Impact of state of arousal and stress neuropeptides on urodynamic function in freely moving rats

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Kiddoo, Darcie A., Rita J. Valentino, Stephen Zderic, Arjunan Ganesh, Steven C. Leiser, Lance Hale, and Dimitri E. Grigoriadis. Impact of state of arousal and stress neuropeptides on urodynamic function in freely moving rats. Am J Physiol Regul Integr Comp Physiol 290: R1697–R1706, 2006. First published January 26, 2006; doi:10.1152/ajpregu.00742.2005.—Corticotropin-releasing factor (CRF) is a neurotransmitter in Barrington’s nucleus neurons. These neurons can coregulate parasympathetic tone to the bladder (to modulate micturition) and brain noradrenergic activity (to affect arousal). To identify the role of CRF in the regulation of micturition, the effects of CRF agonists and antagonists on urodynamics in the unanesthetized rat were characterized. Rats were implanted with bladder and intrathecal or intraperitoneal catheters under isoflurane anesthesia. Cystometry was performed in the unanesthetized, unrestrained state at least 24 h later. In some cases, cortical electroencephalographic activity (EEG) was recorded simultaneously to assess arousal state. During cystometry, the state of arousal often shifted between waking and sleeping and urodynamic function changed depending on the state. Micturition threshold, bladder capacity, and micturition volume were all increased during sleep. The CRF1/CRF2 receptor agonists CRF and urocortin 2 increased bladder capacity and micturition volume in awake but not in sleeping rats. Conversely, the CRF1 receptor antagonists antalarmin and NBI-30775 increased urinary frequency and decreased bladder capacity in awake rats. The present results demonstrate a profound effect of the state of arousal on urodynamic function and suggest that simultaneous monitoring of EEG and cystometry may provide a useful model for studying nocturnal enuresis and other urinary disorders. In addition, the results provide evidence for an inhibitory influence of CRF in the spinal pathway on micturition. Targeting the CRF system in the spinal cord may provide a novel approach for treating urinary disorders.

corticotropin-releasing factor; cystometry; micturition; electroencephalographic activity

CONVERGENT FINDINGS INDICATE that Barrington’s nucleus in the rat is an integral component of the micturition reflex. For example, Barrington’s nucleus lesions disrupt the micturition reflex, and electrical or discrete chemical stimulation of this region elicits bladder contraction (3, 26, 29, 33). The anatomic substrate for this response is a projection from Barrington’s nucleus to the preganglionic neurons in the lumbosacral spinal cord that provide the parasympathetic input to the bladder (10, 19, 21). Transectional tracing studies have verified that Barrington’s nucleus neurons are synaptically linked to the bladder (25, 32, 34).

A major neurotransmitter in Barrington’s nucleus projections to the parasympathetic preganglionic neurons is the stress-related neuropeptide corticotropin-releasing factor (CRF) (39, 42). CRF was initially characterized as the neurohormone released from the paraventricular nucleus of the hypothalamus into the median eminence to elicit release of adrenocorticotropin from the anterior pituitary, a hallmark of the stress response (37). Convergent anatomical, physiological, and behavioral evidence has suggested that CRF also serves as a brain neurotransmitter that may act to modulate autonomic function and behavior in concert with its endocrine effects (27). Many Barrington’s nucleus neurons are CRF immunoreactive, and a dense CRF terminal field targets the preganglionic lumbosacral neurons, suggesting that CRF in this pathway can influence micturition (39, 42). Studies in anesthetized rats have suggested that CRF has an inhibitory influence in the pontine micturition pathway, perhaps by inhibiting the release of an excitatory neurotransmitter from Barrington’s nucleus or by interfering with its postsynaptic effect on preganglionic neurons (29). Thus increases in bladder pressure elicited by discrete chemical stimulation of Barrington’s nucleus were attenuated by intrathecal administration of CRF and enhanced by intrathecal administration of CRF antagonists. Consistent with this, CRF inhibited reflex bladder contractions in anesthetized rats (35). In contrast, recent reports using cystometry in unanesthetized rats have provided evidence that CRF facilitates micturition by decreasing the micturition threshold, micturition volume, and intercontraction interval (17). These discrepant findings may relate to different conditions of anesthesia or specific experimental conditions (e.g., direct stimulation of Barrington’s nucleus vs. bladder filling during cystometry).

Many of the Barrington’s nucleus neurons that project to the lumbosacral spinal cord also project to the major brain noradrenergic nucleus, the locus coeruleus (LC) (38). The LC-norepinephrine system has long been implicated in arousal and attention (1). LC neurons are activated by diverse stimuli, including bladder and colon distension, and this is translated to forebrain electroencephalographic (EEG) activation through the widespread LC-norepinephrine projection system (2, 20, 28, 36). Through its divergent projections to both the LC and preganglionic parasympathetic neurons, Barrington’s nucleus is poised to coregulate visceral and central responses to bladder distension (40). Because CRF is one neurotransmitter contained in Barrington’s neurons that give rise to this divergently projecting pathway, it may play an important role in coordinating micturition and arousal (38).

The present study was designed to identify the potential role of CRF in Barrington’s nucleus spinal projections by charac-

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terizing the effects of CRF analogs on urodynamic parameters measured using in vivo cystometry in the unanesthetized, freely moving rat. Because CRF interacts with two receptor subtypes, CRF₁ and CRF₂, agents with different selectivities for the subtypes were examined (5).

Materials and Methods

Subjects. Adult male Sprague-Dawley rats (~300 g) were housed three to a cage before surgery in a controlled environment (20°C, 12:12-h light-dark cycle, lights on at 7:00 AM). They had free access to food and water in the home cage. Care and use of animals was approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee and was in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Surgery. Rats were anesthetized with isoflurane, and a small cut was made between the scapulae to provide an exit for catheters. A midline incision was made in the abdomen to access the bladder. A 5-French umbilical artery catheter was tunneled subcutaneously from the hole between the scapulae to the abdomen. The catheter end, which had been previously cauterized and flared, was brought intraperitoneally and inserted into the bladder dome and sutured above the flare. A 24-gauge catheter was implanted into the intraperitoneal or intrathecal space during the same surgery. The intrathecal catheter was positioned using a lumbar approach. An incision was made above the lumbar enlargement, and the catheter was threaded through a needle that was inserted into the lumbar intrathecal space. The position was verified during surgery by a tail flick that occurred when the needle was properly positioned. A small drop of superglue placed against the skin anchors the catheter in place while the needle was removed. The catheter was then threaded subcutaneously to exit in the hole between the scapulae. The exposed catheter ends connected to ports that allowed easy connection to syringes used to administer agents or to the syringe pump (for bladder infusion). For some rats, after surgery for catheter implantation, an electrode was implanted into the frontal cortex for recording cortical EEG activity. The recording wire of the electrode was inserted 1.5 mm lateral to the midline and 2.0 mm anterior to bregma. The ground wire of the electrode was placed 1 mm anterior to the recording wire. Both wires were inserted 1 mm ventral to the dural surface.

Protocol. After a 24-h recovery period, rats were placed in a cystometry chamber (Medical Associates, St. Albans, VT) that consisted of a holding cage, a syringe pump for continuous infusion of saline into the bladder, an in-line pressure transducer to monitor bladder pressure, and a urine catch pan positioned on a scale to monitor urine volume. These were situated in a chamber equipped with a house light and fan. There was no access to food or water in the cystometry chamber. Bladder pressure, infusion volume, and urine volume were monitored continuously online on a personal computer with Medical Associates software. EEG was amplified and filtered using an Alpha Omega signal processing system (Nazareth Illit, Israel). Rats were placed in the chamber 24 h after recovery, and if urodynamic parameters were stable throughout a 30-min baseline period, data were collected during this first session (15% of experiments). However, most data were obtained in sessions conducted 48–72 h after surgery (74% experiments) or 96 h after recovery (11% of experiments). Sessions were used for data collection only when urodynamic parameters were stable and within normal limits throughout the baseline session. In addition, data were only collected in sessions in which rats appeared stress free as indicated by normal exploratory movement and/or sleeping in the cystometry chamber and ease with which they could be handled.

After a 30-min baseline recording period, substances were injected intrathecally or intraperitoneally through the appropriate catheters, followed by a saline flush, and the recordings continued for an additional hour. The same rat was usually used to test one or two different conditions and was euthanized by isoflurane overdose at the end of the week of the surgery. Before death, methylene blue was injected into the intrathecal catheter to verify the site of injection. In the early stages of the study, local anesthetics such as lidocaine (4%, 14 μl, intrathecal) were injected, and cystometry was performed to verify reversible disruption of micturition. This provided further evidence that the intrathecally injected agents would reach the region of interest.

Data analysis. The recorded parameters included bladder pressure, bladder capacity (volume of infused saline in a micturition cycle), micturition volume, micturition pressure threshold, and micturition pressure. Because the pressure traces could be affected by movement artifacts or changes in the position of the rat, bladder pressure values could not be compared in all rats. Micturition thresholds were compared by calculating the difference between the actual threshold and the average baseline pressure just after the previous micturition up to the point at which bladder pressure began to rise for the next cycle. This avoided the artifactual impact of drifts in baseline pressure. Urodynamic parameters were compared within the same subject during the awake and sleep state (with treatment condition constant) using the Student’s paired t-test (two-tailed). On average, three micturition cycles were used to compare parameters between sleep and waking in the same subject. Because the state of arousal affected urodynamic parameters in the absence of treatments, care was taken to match pre- and postinjection comparisons by state of arousal (i.e., wake vs. sleep). The effects of treatment (with arousal state constant) on urodynamic parameters were determined by comparing pre- and postinjection values within the same subject using the Student’s paired t-test. On average, three micturition cycles were used to compare parameters between pre- and posttreatment state in the same subject. Data in bar graphs and Table 1 are expressed as means ± SE.

Behavior was continuously observed and noted for each micturition cycle. Rats were determined to be awake as indicated by movement and open eyes or asleep by closed eyes and lack of movement. For some rats, EEG data were continuously monitored online using the Alpha Omega signal processing system. EEG data were subjected to fast Fourier transformation for power spectrum analysis.

Drugs. Ovine CRF and urocortin 2 were obtained from Dr. Jean Rivier (The Salk Institute, La Jolla, CA). Ovine CRF is an agonist that is more potent at CRF₁ vs. CRF₂ receptors, and urocortin 2 is a selective CRF₂ receptor agonist (5, 31). Astressin, a peptide CRF₁/CRF₂ receptor antagonist (30), was obtained from Sigma Chemical. Astressin, CRF, and urocortin 2 were diluted in water; aliquots were made and concentrated and were stored at −70°C until the day of the experiment, when aliquots were diluted in artificial cerebrospinal fluid (aCSF). The dose of CRF (6 μg in 14 μl, intrathecal) was chosen on the basis of its ability to alter Barrington’s stimulated bladder contractions in anesthetized rats and reports of increasing micturition in unanesthetized rats (17, 29). The dose of urocortin 2 (5 μg in 14 μl, intrathecal) was chosen on the basis of its potency at CRF₂ receptors, which is slightly greater than the potency of CRF at CRF₁ receptors (7). The astressin dose is that which has been shown to be an effective CRF antagonist when administered intracisternally (18). Antalarmin (Dr. Kenner Rice, Laboratory of Medicinal Chemistry, NIH National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) and NBI-30775 (Neurocine Biosciences, San Diego, CA) were dissolved in a solution containing 5% ethanol and 5% cremophor. The pH of the vehicle alone was similar to that of vehicle with either antagonist dissolved (pH 4). The antagonists were administered at a dose of 20 mg/kg ip. In 4 experiments, antalarmin was administered at a dose of 20 μg (intrathecal). The dose used for systemic administration was that which had been previously shown to be an effective, selective CRF₁ receptor antagonist in several studies, and for intrathecal injections, the dose was chosen to be 100 times less (45).
**RESULTS**

Effects of arousal state on urodynamics: behavioral and EEG observations during cystometry. Cystometry recordings were made from 45 rats. Behavioral observation throughout the cystometry session revealed that the state of arousal varied between subjects and often within the same session for an individual subject. Initially, when connected to the pump and placed into the chamber, rats were awake and exhibited some exploratory motor activity and/or grooming. They then moved to a corner of the chamber and were either in a quiet waking state or asleep. Within a 90-min session, most animals alternated between waking and sleep. During the awake state, rats sometimes exhibited grooming behavior or crossed to the other side of the chamber. Bladder capacity and micturition volume were related to the state of arousal such that for the same subject, these volumes were greater during sleep (Figs. 1 and 2). Micturition threshold also was greater during sleep. Thus an elevation above baseline of 4.8 ± 0.9 cmH2O was necessary to elicit micturition during sleep, whereas 2.4 ± 0.4 cmH2O was sufficient for the same rats in the awake state (n = 10, P < 0.005). Micturition pressure was slightly greater in the awake state at (38 ± 0.005). Micturition pressure was slightly greater in the awake state compared to the sleep state. In three subjects in which pressure recordings were stable and without movement artifact and that could be matched in the awake state before and after CRF, micturition threshold was increased from 3.1 ± 0.9 to 5.1 ± 0.7 cmH2O above baseline (P < 0.001, Student’s paired t-test) after CRF. Micturition pressure tended to be greater in the same rats after CRF, but this effect was not statistically significant (39 ± 2 vs. 42 ± 7 cmH2O before and after CRF, respectively; P = 0.09, Student’s paired t-test).

The effects of CRF were absent in rats that were sleeping. Bladder capacity before and after CRF in eight rats in which effects could be compared during sleep were 764 ± 82 and 832 ± 81 µl, respectively (P = 0.6, Student’s paired t-test). Micturition volume before and after CRF in the same rats was 821 ± 73 and 836 ± 69 µl, respectively (P = 0.9, Student’s paired t-test).

The selective CRF2 receptor agonist urocortin 2 had effects similar to those of CRF on bladder capacity and micturition volume (Table 1). Like the effects of CRF, the effects of urocortin 2 were not apparent during sleep (n = 3, P > 0.2, Student’s paired t-test).

Effects of CRF antagonists on urodynamics. Astressin (3 µg, intrathecal), a potent and nonselective CRF1/CRF2 receptor antagonist, did not alter urodynamic parameters of awake rats, although there was a tendency for bladder capacity and micturition volume to be decreased with this agent (Table 1). This dose was sufficient to prevent the effects of CRF (e.g., Fig. 3C). The mean bladder capacity before and after CRF administration to astressin-pretreated rats (awake state) was 825 ± 313 and 703 ± 227 µl, respectively (n = 4, P = 0.2, Student’s paired t-test). The mean micturition volume in the same subjects was 721 ± 273 and 609 ± 157 µl, respectively (P = 0.3). Interestingly, in five subjects for which the effects of astressin could be compared during sleep, the antagonist produced a significant decrease in bladder capacity from 522 ± 45 to 487 ± 140 µl (P < 0.05, Student’s paired t-test) and tended to decrease micturition volume (524 ± 140 vs. 495 ± 130 µl; P = 0.09).

The selective and systemically active CRF1 receptor antagonist antalarmin had striking effects on micturition whether administered systemically (n = 6) or intrathecally (n = 2). In all cases, baseline bladder pressure increased, micturition frequency increased, and bladder capacity and micturition volume decreased after antalarmin injection (Fig. 4, Table 1). In three of the six subjects, antalarmin altered the behavioral state such that rats were immobile and appeared to be asleep immediately after injection. Nonetheless, they exhibited frequent micturition episodes during this time, and even compared with the awake predrug state, bladder capacity and micturition volume were substantially reduced by antalarmin (Table 1). The effects produced by antalarmin could not be attributed to the pH or other property of the vehicle, because this had no effect when the same volume was administered intraperitoneally (Fig. 4, Table 1). NBI-30775, another systemically active, selective CRF1 receptor antagonist, had a urodynamic profile similar to that of antalarmin when administered systemically, although the tendency to decrease micturition volume did not reach statistical significance (P < 0.07) (Fig. 4C, Table 1).

**DISCUSSION**

A major finding of the present study is the profound effect of arousal state on urodynamic function. This was apparent with...
intrathecally. *urocortin 2-treated, and 4 astressin-treated rats. All agents were administered spinal fluid (aCSF)-treated, 10 corticotropin-releasing factor (CRF)-treated, 6 experiments. Bars represent means indicates the condition under which the values were obtained: baseline indi-

Fig. 1. Effects of state of arousal on bladder urodynamics. 

A

B

Fig. 2. Bladder capacity and micturition volume are consistently elevated during sleep, regardless of drug condition. Data indicate micturition volume (A) or bladder capacity (B) in rats during waking (open bars) and sleep (solid bars) for the same rats under the same drug condition. Thus comparisons are between awake vs. sleep under the same treatment condition. The abscissa indicates the condition under which the values were obtained: baseline indicates no treatment and occurred during the preinjection period of different experiments. Bars represent means ± SE of 37 baseline, 8 artificial cerebrospinal fluid (aCSF)-treated, 10 corticotropin-releasing factor (CRF)-treated, 6 urocortin 2-treated, and 4 astressin-treated rats. All agents were administered intrathecally. *P < 0.05, **P < 0.01, ***P < 0.001.

Impact of the state of arousal on urodynamic function. Studies that have examined the relationship between arousal and urodynamics in human subjects have been performed primarily in patients with nocturnal enuresis (14, 43), and this has not been explored in rodent cystometry models. The present observation that urodynamics are altered during sleep has important technical, biological, and clinical implications. At a technical level these findings underscore the importance of considering the state of arousal when interpreting results of cystometry studies. For example, drugs or experimental conditions may alter urodynamic parameters indirectly by altering the state of arousal. Conversely, an effect of a drug or condition on urodynamic parameters may be masked if it is studied under a state of arousal different from that of the control condition. To control for this, the state of arousal should be matched between control and experimental conditions.

Changes in bladder capacity in the absence of pressure changes would be indicative of a change in bladder compli-

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should advance our knowledge of how sleep-related processes modulate circuits controlling urination.

To our knowledge, this is the first report to combine simultaneous EEG recordings with cystometry in nonhuman subjects. Using this approach, we were able to obtain fine temporal resolution of the relationship between arousal and micturition. The recordings verified an offset between the central and visceral responses to bladder distension in the rat. The identification of this offset in the rodent suggests that simultaneous EEG and cystometry recordings may be useful in studying enuresis and in the development of therapies to treat enuresis.

**CRF regulation of urination.** Initial studies of the role of CRF in micturition demonstrated that intrathecal administration of CRF agonists and CRF antagonists decreased and increased, respectively, the magnitude of bladder contractions evoked by discrete chemical stimulation of Barrington’s nucleus in the anesthetized rat (29). Consistent with this, other studies in anesthetized rats demonstrated that CRF inhibited reflex bladder contractions (35). Because excitatory amino acid projections from Barrington’s nucleus to the spinal cord are thought to mediate excitation of the parasympathetic preganglionic neurons (23, 24, 44), CRF was hypothesized to interfere with release or the postsynaptic action of the excitatory neuromediator (29). Although informative, the focus of these studies was limited to the impact of CRF within specific limbs of the micturition reflex. In addition, the use of anesthesia in these studies is a possible confounding factor. Cystometry provides the advantages of characterizing the net impact of agents on micturition in the anesthetized state. Recent preliminary reports using cystometry in unanesthetized rats have suggested that CRF decreases micturition threshold and increases micturition frequency (17). These findings contrast with those of the present study. One critical difference is that the present study controlled for the effects of arousal state. It is possible that the discrepant results of the previous study related to changes in the state of arousal during the cystometry session. Alternatively, differences in rat strain or supplier could account for discrepant results between the studies.

The effects of the non-peptide-selective CRF₁ antagonists were consistent with an inhibitory influence of CRF in the spinal cord on micturition. However, the lack of effect of astressin, the peptide analog of CRF that is an effective antagonist at both CRF₁ and CRF₂ receptors, was surprising. Nonetheless, little is known regarding the CRF receptor protein within the spinal cord or its cellular transduction mechanisms, and there is substantial evidence for differential interactions of peptide and nonpeptide CRF antagonists with the CRF receptor protein in brain and cell culture (11–13). Further investigations into the differences between CRF antagonists in the spinal cord are warranted, given the therapeutic potential for these agents in urinary disorders.

**Potential functions of CRF in the micturition circuit.** An inhibitory influence of CRF on micturition is somewhat counterintuitive, because acute stress and fear are often associated with urination. However, chronic stress, particularly social stress, results in urinary retention. Although this may be naturally adaptive for hiding from predators, it can be sufficiently severe to produce nephropathy as a result of reflux (8, 9, 22). Chronic stress also increases CRF mRNA expression in Barrington’s nucleus neurons (15, 16). Given the present findings, it is possible that chronic stress-induced increases in CRF mRNA expression in Barrington’s nucleus neurons favor an inhibitory influence on micturition that results in the observed urinary retention.

Many of the spinal projecting neurons of Barrington’s nucleus, some of which contain CRF, also project to the LC (38), and CRF activates LC neurons (6). LC activation by diverse stimuli, including bladder distension, results in forebrain EEG activation and behavioral indexes of arousal (4, 41). Thus this anatomic arrangement allows for bladder distension to elicit an arousal response with bladder contraction. Nonetheless, this arrangement must account for a temporal offset between arousal and micturition, with arousal occurring first. The present findings lead to the speculation that as bladder pressure begins to rise, CRF release in LC projections of Barrington’s nucleus neurons facilitates arousal and a simultaneous release in spinal projections inhibits bladder contraction. As bladder pressure continues to rise, recruitment of excitatory amino acid neurotransmission from Barrington’s nucleus may overcome the inhibitory influence of CRF.

Together, the present results lead us to speculate that an acute function of CRF in Barrington’s nucleus projections may be to allow arousal to occur before micturition to facilitate preparative behaviors. A consequence of CRF overexpression in the circuit as a result of chronic stress may contribute to stress-induced urinary retention, which is adaptive but also can be pathological. Future studies that combine EEG recordings with cystometry and that investigate urodynamics in animals with a history of stress will ultimately test this hypothesis.
Fig. 4. CRF₁ receptor antagonists cause robust changes in urinary parameters. Traces show the effects of intraperitoneal antalarmin (20 mg/kg; A), intrathecal antalarmin (20 μg; B), intraperitoneal NBI-30775 (20 mg/kg; C), and intraperitoneal vehicle injection (D) on bladder pressure (cmH₂O), bladder capacity (μl), and micturition volume (ml). Arrows indicate time of injections; i.p., intraperitoneal; i.t., intrathecal. Antalarmin produced a robust increase in urinary frequency, increased bladder pressure, and decreased bladder capacity and micturition volume. These effects were produced with both systemic and intrathecal injection and were not apparent with vehicle injection. The effects of NBI-30775 were similar, although the trend to decrease micturition volume was not statistically significant (P < 0.07). Rats were awake during micturition cycles unless indicated by an asterisk above the cycle. Irregularities in the bladder pressure trace in C are associated with movement.
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DISCLOSURES

D. E. Grigoriadis is an employee of Neurocrine Biosciences, Inc.

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