Perhaps this very weak labeling represents terminals synapsing on noradrenergic neurons within the dorsolateral pontine tegmentum, not only located in the locus coeruleus and
Figure 8. Schematic overview of tritiated leucine injection sites that did not result in anterograde labeling in the PMC.
nucleus subcoeruleus but, to a limited extent in the PMC as well (Swanson and Hartman, 1975; Jones and Friedman, 1983). This means that also the results of the anterograde tracing studies will have to be interpreted with caution. As always only ultrastructural studies can verify whether the positive findings in this study are true. In rat such studies have been done for the ventrolateral PAG and the MPO (Ding et al., 1998; Ding et al., 1999), but in cat such studies do not exist. The results from the present study are in agreement with earlier anatomical studies that have shown projections from the lateral part of the MPO to the PMC (Holstege, 1987; Valentino et al., 1994; Marson and Foley, 2004) and the dorsomedial and ventrolateral PAG (Blok and Holstege, 1994) but this study for the first time shows PMC afferents from the caudal brainstem medial tegmentum, the perifornical posterior hypothalamus and the medial part of the MPO to the PMC in the cat. It is important to emphasize that the local short afferent connections to the PMC were not examined in this study, because the tracers used did not allow to investigate these short fiber pathways. In all likelihood, such local connections do exist.

In rat (Valentino et al., 1994), using retrograde and anterograde tracing, showed that the lateral part of the preoptic hypothalamus, the perifornical part of the posterior hypothalamus and the ventrolateral PAG project to the PMC. However, they did not show a projection from the medial MPO, the dorsomedial PAG, and from the pontine and medullary ventromedial tegmentum. Also in their retrograde tracing study they did not find any retrogradely labeled neurons in any part of the brainstem tegmental field. Interestingly (Valentino et al., 1994) demonstrated a projection from the Kölliker-Fuse nucleus, while in this study no such projection was found. Finally, these authors suggest, on the basis of retrograde tracing, that various other regions in the brainstem, such as the nucleus of the solitary tract, the nucleus paragigantocellularis, the parabrachial nucleus, cuneiform nucleus and dorsal raphe nucleus would project to the PMC. The present results show that it is unlikely that there are projections from NTS or the dorsal raphe to the PMC in the cat. It is hard to draw conclusions about projections from the parabrachial nuclei to the PMC because the injection sites in this study are too large to reliably study such a local connection.

An other important observation of this study is that cortical and limbic regions as the dorsomedial prefrontal cortex, the prelimbic cortex, the infralimbic cortex, the bed nucleus of the stria terminalis and the central nucleus of the amygdala, do not send direct projections to the PMC. This is in agreement with earlier studies of efferents to the brainstem from the pre- and infralimbic cortices (Room et al., 1985), the bed nucleus of the stria terminalis (Holstege et al., 1985) and the amygdala (Hopkins and Holstege, 1978).
Functional considerations
This study shows that the medial pontomedullary tegmental field projects to the PMC, albeit very diffusely, involving the whole dorsolateral pontine tegmentum. The medial tegmentum is known to send diffuse projections throughout the central nervous system including the spinal cord and is thought to be part of a general level setting system (Holstege, 1991). The present results indicate that this level setting system may also involve the PMC.

The present study shows that both the dorsomedial and ventrolateral parts of the PAG project to the PMC but whether these two regions have a different influence on micturition is not known. In cat, stimulation of the ventrolateral PAG can elicit micturition (Kabat et al., 1936; Taniguchi et al., 2002), but it is not known whether the dorsomedial PAG has a similar effect on micturition.

Projections from MPO to PMC have first been published by Holstege (1987) in cat and later by others in rat (Valentino et al., 1994; Rizvi et al., 1994; Ding et al., 1999; Marson and Foley, 2004). The precise function of the MPO-PMC pathway is not known. The MPO is a sexual dimorphic area and is well known to be involved in sexual function (Sachs and Meisel, 1988). On the basis of their finding that chemical and electrical stimulation of the MPO fails to produce FOS expression in the PMC, Rizvi et al., (1998) suggested that this pathway inhibits micturition during sexual behavior. This hypotheses, however, is not in agreement with the much earlier finding of Gjone (1966), that stimulation of the MPO can elicit micturition.

With respect to the PMC projection from the perifornical area, a large cluster of orexin (hypocretin) synthesizing neurons is located in this part of the posterior hypothalamus in both rat and cat (Gautvik et al., 1996; Peyron et al., 1998; Zhang et al., 2001). On the basis of the finding that numerous orexin/hypocretin immunoreactive fibers and terminals (Peyron et al., 1998; Zhang et al., 2004) and HCRT-1 and HCRT-2 receptors on which orexins act (Greco and Shiromani, 2001) are present in the PMC one might speculate that these orexinergic neurons project to the PMC. The posterior hypothalamic orexin synthesizing neurons have widespread projections throughout the central nervous system (Peyron et al., 1998) and are thought to play a role in the integration of several homeostatic systems such as sleep, feeding, energy metabolism, cardiovascular function, hormone homeostasis and fluid balance (Sutcliffe and de Lecea, 2000). Perhaps the orexinergic neuronal projections to the PMC play a role in the integration of fluid balance with the other homeostatic functions. The hypothalamus is also likely to be involved in micturition in humans: adenomas which compress the hypothalamus result in problems with micturition, mainly urge-incontinence (Yamamoto et al., 2005). Thus, with the exception of the hypothalamus and PAG
Figure 9. Schematic depiction of the results of this tracing study showing all PMC afferents found in this study.
no other brain structures have direct monosynaptic access to the PMC, although several of them can influence micturition. One of these regions is the anterior cingulate gyrus, often considered the ‘visceromotor’ cortex (Hurley et al., 1991) and stimulation in the anterior cingulate gyrus is known to influence bladder function (Gjone and Setekleiv, 1963). Neuroimaging studies in humans (Blok et al., 1997b; Blok et al., 1998a) have shown that this part of the cortex is activated during micturition. Other structures, as the amygdala and the bed nucleus of the stria terminalis, have also been shown to influence bladder function (Gjone, 1966). The present study shows that these brain structures can only influence micturition via indirect pathways either through the hypothalamus or through the PAG.