Chapter 6

Barrington’s nucleus in the guinea pig (cavia porcellus): location in relation to noradrenergic cell groups and connections to the lumbosacral spinal cord

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Brain Research Bulletin; Submitted

Abstract
Micturition is largely controlled by Barrington’s nucleus in the dorsolateral tegmentum of the pons. This nucleus coordinates simultaneous bladder contraction and external urethral sphincter relaxation by means of a specific pattern of projections to the lumbosacral spinal cord. The majority of small animal neurourological research uses the rat as an animal model. However, urodynamic studies suggest that, in comparison to rat, guinea pig micturition is more similar to human micturition. Therefore, the present study, using retrograde and anterograde tracing and double immunofluorescence, was designed to investigate the location of Barrington’s nucleus in the guinea pig, to identify Barrington’s nucleus projections to the spinal cord and to clarify the relationship of Barrington’s nucleus to pontine noradrenergic cell groups. Results show that Barrington’s nucleus is located in the dorsolateral pons, projects to the intermediolateral and intermediomedial cellgroups of the lumbosacral spinal cord and is clearly distinct from the pontine noradrenergic cellgroups.

Introduction
In recent years, rat has become the most widely used animal in neurourology. This is surprising because the urodynamic profile of rat micturition differs fundamentally from human micturition (Van Asselt et al., 1995). The urodynamic profile of the guinea pig on the other hand resembles the human profile (Van Asselt et al., 1995; Walters et al., 2005) and may be better animal model in neurourological research. However little is known about the neuroanatomical substrates controlling micturition in guinea pig. Previously we reported the location of bladder and external urethral motoneurons in guinea pig (Kuipers et
In the present study the location and projections to the lumbosacral spinal cord of Barrington’s nucleus in the guinea pig will be investigated. Micturition or voiding, normally occurs when the bladder is full, and consists of contraction of the detrusor smooth muscle of the bladder with simultaneous relaxation of the striated muscle of the external urethral sphincter (EUS) and relaxation of the circular smooth muscle of the bladder neck and proximal urethra. Barrington (1925) recognised that this coordination between bladder and urethral musculature takes place in the brainstem, more specifically in the pons. It has since been established that stimulation of Barrington’s nucleus, a nucleus in the dorsolateral pons named after Barrington, can elicit micturition in rat (Noto et al., 1989), cat (Holstege et al., 1986) and dog (Nishizawa et al., 1988). Barrington’s nucleus, in turn, is under control of the Periaqueductal Gray matter (PAG) and different parts of the hypothalamus (Valentino et al., 1994; Kuipers et al., 2006). Furthermore, neuroanatomical studies, in rat (Loewy et al., 1979), opossum (Martin et al., 1979), cat (Holstege et al., 1979; Holstege et al., 1986) and monkey (Westlund and Coulter, 1980), have shown that Barrington’s nucleus can elicit micturition through a specific pattern of projections to the lumbosacral spinal cord. Bladder contractions can be elicited by direct projections to parasympathetic preganglionic bladder motoneurons in the lumbosacral intermediolateral cell column (IML) while sphincter relaxation is mediated by projections to the lumbosacral intermediomedial cell column (IMM). Sphincter relaxation can be elicited by Barrington’s nucleus because it projects to inhibitory GABA-ergic and glycinergic interneurons in the IMM (Blok et al., 1997; Sie et al., 2001). Since electrical stimulation of these IMM neurons results in sphincter relaxation (Blok et al., 1998), it is thought that IMM inhibitory interneurons project to external urethral sphincter motoneurons which are located in the ventromedial ventral horn of the lumbosacral spinal cord in rat (Schroder, 1980), hamster (Gerrits and Holstege, 1996), guinea pig (Kuipers et al., 2004), cat (Sato et al., 1978; Kuzuhara et al., 1980) dog (Kuzuhara et al., 1980) and monkey (Roppolo et al., 1985).

Although Barrington’s nucleus nucleus plays a crucial role in the control of micturition it is not the only function of the nucleus. It has been suggested that Barrington’s nucleus is also involved in the control of other pelvic viscera (Pavcovich et al., 1998) and stress (Sved et al., 2002). In respect to the latter, it has been shown that neurons in Barrington’s nucleus express the stress related neuropeptide corticotrophin releasing factor (CRF; Swanson et al., 1983; Sutin and Jacobowitz, 1988; Valentino et al., 1994). Barrington’s nucleus is often defined solely as the labeled neurons in the dorsolateral pontine tegmentum after retrograde tracer injections in the lumbosacral spinal cord. This is problematic because Barrington’s nucleus is
surrounded by noradrenergic neurons which form the locus coeruleus and locus subcoeruleus (Palkovits and Jacobowitz, 1974; Jones and Friedman, 1983). Importantly, neurons from these anatomical areas project to almost the entire nervous system and have classically been associated with arousal, but more recently this view has been widened to incorporate more complex attentional processes (Berridge and Waterhouse, 2003; Aston-Jones and Cohen, 2005). Some of these noradrenergic neurons also project to the entire spinal cord (Westlund et al., 1982) and therefore they are labeled together with Barrington's nucleus when retrograde tracers are injected in the lumbosacral spinal cord. Therefore, the objective of this study was to determine the location and projections to the lumbosacral spinal cord of Barrington's nucleus in the Guinea Pig, using wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP) as a retrograde and anterograde tracer. Furthermore, the study aimed to define Barrington’s nucleus as the non-adrenergic neurons in the dorsolateral pons that project to the lumbosacral spinal cord by applying double immunofluorescence for the retrograde tracer cholera toxin beta subunit (CTB) and tyrosine hydroxylase (TH).

Materials and methods

Surgical procedures
Surgical procedures, pre- and postoperative care and handling and housing of the animals were approved by the Ethical Commitee of the Faculty of Medical Sciences of the University of Groningen, The Netherlands. Seven adult female guinea pigs (Cavia porcellus, Dunkin-Hartley, Harlan Nederland bv) weighing 300-500 g. were used. Prior to surgery and injection of neuronal tracers, animals were anesthetized with a combination of xylazine (5 mg/kg i.m.) and ketamine (40 mg/kg i.m.). Following surgery, buphrenorphine was administered (0.1 mg/kg s.c.) to provide analgesia. During surgery body temperature was maintained using a heating pad.

In the anterograde and retrograde WGA-HRP tracing experiments, following recovery from surgery and after a survival time of 48 h, the animals were deeply anesthetized with Pentobarbital. Subsequently, the animals were perfused transcardially with 800 ml of saline followed by 800 ml of 0.1 M phosphate buffered fixative containing 2% glutaraldehyde, 1% paraformaldehyde and 4% sucrose. Brain, brainstem and spinal cord were removed, postfixed for 2 hrs in the same fixative and cryoprotected by overnight storage in 0.1 M phosphate buffered 25% sucrose at 4°C. The next day tissue was frozen in an isopentane bath (-55°C).
In the doublestaining experiments in which CTB was used as a retrograde tracer, following recovery from surgery and a survival time of 168 hrs, animals were deeply anesthetized with pentobarbital and perfused transcardially with 800 ml of saline followed by 0.1 M phosphate buffered fixative of 4% paraformaldehyde. Tissue handling and freezing procedures were identical to those described for the WGA-HRP experiments.

**Injections**

For retrograde labeling of Barrington’s nucleus, after laminectomy, approximately 100 nl of 2.5% WGA-HRP (Sigma) in saline (n=2 animals; GP16, GP17) or 100 nl of CTB (2%; List Biological Laboratories; n=2 animals; GP32, GP37) was injected bilaterally into the lumbosacral spinal cord using a glass micropipette with a pneumatic picopump (World Precision Instruments PV 830). CTB injected animals were utilized for immunofluorescent double staining with TH in order to study the location of Barrington’s nucleus in relation to noradrenergic neurons that project to the lumbosacral spinal cord. In order to identify projections to the lumbosacral spinal cord from Barrington’s nucleus and the area just ventral to it, 2.5% WGA-HRP injections were made either in Barrington’s nucleus (n=1 animal) or just ventral to Barrington’s nucleus (n=2 animals). These injections were made stereotactically using iontophoresis through a glass micropipette (10 minutes, 5μA positive alternating current with a 7s cycle followed by 10 minutes 5μA negative alternating current with a 7s cycle; internal tip diameter of the micropipette 25μm). Stereotactic coordinates were determined using a stereotactic atlas of the guinea pig brain (Rapisarda and Bacchelli, 1977).

**Histological procedures**

**WGA-HRP experiments**

Serial 40μm frozen transverse sections of brainstem and lumbosacral spinal cord segments were cut using a cryostat. Every second 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 in graded alcohols, cleared in xylene and coverslipped with Permount mounting medium. In order to define the extent of the injection site an extra series of sections containing the injection site was incubated with diaminobenzidine (DAB).

**CTB-TH double staining experiments**

Serial 40μm frozen transverse sections of lumbosacral spinal cord segments
and 30µm transverse sections of the brainstem were cut using a cryostat and collected in 0.05 M tris buffered saline (pH 7.6; TBS).

For immunolocalization of CTB in lumbosacral spinal cord, every second section was incubated for 1hr at room temperature in 1% H\textsubscript{2}O\textsubscript{2} in TBS, followed by 30 minutes incubation in 5% normal rabbit serum (Vector Laboratories) in 0.3% Triton X100/TBS. Subsequently sections were incubated for 48 hrs at 4°C in goat anti-CTB (1:25000; List Biological Laboratories) with 1% normal rabbit serum in 0.3% Triton X100/TBS, rinsed for 90 minutes in TBS and then incubated for 60 minutes at room temperature with rabbit anti-goat (1:250; Dakocytomation) and 1% normal rabbit serum in 0.3% Triton X100/TBS. Sections were then rinsed for 90 minutes in TBS followed by 60 minutes incubation with PAP (peroxidase anti peroxidase) goat (1:250; Dakocyтомation) in 0.3% Triton X100/TBS before being rinsed for 90 minutes in TBS. The bound peroxidase was visualized with 0.025% DAB and 0.004% H\textsubscript{2}O\textsubscript{2} in TBS. Sections were mounted on chromalum-gelatine coated slides, dried, dehydrated in graded alcohols, cleared in xylene and coverslipped with DEPEX mounting medium.

Fluorescent double immunohistochemical staining for CTB and TH in the brainstem was performed using the following protocol. Every second section was incubated for 30 minutes in 5% normal donkey serum (Vector laboratories) in 0.3% Triton X100/TBS.
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X100/TBS at room temperature. This was followed by an 48hrs incubation at 4°C in a cocktail of goat anti-CTB (1:8000; List Biological Laboratories), mouse anti-TH (1:3000; Diasorin/Immunostar) and 1% normal donkey serum in 0.3% Triton X100/TBS. Sections were then rinsed in TBS for 90 minutes at room temperature and subsequently incubated for 1hr (at room temperature, in the dark) with a cocktail of Alexa 568 donkey anti-goat (1:1000; Molecular Probes) and Alexa 488 donkey anti-mouse (1:1000; Molecular Probes) in TBS. Finally, sections were rinsed for 60 minutes in TBS (in the dark), mounted on APES coated glass slides, dried and coverslipped with Permount mounting medium.

Antibody specificity was verified by Control experiments, omitting the anti-CTB antibody and substituting the anti-TH antibody with a non-specific mouse IgG control antibody (Vector laboratories).

Visualization of WGA-HRP anterograde and retrograde labeling

To visualize retrogradely labeled neurons in the brainstem and anterogradely labeled fibers and terminals in the lumbosacral spinal cord photomicrographs of the relevant sections were taken using a Leica DC500 digital camera, connected to a Leica DM500 microscope with darkfield polarized illumination, using Leica Qwin software. The photomicrographs were further processed using Adobe Photoshop. Schematic drawings of all retrogradely labeled neurons in one out of every 4 pontine sections containing Barrington’s nucleus and anterograde labeling in one out of every 4 lumbosacral spinal sections were made using a Neurolucida System (MicroBrightField Inc., Colchester, Vermont, USA) connected to a Zeiss Axioplan microscope with darkfield polarized illumination. A retrogradely labeled neuron was identified as such when both a stained cell body and one or more stained dendrites could be distinguished.

Visualization and counting of fluorescent CTB and TH double immuno-histochemistry

Pontine sections containing Barrington’s nucleus were studied using a Leica DM500 microscope with Leica L5 and TX2 filterblocks and relevant sections were photographed. Combined RGB photographs of both CTB and TH fluorescent staining were created using Adobe Photoshop software. On all photomicrographs on which Barrington’s nucleus could clearly be distinguished as a compact, oval shaped, Barrington’s nucleus was delineated (figure 5) and single labeled CTB and TH and double labeled CTB/TH immunoreactive neurons within and outside Barrington’s nucleus were counted bilaterally in all sections.
Figure 2. Drawings showing the distribution of retrogradely labeled neurons in the pons after WGA-HRP injection in the lumbosacral spinalcord in case GP16.
Results

Retrograde tracing
In both cases in which WGA-HRP was injected in the spinal cord, the injection sites included the lumbosacral spinal cord. In one animal (case GP16) the center of the injection was located in the S1 segment and extended into parts of L6 and S2, while in a second animal (case GP17) the injection was larger and included the complete L6-S2 segments (figure 1). Both injections included both dorsal and ventral horns bilaterally.

In both cases a similar pattern of retrogradely labeled neurons was observed in the pons (figures 2 and 3). A compact, oval shaped, group of labeled neurons just medial to the mesencephalic trigeminal tract was observed in the dorsolateral pontine tegmentum just caudal to the inferior colliculus (figures 2 and 3). This nucleus most likely corresponds to Barrington’s nucleus. The core of the nucleus...
Figure 5. RGB compilation of fluorescent photomicrographs of retrogradely labeled CTB immunoreactive neurons (red; asteriks with arrow), TH immunoreactive neurons (green; closed arrow) and CTB/TH double labeled immunoreactive neurons (orange; open arrow) at rostral (A), mid (B) and caudal (C) levels of Barrington’s nucleus. Barrington’s nucleus is delineated with dotted lines on all 3 photomicrographs. Scale bars represent 100μm
is located dorsal and scattered labeled neurons could be observed ventrolateral to the core of the nucleus (figures 2 and 3). These scattered neurons are likely to be spinally projecting noradrenergic neurons that do not belong to Barrington's nucleus. These results indicate that Barrington's nucleus in the guinea pig is located in the dorsolateral pontine tegmentum, however the exact boundaries of the nucleus cannot be determined on the basis of these retrograde tracing results alone.

**CTB and TH double immunofluorescent staining**
In both animals (GP32, GP37) in which CTB was injected, the injection sites included the lumbosacral spinal cord (figure 4). The injection was centered in the S1 segment and included both dorsal and ventral horns bilaterally and extended into the ipsilateral dorsal horn of L6 and bilaterally into the dorsal horn of S2. In both cases, a similar distribution of CTB and TH immunoreactivity was observed in the dorsolateral pontine tegmentum bilaterally (figure 5). The distribution of CTB immunoreactivity was similar to the retrograde labeling seen in the WGA-HRP experiments. A dense cluster of single labeled CTB immunoreactive neurons (red), probably corresponding to Barrington's nucleus, was observed dorsally (figure 5). The nucleus could clearly be distinguished as an oval shaped nucleus located dorsomedial from the large group of TH-immunoreactive neurons (green) which correspond with the nucleus subcoeruleus. Scattered CTB immunoreactive neurons (red) were observed ventrolateral from the dense cluster and many but not all of these were immunoreactive for both CTB and TH (orange; figure 5; table 1). Within Barrington’s nucleus, almost all (94.9%; table 1) immunoreactive neurons were CTB immunoreactive. A small amount of neurons within Barrington’s nucleus were only TH immunoreactive (4.7%; table 1), these neurons were located mainly in the ventral part of the nucleus at caudal levels (figure 5C). Almost no double labeled (CTB/TH) neurons were observed within Barrington’s nucleus (0.4%; table 1). On the ventrolateral side, outside Barrington’s nucleus, most immunoreactive neurons were either TH (55.4%; table 1) or CTB/TH double immunoreactive (31.2%; table 1). These neurons correspond most likely with noradrenergic nucleus subcoeruleus neurons. However, significant numbers of immunoreactive neurons outside Barrington’s nucleus were only CTB immunoreactive (13.4%; table 1) and are thus non-noradrenergic but do project to the lumbosacral spinal cord. Finally, CTB labeled neurons were surrounded by TH-immunoreactive fibers (figure 5) and at higher magnifications, close approximations between TH-immunoreactive fibers and CTB immunoreactive neurons could be observed (figure 6).
**Anterograde tracing**

In three animals WGA-HRP injections were placed either in or just ventral to Barrington's nucleus (GP36, GP25, GP28; figure 7) to study its projections to the lumbosacral spinal cord. When the injection was placed in Barrington's nucleus (GP36; figure 7A) anterogradely labeled fibers and terminals were found bilaterally in the IML and in the IMM between L6 and S2. Additionally some, but very little, anterograde labeling was found in the ventral horn. In the two cases in which the injections were placed just ventral to Barrington's nucleus and included the nucleus subcoeruleus (GP25, GP28) a completely different pattern of projections to the lumbosacral spinal cord was observed (figure 7B). Diffusely distributed labeled fibers were observed throughout the ventral horn but very few fibers were found in the IML and IMM. These results indicate that Barrington's nucleus, in contrast to the more ventrally located nucleus subcoeruleus, projects to IMM and IML in the lumbosacral spinal cord.

**Discussion**

The results of the present study indicate the presence of a distinct nucleus in the dorsolateral pontine tegmentum that projects to the IMM and IML in the lumbosacral spinal cord and may represent Barrington's nucleus in the guinea pig. In addition, this nucleus is clearly distinct from the more ventral noradrenergic nucleus subcoeruleus which projects diffusely to the ventral horn and intermediate zone of the lumbosacral spinal cord. These results are in agreement with earlier studies on Barrington's nucleus in other species (Holstege et al., 1986; Ding et al., 1995; Ding et al., 1999) and suggest a role in the control of pelvic viscera. The fact that neurons within Barrington's nucleus are non-noradrenergic and that the nucleus is clearly separated from the noradrenergic locus coeruleus and subcoeruleus is in agreement with studies in rat which have shown that neurons in Barrington's nucleus are immunoreactive for CRF and distinct from noradrenergic locus coeruleus and subcoeruleus neurons (Swanson et al., 1983; Sutin and Jacobowitz, 1988; Valentino et al., 1994).

Interestingly the present study shows that the region ventral to Barrington's nucleus contains non-noradrenergic neurons that also project to the lumbosacral spinal cord. It is possible that this neuronal population corresponds with the so-called L-region in the cat. In cat this region is located lateral within the pons and maintains direct projections with Onuf's nucleus in the lumbosacral spinal cord, suggesting that it functions as a continence maintaining center that can induce contraction of the external urethral sphincter (Holstege et al., 1986). Similarly, in rat the region just ventral to Barrington's nucleus also seems to project to Onuf's nucleus (Ding et al., 1995), supporting the hypothesis that the area ventral
to Barrington’s nucleus may be a continence maintaining center. It should be noted however that in the present study the projections from the area ventral to Barrington’s nucleus to the ventral horn of the lumbosacral spinal cord are diffuse, which may conflict with this hypothesis. It is possible that the non-adrenergic neurons in this area do project specifically to Onuf’s nucleus but are intermingled with diffusely projecting noradrenergic neurons of the nucleus subcoeruleus to such an extent that it is not possible to visualize the two projections separately with a single tracer injection.

In conclusion this study has identified the location of Barrington’s nucleus relative to noradrenergic cell groups within the pons, and it’s projections to the lumbosacral spinal cord in guinea pig. This study can serve as a basis for further neurourological studies in guinea pig which may support it’s utilization as an animal model of urological function.

Figure 6. High magnification photomicrograph of TH immunoreactive dendritic arborizations (green; closed arrow) in close approximation with CTB immunoreactive neurons (red; open arrow). Scale bar represents 25μm.
Figure 7. Drawings showing the distribution of anterogradely labeled terminals in the lumbosacral cord after WGA-HRP injection in Barrington’s nucleus (A) and in the area ventral to Barrington’s nucleus (B). Corresponding schematic depictions of the injection sites are depicted in the insets (a, b). Note that after injections in Barrington’s nucleus numerous labeled fibers are present in the IML (light gray) and IMM (dark gray) whereas after injections ventral to Barrington’s nucleus, few labeled fibers are present in IML and IMM but many fibers can be observed in the ventral horn.
Acknowledgement
This research was sponsored by a grant from Pfizer Global Research and Development, Sandwich, UK.