Volume-evoked micturition reflex is mediated by the ventrolateral periaqueductal gray in anesthetized rats

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Matsuura, Shinobu, Gary V. Allen, and John W. Downie. Volume-evoked micturition reflex is mediated by the ventrolateral periaqueductal gray in anesthetized rats. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R2049–R2055, 1998.—The central pathway of the micturition reflex in the rat was investigated functionally by acute blockade of synaptic neurotransmission using microinjection of cobalt chloride into the periaqueductal gray or pontine tegmental region. In 27 urethan-anesthetized (1.2 g/kg ip) rats, the bladder pressure response to continuous infusion of the bladder with saline (0.1–0.25 ml/min) was assessed. Electromyographic activity of external urethral sphincter and arterial blood pressure were also monitored. Bladder contractions and external urethral sphincter activity were reversibly attenuated after unilateral or bilateral stereotaxic injections of 10 mM cobalt chloride into the caudal (bregma –7.80 to –8.80) ventrolateral periaqueductal gray as well as into Barrington’s nucleus. Blood pressure was not affected by injection into either area. The results demonstrate that the caudal ventrolateral periaqueductal gray, in addition to Barrington’s nucleus, is a critical part of the long-routed micturition reflex circuitry in the anesthetized rat.

Barrington’s nucleus; cystometry; dorsolateral pontine tegmentum

THE BASIC MICTURITION REFLEX in the adult mammal is controlled supraspinally. On the basis of lesioning studies, a pontine micturition reflex center (Barrington’s nucleus) responsible for coordinating bladder and sphincter function has been identified in the dorsolateral pontine tegmentum in the rat (23). Neuroanatomical (13, 16), electrophysiological (19), and neuropharmacological (21, 28) studies have characterized the pathways and have confirmed the involvement of Barrington’s nucleus in the basic micturition reflex. Recent tracing studies in the rat indicate that ascending pathways from the lumbosacral spinal cord project to the periaqueductal gray (PAG) as well as Barrington’s nucleus (9) and that the PAG has dense projections to neurons in Barrington’s nucleus that project directly to the lumbosacral spinal cord (8). In addition, neuroanatomical evidence in the cat suggests that ascending pathways terminate primarily in the PAG compared with Barrington’s nucleus (5, 26). These data raise the possibility that ascending pathways of the basic micturition reflex primarily terminate in the PAG rather than Barrington’s nucleus.

Clearly, neuroanatomical evidence for connections between various structures and Barrington’s nucleus (1, 9, 25) does not necessarily indicate a role for these structures in micturition. This is particularly so because Barrington’s nucleus has been implicated in functions other than micturition (1, 22). Thus we sought functional evidence that the PAG has a role in micturition. Testing consisted of attempting to block ongoing bladder distension-evoked micturition by injection of a blocker of synaptic neurotransmission, cobalt chloride (CoCl2), into the areas of interest in the urethan-anesthetized rat. It was reasoned that if blockade of synaptic transmission in a particular site interrupted micturition, that site must play a critical role in micturition. The blocking action of CoCl2 was confirmed at Barrington’s nucleus, a site known to be important in coordinating micturition. The PAG was then searched for sites at which CoCl2 was effective in interrupting micturition. A preliminary report of these results has been presented (11).

EXPERIMENTAL PROCEDURES

Animals. Male Sprague-Dawley rats (n = 27) weighing 285–516 g were used in this study. The experimental protocol was approved by the University Committee on Laboratory Animals, Dalhousie University.

Experimental preparation. Animals were anesthetized with urethan (1.2 g/kg ip), and a cannula (PE-50, Clay Adams, Parsippany, NJ) was placed in the femoral artery for monitoring blood pressure. The bladder and proximal urethra were exposed through a midline abdominal incision, and a cannula (PE-60) was inserted into the bladder via the dome and secured with a ligature. The bladder was constantly infused with room temperature 0.9% saline (0.10–0.25 ml/min) with the use of a syringe pump (model 355, Orion Research, Boston, MA). The infusion rate was chosen to elicit micturition at ~90-s intervals. Both the arterial line and a sidearm of the bladder infusion line were attached to pressure transducers (Statham P-23, Gulton Statham Transducers, Costa Mesa, CA). Bladder infusion evoked reflex micturition contractions that were accompanied by fluid flow from the urethral meatus. To evaluate urethral activity, electromyography (EMG) of the external urethral sphincter (EUS) was also performed. For EUS-EMG recording, the rostral pubic bone was removed and two fine silver wire electrodes (0.25 mm diameter, Teflon coated, A-M Systems, Carlsborg, WA) were inserted bilaterally into the EUS. The amplified EMG and pressure signals were digitized with an MP 100 acquisition unit and displayed, stored, and analyzed with the use of a computer (AcqKnowledge Software, BIOPAC Systems, San Diego, CA).

With the rat’s head held in a stereotaxic frame, minimal craniotomies were performed to expose the dorsal surface of the brain. After surgery, the animals were allowed to stabilize for 30 min before chemical testing was begun. Body tempera-
ture was monitored by means of a rectal thermometer and maintained at 37.0 ± 1.0°C with a heating pad.

CoCl₂ blockade of the micturition reflex. A glass micropipette (20–40 μm tip diameter, A-M Systems) fitted to a 1.0 μl syringe (Hamilton, Reno, NV) was filled with 10 mM CoCl₂ (Sigma Chemical, St. Louis, MO) dissolved in artificial cerebrospinal fluid (aCSF, pH 7.4) of the following composition (in mM): 2.5 potassium chloride, 2.0 magnesium chloride, 1.26 calcium chloride, 1.3 sodium phosphate (dibasic), and 125 sodium chloride in distilled water. The pipette was lowered into the PAG and lateral pontine tegmentum region in 0.5-mm steps with the use of a microdrive mounted on a stereotaxic frame. Changes of bladder and arterial blood pressure and EUS-EMG activity were continuously monitored during injections of CoCl₂. Four rats were used in an initial survey of potential blocking sites in the PAG. Injection volumes of 100–200 nl of CoCl₂ per site were made unilaterally or bilaterally in dorsoventral tracks from bregma –5.60 to –11.00. In the rest of the experiments, smaller volumes of CoCl₂ (25 or 50 nl) were adopted for the injections to more precisely localize the effective sites (n = 23).

An effective site was identified when CoCl₂ injection produced blockade of infusion-induced micturition contractions and EUS-EMG activity for a period at least equal to double the interval between two micturition contractions. Minimal increases of the volume threshold to induce micturition contraction (corresponding to <2 intercontraction intervals) with preservation of rhythmic micturition contractions and no overflow incontinence were not considered to indicate micturition blockade. Injections of CoCl₂ were also made 0.5–1.0 mm dorsal, ventral, medial, and lateral to the effective sites to determine the effective diffusion zone of CoCl₂. Control injections of aCSF (50 nl) were injected into effective sites. Effective sites were marked by 25 or 50 nl injections of CoCl₂ (10 mM) containing 2% Fluoro-Gold (FLUOROGOLD, Englewood, CO) or 10% India ink.

At the end of each experiment, the animals were overdosed with anesthetic and transcardially perfused with 0.05 M phosphate-buffered saline followed by 10% formalin in 0.1 M phosphate buffer (pH 7.4). The brains were removed, postfixed in 10% formaldehyde for 2 days, and then transferred to 20% sucrose phosphate buffer (0.1 M) overnight. Frozen sections (40 μm) were cut in the coronal plane with the use of a freezing microtome. The sections were mounted on albumin-subbed glass slides and stained with 0.1% thionin. Pipette tracks and injection sites were identified using bright-field or fluorescence microscopy with an Olympus BH-2 microscope. The locations of the injection sites were plotted on representative sections from a stereotaxic atlas (20).

RESULTS

Effects of CoCl₂ injection on the micturition reflex. In the urethan-anesthetized rat, continuous infusion of saline into the bladder produced volume-evoked micturition contractions that were evident as rhythmic, large-amplitude increases in bladder pressure accompanied by fluid flow from the urethral meatus (Fig. 1). The amplitude of the pressure response and intermicturition interval were stable (Fig. 1). The threshold volume for induction of a micturition reflex ranged from 0.13 to 0.60 ml (mean = 0.29 ml, n = 23), and the peak amplitudes of bladder contractions ranged from 20.3 to 31.7 cmH₂O (mean = 26.0 cmH₂O, n = 23). EUS-EMG exhibited a low level of activity during bladder filling that changed to large-amplitude bursts at the peak of bladder contractions (Figs. 1 and 2) as described in other studies (14). Mean femoral arterial blood pressure was in the range of 80–120 mmHg and remained stable during bladder filling. However, during micturition, small biphasic changes of blood pressure (−2–8 mmHg) were often observed (Figs. 1 and 3). In the initial experiments, unilateral as well as bilateral injections using large volumes of CoCl₂ (100 nl, n = 4) were found to block micturition when injected into a restricted area of midbrain and pons (bregma –7.60 to –11.00).
Therefore, in subsequent experiments we used smaller volume unilateral injections of CoCl₂ (25 or 50 nl) in this area.

Blockade of micturition after CoCl₂ injection was characterized by blocking of the rhythmic, large-amplitude increases in bladder pressure and EUS-EMG activity, resulting in urinary distention, continuously high bladder pressure, and overflow incontinence (Fig. 2). The small blood pressure changes accompanying micturition contractions disappeared after injection of CoCl₂ into effective sites, but CoCl₂ itself did not cause cardiovascular changes (Fig. 3). Blockade of the micturition contractions was usually successfully repeated after the second CoCl₂ injection with Fluoro-Gold at the same sites. Injections of aCSF had little or no effects on the cystometrogram or EUS-EMG activity. There was little or no effect on micturition after CoCl₂ injection (50 nl) 0.5 mm dorsal to the effective site (Fig. 4). CoCl₂ injections into the cerebral aqueduct or the fourth ventricle had no effect on the micturition reflex (Fig. 5).

CoCl₂ injections into the pontine tegmental region. Unilateral CoCl₂ injections into the pontine tegmentum at 20 sites in 17 rats resulted in urinary retention (Figs. 5 and 6A). CoCl₂-induced blockade of reflex micturition was demonstrated in Barrington’s nucleus (Figs. 2A, 5, and 6A). Injections into the dorsolateral tegmental nucleus or the locus ceruleus next to Barrington’s nucleus did not influence micturition (Fig. 5). Analysis of injection sites using Fluoro-Gold as a marker showed that injection sites made at least 0.5 mm from an effective site were ineffective in producing blockade (Fig. 4A).

Micturition blockade usually occurred immediately after the CoCl₂ injection and dissipated in 3–21 min (mean = 5.4 min, n = 16). Four effective injections in the tegmental region, after which the micturition blockade continued over 30 min, were not considered in calculating this blocking time, because the experiments were not carried on long enough to see spontaneous recovery of micturition.

CoCl₂ injections into the PAG. Blockade of reflex micturition after CoCl₂ injections was demonstrated in 12 sites (11 rats) in the PAG region (Figs. 2B, 5, and 6B). These effective sites in the PAG showed a confined distribution along the rostrocaudal axis of the PAG (Fig. 5). Most effective sites for blocking reflex micturition were located in the caudal part of the PAG (approximately at bregma −7.80 to −8.80), especially near the lateral border of the ventrolateral region of the PAG (PAGvl) (Figs. 5 and 6B). Some effective sites were identified in lateral regions adjacent to the PAGvl (Fig. 5). No effective sites were identified in other regions in the midbrain, including rostral and intermediate PAG. Injection of CoCl₂ into the dorsal PAG had no effect on micturition, and CoCl₂ injection into the intermediate region of the PAG sometimes produced short intermicturition elongations that were not considered to be a blocked micturition reflex (Fig. 4B). Effective injections in the PAGvl blocked rhythmic micturition contractions.
and EUS-EMG activity (Fig. 2B) for 3–23 min (mean = 5.9 min, n = 9). The mean duration of blockade by the PAGvl injections was not different from that produced by injections into Barrington’s nucleus (P > 0.05 by Mann-Whitney U test). Three effective injections in the PAG blocked micturition for >30 min. Because we did not wait for spontaneous recovery of micturition in these three experiments, they were not considered in calculating the blocking time.

DISCUSSION

The present experiments show that micturition reflex activity in the urethan-anesthetized rat is interrupted by blockade of synaptic transmission in the PAGvl as well as in Barrington’s nucleus. Lumbosacral neurons project to both areas in the rat (9). However, our findings provide functional evidence that PAGvl is critical to micturition, despite the presence of the direct projection from spinal cord to Barrington’s nucleus.

Fig. 6. Thionin-stained sections showing location of CoCl₂ injection tracks (arrows) in Barrington’s nucleus (A) and the PAG (B). Scale bar, 500 µm (A), 200 µm (B). DTN, dorsal tegmental nucleus.

Fig. 5. Schematic diagram showing location of CoCl₂ injection sites in the pontine tegmentum and midbrain. ● Effective sites where micturition contractions were blocked; ○, ineffective sites. Note that micturition was blocked after injection of CoCl₂ into Barrington’s nucleus and the caudal part of the PAGvl. Markers on the left indicate distance (mm) caudal to bregma. Aq, cerebral aqueduct.
Technical considerations. CoCl₂ reversibly suppresses synaptic transmission in two ways: 1) by inhibiting neurotransmitter release, presumably by binding to presynaptic calcium channels (12, 27); and 2) by blocking ligand-gated channels postsynaptically (2, 12). We have used CoCl₂ in preference to local anesthetic because the former is reported to have no effect on fibers of passage (15). However, it is also suggested that repeated injections or high concentration of CoCl₂ could induce permanent damage to both neural fibers and somata (15). In our study, the micturition reflex recovered after CoCl₂-induced blockade. Therefore, it is reasonable to assume that blockade of micturition produced by this agent is related to reversible interruption of synaptic transmission in the restricted injection zone. Because the localization of sites contributing to micturition is important as well as prevention of excessive dose injection, we adopted small injection volumes in this study. As CoCl₂ injection (50 nl) 0.5 mm dorsal to the effective site had little or no effect on micturition, it is concluded that the functional spread zone of CoCl₂ injections was <1.0 mm in diameter.

The effectiveness of unilateral microinjection of CoCl₂ in blocking micturition in the anesthetized rat was unexpected, and we do not have a clear explanation for this finding. Urethan anesthesia is known to be able to preserve various kinds of reflexes, including micturition, at the dose used in these experiments (17). In three chronic cannula-implanted rats, we found that bladder capacity was 1.0–1.3 ml in the conscious state but fell to 0.2–0.4 ml under urethan anesthesia (1.2 g/kg ip; data not shown). The facilitation of micturition under urethan anesthesia may be due to release of the basic micturition reflex from rostral inhibitory modulation (24). On the other hand, urethan has inhibitory effects on micturition as the dose is increased (17). It is conceivable that the micturition reflex pathway normally has a large safety factor for transmission but that this is reduced during urethan anesthesia. The reduced safety factor may make the pathway sensitive to relatively modest degrees of interference with synaptic transmission such as would occur with unilateral administration of CoCl₂.

Blood pressure per se was not altered during bladder filling or by injection of CoCl₂ in the sites examined in our study. This result, although not definitive, at least suggests that the modest rate of bladder filling used is probably not noxious.

Micturition blockage after CoCl₂ injections in Barrington’s nucleus. It is believed that the basic micturition reflex is supraspinally regulated in Barrington’s nucleus. Volume-evoked bladder contractions in the urethan-anesthetized rat induced by bladder filling through a cannula in the dome of the bladder have been shown to depend on this spino-bulbo-spinal reflex circuit (18). Neuroanatomical studies have demonstrated neural connections between Barrington’s nucleus and the intermediolateral cell column of the lumbosacral spinal cord, which contains preganglionic neurons of the pelvic nerve (13, 16). Electrical and chemical stimulation of Barrington’s nucleus elicit bladder contraction (19, 21, 28), whereas lesions of Barrington’s nucleus induce urinary retention (23). Injection of CoCl₂ into Barrington’s nucleus blocked the micturition reflex in this study, also supporting the critical involvement of this nucleus in the basic micturition reflex. By contrast, injection of CoCl₂ into the locus ceruleus located close to Barrington’s nucleus failed to block micturition in this study. These results confirm the selectivity of block and reliability of the microinjection technique used in addition to confirming the lack of a critical role for the locus ceruleus in the basic micturition reflex.

Micturition blockage after CoCl₂ injections in the PAG. In the rat (9, 13), unlike the cat (5), Barrington’s nucleus receives dense neural projections from the lumbosacral spinal cord (9, 13), but the PAG is also a major target of ascending projections in both species (9, 26, 29). Both the dorsal part of the PAG and the PAGvl receive neural projections from the lumbosacral spinal cord (9, 10, 19, 27). Barrington’s nucleus receives a denser projection from the dorsolateral PAG than the PAGvl in the rat (6), and electrical stimulation of bladder afferents in the pelvic nerve results in short-latency potentials in the caudal part of the dorsolateral PAG than those observed in Barrington’s nucleus (19). However, stimulation of the dorsal PAG evokes no micturition contractions or pelvic nerve discharge (19) and in our experiments micturition was not blocked by CoCl₂ injections in this area. Therefore, the dorsal PAG appears not to be an indispensable component of the basic micturition reflex circuitry, although it may have some other influence on micturition.

The PAG is known to play an important role in pain modulation, vocalization, reproductive behavior, and autonomic regulation (3, 4). It appears to be functionally and anatomically organized in distinct longitudinal neural columns extending for varying distances along the rostrocaudal axis of the PAG (3, 4). Activation of neurons in the PAGvl column results in opioid-mediated analgesia and behavioral quiescence, hypotension, and bradycardia (3, 4). Various kinds of noxious manipulations, including noxious visceral stimuli, e.g., intraperitoneal acetic acid, elicit a significant increase in the number of Fos-positive neurons in the area including, but not restricted to, the PAGvl (3, 4). Also, a tracing study demonstrated that neural projections from the lumbosacral spinal cord are widely distributed throughout the PAGvl, resulting in a column-like distribution (9). PAGvl neurons projecting densely to Barrington’s nucleus (8, 9, 25) originate from all rostrocaudal levels of the PAG, although they may be more numerous in the caudal regions (25). By contrast, functional evidence suggests that the area related to bladder function seems to be more restricted. Previous experiments in rats showed that neurons of the caudal part of the ventral PAG respond to electrical stimulation of bladder afferent nerves and that electrical stimulation of the caudal part of the ventral PAG elicited micturition (19). In our experiments, the sites of CoCl₂-induced blockage of micturition were restricted to the ventral border of the caudal region of the PAGvl (Fig. 5). Thus, despite a fairly broad projection of neurons from the

PAG vl.
lumbosacral spinal cord to the PAGvl and from the PAGvl to Barrington’s nucleus, the area critical for the basic micturition reflex function resides in a small region in the caudal part of the PAGvl. Some effective injection sites were located outside of the PAGvl. Among explanations for this observation we must consider that 1) CoCl₂ may reflux a short distance along the pipette track and reach the area responsible for the micturition reflex in the PAGvl, 2) effective injection sites may lie in the dendritic fields of the PAGvl neurons, or 3) the population of midbrain cells essential for micturition does not conform to the boundaries of the PAGvl.

In the rat, urethral sphincter activity is low during bladder filling, but during micturition it exhibits brief but intense bursts that are part of the coordinated pattern of micturition, as they appear to be necessary to produce complete emptying (14). In the experiments reported here, blockade of reflex micturition by CoCl₂ was accompanied by loss of the EUS-EMG burst. It is likely that interference with the basic reflex pathway for bladder contraction would disrupt the coordinated sphincter response as well. However, we cannot eliminate the possibility of a direct action of CoCl₂ on neurons concerned with sphincter control because 1) neurons projecting directly to the spinal nucleus containing EUS motoneurons are located immediately ventral to Barrington’s nucleus (D region) (7) and thus may be reached by CoCl₂ injections into the region of Barrington’s nucleus and 2) there is a dense neuronal projection to the D region from the PAGvl (8), and thus CoCl₂ injections into the PAGvl may block bladder-related and sphincter-related neurons concurrently.

Perspectives

Recent neural tracing studies in the cat suggest that afferent input from the bladder is processed in the PAG before reaching Barrington’s nucleus and that the PAG is an integral part of the basic micturition reflex. In rats, however, there is little difference in the strength of the projections from lumbosacral spinal cord to the PAG and to Barrington’s nucleus (9). Thus there is great need to identify the functional role of the PAG in the mediation of the micturition reflex. The present work has provided functional evidence that the caudal part of the PAGvl, in addition to Barrington’s nucleus, plays an integral role in micturition in the urethane-anesthetized rat. This finding broadens the concept of a “pontine micturition center” to include an essential region of the PAG and implies that distributed processing of bladder afferent information is required to support the basic micturition reflex.

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