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Corticotropin Releasing Factor (CRF) Expression in Postnatal and Adult Rat Sacral Parasympathetic Nucleus (SPN)

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Abstract

The neural control of micturition undergoes marked changes during the early postnatal development. During the first few postnatal weeks, the spinal micturition reflex is gradually replaced by a spinobulbospinal reflex pathway that is responsible for micturition in adult animals. Upregulation of brainstem regulation of spinal micturition pathways may contribute to development of mature voiding patterns. We examined the expression of corticotropin releasing factor (CRF), present in descending projections from Barrington's nucleus to the sacral parasympathetic nucleus (SPN), in postnatal (P0-P36) and adult Wistar rats (P60–90). CRF-IR was present predominantly in the SPN region although some staining was also observed in the dorsal horn and dorsal commissure in L5-S1 spinal segments. CRF-IR in spinal cord regions was age dependent ($R^2 = 0.87-0.98$). The majority of the CRF-IR in the lumbosacral spinal cord was eliminated by complete spinalization (2–3 weeks). Double-label immunohistochemistry was combined with quantitative confocal laser scanning microscopy to quantify the number, percentage of preganglionic cell body or proximal neurite occupied and percentage of colocalization between CRF-immunoreactive varicosities and preganglionic cells or proximal neurites in the SPN in postnatal and adult rats. Results demonstrate an age dependent upregulation of CRF-IR in the SPN region and specifically in association with preganglionic parasympathetic neurons identified with neuronal nitric oxide synthase (nNOS)-IR. CRF-immunoreactive varicosities on or within a 1 μ m perimeter of nNOS-immunoreactive cells or proximal neurites also increased with postnatal age. The upregulation of CRF-IR in bulbospinal projections to the SPN may contribute to mature voiding reflexes.

Keywords

spinal cord; nNOS; micturition reflexes; postnatal development

Introduction

The mechanisms involved in the storage and periodic elimination of urine exhibit marked changes during prenatal and postnatal development (de Groat and Araki, 1999; de Groat et al., 1998). In the young fetus, prior to maturation of the nervous system, urine is presumably eliminated from the urinary bladder by nonneural mechanisms. At later stages of development, micturition is regulated by primitive spinal reflex pathways. As the central nervous system continues to mature during the postnatal period, reflex voiding is brought under voluntary control involving higher brain centers (de Groat and Araki, 1999; de Groat et al., 1998). In

adults, injuries or diseases of the nervous system can lead to the reemergence of primitive functions that were prominent early in development but then were suppressed during neural maturation (de Groat et al., 1998). Therefore, developmental studies of micturition reflex pathways are likely to provide key insights into the mechanisms underlying neurogenic disorders of urinary bladder function in adults.

Marked changes (Capek and Jelinek, 1956; de Groat, 1975; de Groat et al., 1993; Maggi et al., 1988; Thor et al., 1990; Thor et al., 1986) in the neural control of voiding function that occur during early postnatal development may be mediated by multiple factors, including: (1) upregulation of brainstem regulation of spinal micturition pathways; (2) changes in the properties of urinary bladder and perineal afferent neurons induced by peripheral organ maturation and (3) changes in the properties of interneuronal populations in the spinal cord. Electrophysiological studies in spinal cord slice preparations from younger and older neonatal rat pups have demonstrated changes in synaptic efficiency between interneurons and preganglionic neurons in the sacral parasympathetic nucleus (SPN) (Araki and de Groat, 1996; Araki and de Groat, 1997; de Groat and Araki, 1999) that may contribute to the down-regulation of the perineal-to-bladder reflex during postnatal development. It has been hypothesized that up-regulation of bulbospinal projections to the SPN region plays a key role in the down-regulation of the perineal-to-bladder reflex due to competition between supraspinal and segmental inputs (Araki and de Groat, 1996; Araki and de Groat, 1997; de Groat and Araki, 1999). Neuronal connections at developing synapses appear to be refined by competition between multiple synaptic inputs converging on the same target cells (Goodman and Shatz, 1993). Thus, segmental autonomic reflexes may be suppressed by competition from bulbospinal projections for synaptic connections to neurons in the SPN.

Corticotropin releasing factor (CRF) is of particular relevance in the rat, since it is present in descending projections from the PMC or more specifically from Barrington's nucleus to the SPN (Merchenthaler, 1984; Merchenthaler et al., 1982; Suzuki et al., 1990; Vincent and Satoh, 1984a). Barrington's nucleus has historically been viewed as the supraspinal switching center that regulates storage and elimination of urine (Loewy et al., 1979; Noto et al., 1989; Satoh et al., 1978). More recent studies have led to the suggestion that Barrington's nucleus may contain neurons that control a broad range of pelvic organ functions, not exclusively micturition (Marson, 1997; Marson et al., 1993; Pavcovich et al., 1998; Rouzade-Dominguez et al., 2003; Vincent and Satoh, 1984b). Numerous neurons in Barrington's nucleus express CRF-immunoreactivity (IR) and CRF-IR is used to identify Barrington's nucleus (Vincent and Satoh, 1984b). CRF is prominently expressed in the descending pathway from Barrington's nucleus to the SPN in the lumbosacral spinal cord and prominent CRF-IR is expressed in the SPN of adult rats (Puder and Papka, 2001; Valentino et al., 1996; Valentino et al., 1995). Opposing roles for CRF in bladder function have been proposed (Pavcovich et al., 1998) (Klausner and Steers, 2004).

The present studies were designed to examine the maturation of descending projections from Barrington's nucleus to the SPN region, identified by CRF-IR, in the lumbosacral spinal cord in postnatal and adult rats. The studies used spinal cord tissue from postnatal and adult rats with the following aims: 1) identify CRF-IR in the SPN with preganglionic parasympathetic cells identified by neuronal nitric oxide synthase (nNOS)-IR using double-labeling immunostaining techniques; 2) quantify CRF-immunoreactive varicosities in close apposition to nNOS-immunoreactive cells and proximal neurites in the SPN and 3) determine the percentage of overlap between CRF-immunoreactive varicosities and nNOS-immunoreactive cell bodies or neurites in the SPN using quantitative confocal analysis. Preliminary reports of portions of this work have appeared in abstract (Malley et al., 2003).

Material and Methods

Experimental animals

Rat pups (Charles River, Canada; postnatal day (P)0, P3, P7, P9, P14, P21, P27, P36; n=9 each) and adult rats (P60–90; n=9) were used for this study. Rat pups were born to timed-pregnant rats and several postnatal ages were studied from each litter born. Developmental ages, prior to (P0–P14) or following (P21–adult) the development of the spinobulbospinal micturition reflex were selected and analyzed (Capek and Jelinek, 1956). In these studies, day of birth is referred to as P0. All experimental protocols involving animal use were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC; 05–026). Animal care was under supervision of the University of Vermont Office of Animal Care Management in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health guidelines. All effort was made to minimize the potential for animal pain, stress or distress.

Tissue harvest

Both male and female rat pups (P0, P3, P7, P9, P14, P21, P27, P36; n=9 each) and female adult rats (P60–90; n=9) were deeply anesthetized with isoflurane (4%) and then euthanized by decapitation (pups) or thoracotomy (adult rats). The spinal cord (cervical to sacral) was removed and placed in paraformaldehyde (4%) for 2 hours. After fixation the spinal cord was placed and pinned on Sylgard dish and segmented. Spinal segments (L6–S1) were identified and then placed in sucrose (20%) for 24 hours for cryoprotection and then sectioned (20 μ m) in the transverse plane using a cryostat. Spinal cord segments were identified based upon at least two criteria: (1) the T13 dorsal root ganglion (DRG) exists after the last rib and (2) the L6 vertebra is the last moveable vertebrae followed by the fused sacral vertebrae. Another less precise criterion is the observation that the L6 DRG are the smallest ganglia following the largest, L5 DRG. In rat pups younger than P14, segmentation using guidance from DRG, vertebrae and peripheral roots was not possible. Thus, segmentation was based on locating the lumbar enlargement and then estimating the location of the L6–S1 spinal cord caudal to this. This estimation was later confirmed by immunostaining the autonomic nucleus in the L6–S1 spinal segments with neuronal nitric oxide synthase (nNOS)-IR (Vizzard et al., 1995b)(see below). Our previous work (Vizzard et al., 1995b) demonstrated that all nNOS-IR cells in the rat SPN were also retrogradely labeled from the major pelvic ganglion and therefore represented preganglionic parasympathetic neurons. Spinal cord sections from each animal were processed for corticotropin releasing factor (CRF)- and nNOS-IR.

Spinalizations

We sought to determine if CRF-IR in the lumbosacral spinal cord originated from primarily supraspinal locations and evaluated CRF-IR after a 2–3 week period of spinalization in adult, female Wistar rats (n = 3). Spinal cord transections (Zvarova et al., 2005) were performed under isoflurane anesthesia (2%) 2–3 weeks prior to intracardiac perfusion as previously described (Vizzard, 1997; Vizzard, 2000). Briefly, the dorsal T8–T10 vertebrae were removed and the spinal cord was completely transected. The space between the retracted ends of the spinal cord was packed with Gelfoam (Upjohn Company of Canada, Ontario) and the incision sutured. Complete spinal transection was visually confirmed at the time of euthanasia and tissue dissection. One-day prior to surgery and for one week following, ampicillin (0.1g/kg, s.c.) was given once a day. Following surgery, the animals were given buprenorphine (0.05 mg/kg, s.c.) every twelve hours for a total of 4 doses, and housed in Alpha-Dri (Shepherd Specialty Papers, Kalamazoo, MI) lined cages and bladders were manually expressed three times a day. Studies were restricted to female animals because manual expression of the bladder is more easily accomplished because of the shorter and concomitantly smaller region occupied by the external

urethral sphincter compared to male rats. All spinalization protocols were approved by the IACUC (03–148).

Immunohistochemistry

Spinal cord sections were processed using an on-slide processing technique. Groups of experimental animals were processed simultaneously to decrease the incidence of variation in staining and background that can occur between animals and on different processing days. Slides with spinal cords tissue were incubated overnight at room temperature with primary antisera (Table 1). Slides were then washed (3 x 10 minutes) with 0.1 KPBS (pH 7.4) and then incubated (2 hours at room temperature) in species-specific secondary antisera (Table 1). Slides were again washed (3 x 10 minutes) with 0.1 KPBS (pH 7.4) and placed under coverslips using Citifluor (Citifluor, London, UK). Control tissues incubated in the absence of primary and secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of antibody, no positive immunostaining for nNOS or CRF was observed.

Spinal Cord Densitometry

The density of CRF-IR in specific regions of spinal cord in postnatal and adult rats ($n = 6$) was determined with densitometry analysis (Image-Pro express, version 4.0, Media Cybernetics, L.P.) as previously described (Vizzard, 1999; Vizzard, 2000; Zvarova et al., 2004). Spinal cord segments were sectioned entirely from rostral to caudal. Every third and sixth tissue section was then processed for CRF-IR. Of these tissue sections, every first and fifth tissue section was then used for semi-quantitative analysis of CRF-IR. We did not select sections based upon staining intensity and no sections were discarded from analysis because of low staining. The following regions of spinal cord from both, experimental and control animals were analyzed: superficial, lateral dorsal horn (LDH), dorsal commissure (DCM) region, the region of sacral parasympathetic nucleus (SPN; L6, S1), and the region of lateral collateral pathway (LCP; L6, S1). Seven randomly chosen sections from each spinal segment examined were viewed with a 4x objective and captured through a video camera attachment to the microscope with exposure time, brightness and contrast being held constant. The image was converted into pixels on the computer monitor according to a gray scale that ranges in intensity from 0 (white) to 255 (black). The spinal cord section was centered in the field and a standard size square was overlaid on the areas of interest (LDH, DCM, SPN, LCP regions). The labeled area within the square was measured. Transmittance (t) was calculated as $t = (\text{gray level} + 1/256)$. Optical density (OD) was derived from $OD = -\log t$.

Image Capture

Confocal scanning laser microscopy (Bio-Rad MCR 1024 ES confocal scanning microscopy system; Bio-Rad Laboratories, Hercules, CA, USA) was used to determine the anatomical relationship between CRF-immunoreactive varicosities and nNOS-immunoreactive cells bodies and neuritis because of its excellent spatial resolution ($< 1 \mu\text{m}$) as has been shown for other studies examining nerve varicosities in close apposition to cell bodies (Ginsberg et al., 1994; Todd et al., 2002). Studies demonstrate that the combination of high spatial resolution and narrow depth of focus of the confocal microscope enables one to reliably detect immunoreactive varicosities on immunoreactive cells bodies or dendrites (Ginsberg et al., 1994). The resolution of the system used is approximately $0.5 \mu\text{m}$ laterally and $1 \mu\text{m}$ axially. The confocal scanning laser microscope consists of a mixed argon/krypton laser (with precisely defined lines of excitation at 488, 568 and $647 \mu\text{m}$) with a scanning head mounted on an Olympus BX 50 upright laser microscope. The entire system is controlled by Bio-Rad Lasersharp 2000 software (version 5). For each z-axis interval ($1 - 2 \mu\text{m}$), tissue sections are Kalman scanned three times using Krypton-argon laser with specific excitation wavelengths and sequential images are captured for subsequent computer-generated overlay and analysis.

CRF-IR (Cy3-conjugated secondary antibody) was visualized with an excitation wavelength of 647 nm. nNOS-IR (Cy2-conjugated secondary antibody) was visualized with an excitation wavelength of 488 nm. Images were captured with the use of a 20x and 60x objective lens in 1024 x 1024 pixel, 8-bit grayscale format. A red, green image was assembled from two grayscale images with the use of the color merge software function. Captured images were transferred to a Dell 400 NT workstation.

Data analysis

Digital images were opened in MetaMorph Image Analysis software (Universal Imaging, West Chester, PA, USA) for analysis (image analysis system with morphometry software). After reconstructing the selected nNOS-immunoreactive cells, CRF-immunoreactive neurites and CRF-immunoreactive varicosities, three types of measurements were made to evaluate developmental changes in the anatomical relationship between CRF-immunoreactive varicosities and nNOS-immunoreactive cells and proximal neurites in the SPN of the lumbosacral spinal cord.

1. Numbers of CRF-Immunoreactive Varicosities Apposed to nNOS-Immunoreactive Cells in SPN—

The image was calibrated for the objective used (20x and 60x), red and green colors of the merged picture were digitally separated and the spectrum of both red and green colors was converted to gray scale. The pixel intensity of the grey scale was thresholded. Color threshold is defined by pixel hue, saturation, and intensity. The hue and saturation pixel ranges were set as inclusive from 0 to 255, and intensity range was set at 1–184 for red and 1–107 for green inclusive. The brightness threshold was adjusted for each experimental series using concomitantly processed negative controls as our guide for setting background fluorescence. For all CRF- as well as for all NOS-immunoreactive images in each experimental series, the same threshold was used. We considered CRF (or NOS) positive only those varicosities (or cells or neurites) with an intensity that exceeded the established thresholds, distinctive for CRF and nNOS. CRF-immunoreactive varicosities were counted in a 1 μ m area surrounding a nNOS-immunoreactive cell surface. CRF-immunoreactive varicosities met these criteria: color intensity exceeded threshold, one terminal could be separated from another by clearly negative space. CRF-immunoreactive varicosities were counted irrespective of their size and direct or indirect contact with nNOS-immunoreactive cells. CRF-immunoreactive varicosities less than 1 μ m from a nNOS-immunoreactive cell were considered in functional contact. Results presented are the average number of CRF-immunoreactive varicosities within a 1 μ m perimeter of 30 nNOS-immunoreactive cells in the SPN per animal (n = 3) in each age group (P3, P9, P14, P21, P27, Adult). Twenty spinal cord sections through the rostral-caudal extent of the SPN in the L6-S1 spinal cord were examined. No differences were observed along the rostral-caudal extent of the SPN. Thus, data were pooled from the entire SPN.

2. Percentage of nNOS-Immunoreactive Cell Area Occupied by CRF-

immunoreactive Varicosities in SPN—Images were calibrated, red and green colors were separated, transferred to gray color and thresholded in the same way as described above. The total area (cumulative area) of all CRF-immunoreactive varicosities (on biplanar pictures), localized between a nNOS-immunoreactive cell surface and a 1 μ m distance from cell surface was quantified. The percentage of the nNOS-immunoreactive cell area occupied by CRF-immunoreactive varicosities was then calculated. Results presented are the average percentage area of 30 nNOS-immunoreactive cells occupied by CRF-immunoreactive varicosities in the SPN per animal (n = 3) in each age group (P3, P9, P14, P21, P27, Adult). Twenty spinal cord sections through the rostral-caudal extent of the SPN in the L6-S1 spinal cord were examined. No differences were observed along the rostral-caudal extent of the SPN. Thus, data were pooled from the entire SPN.

3. Overlap of nNOS-Immunoreactive Cells and CRF-Immunoreactive

Varicosities in the SPN—Images were calibrated, red and green colors were separated, transferred to gray scale and thresholded in the same way as described above. A determination of overlap (colocalization) of all CRF-immunoreactive varicosities on a nNOS-immunoreactive cell was made. Data are presented as the average percentage of colocalization from 30 nNOS-immunoreactive cell surfaces that exhibit colocalization with CRF-immunoreactive varicosities (red + green) per animal ($n = 3$) per age group (P3, P9, P14, P21, P27, Adult). Twenty spinal cord sections through the rostral-caudal extent of the SPN in the L6-S1 spinal cord were examined. No differences were observed along the rostral-caudal extent of the SPN. Thus, data were pooled from the entire SPN.

4. CRF-immunoreactive terminal density, terminal size and overlap on nNOS-immunoreactive proximal neurites in the SPN—To measure anatomical apposition of CRF-immunoreactive varicosities to proximal neurites of nNOS-immunoreactive cells in SPN, we used the same 3 measurements (size, density, overlap) that were used for CRF-immunoreactive varicosities apposed to nNOS-immunoreactive cells. In all 3 measurements (size, density, overlap), a 20 μm segment of proximal neurite was used and met the following criteria: the neurite clearly originated from a nNOS-immunoreactive cell body that was localized in the SPN and the neurite was longitudinally sectioned in tissue section. All 3 measurements were performed on 10 proximal neurites of nNOS-immunoreactive cells per animal ($n = 3$) per age group (P3, P9, P14, P21, P27, Adult). Fifteen spinal cord sections through the rostral-caudal extent of the SPN in the L6-S1 spinal cord were examined. No differences were observed along the rostral-caudal extent of the SPN. Thus, data were pooled from the entire SPN.

Statistical Analysis

Animals, processed and analyzed on the same day, were tested as a block in the analysis of variance. Percentage data were arcsin transformed to meet the requirements of this statistical test. Two variables were being tested in the analysis: (1) age and (2) the effect of day (i.e., tissue from different postnatal or adult groups of animals were processed on different days). When F ratios exceeded the critical value ($p \leq 0.05$), the Newman-Keuls test was used for multiple comparisons among experimental means. No differences in CRF- or nNOS-IR in the spinal cord were observed between male or female postnatal rats with immunostaining, densitometry and quantitative confocal analyses. Thus, these data from male and female postnatal rats were pooled. To test for the effect of age on CRF expression (size, density, overlap), linear regression analysis was performed. Results are expressed as mean $\pm$ S.E.M.

Results

CRF-IR in Lumbosacral Spinal Cord

Distribution and General Characteristics

In postnatal (P3-P36) and adult animals, CRF-IR was expressed in distinct regions in lumbosacral spinal levels (L5-S1) (Figure 1). CRF-IR was most intense in the region of the sacral parasympathetic nucleus (SPN) but less intense CRF-IR was also present in the medial and lateral dorsal horn (laminae I–II), dorsal commissure (DCM) and in a small fiber bundle extending ventrally from Lissauer's tract in lamina I along the lateral edge of the dorsal horn into the dorsal part of the SPN (Figure 1, 2). The general location of the CRF-IR bundle in lamina I resembles the central projections of visceral afferents in the pelvic nerve which have been labeled in the rat and cat by axonal transport of horseradish peroxidase and designated the lateral collateral pathway of Lissauer's tract (LCP) (Morgan et al., 1981; Steers et al., 1991; Steers et al., 1996; Steers and de Groat, 1988). CRF immunostaining

in the LCP, DCM and LDH was less intense than that observed for CRF-IR in the SPN regardless of the postnatal age examined (Figure 2). CRF-IR was observed in the ventral horn motoneurons of younger postnatal rats (P0-P7; data not shown) but this staining was not present in older postnatal or adult rats. CRF-IR was primarily expressed in nerve fibers in specific regions of the spinal cord and had a punctate staining quality.

CRF-IR in the SPN, LDH, DCM and LCP

Age Dependence

Densitometry analysis of the CRF-IR in the lumbosacral spinal cord confirmed that the SPN exhibited the most intense CRF-IR of the spinal cord regions examined (SPN, LDH, DCM) (Figure 2) in all postnatal ages examined. Some CRF-immunoreactive fibers projected from the SPN to the region of the dorsal commissure as previously described (Figure 1D,G). CRF-IR in the SPN was observed to form pericellular networks around cells in these regions (Figure 1I-K). We confirmed that CRF-immunoreactive nerve fibers formed pericellular networks around preganglionic parasympathetic neurons identified by neuronal nitric oxide synthase (nNOS)-IR (Figures 3–5)(see below).

CRF-IR in the SPN was observed to increase with postnatal age (P0-P36 and adult) ($R^2 = 0.89$, $p \leq 0.001$)(Figure 2). Little, if any, CRF-IR was present in the SPN at birth (P0) (Figure 2, 4 A, D, J) but CRF-IR continued to increase with age (P3-adult) (Figure 2, 4, C, F, I). By P14, prominent CRF-IR was observed in the SPN of the lumbosacral spinal cord (Figure 4). Dense CRF-IR continued to be observed with increasing age (P21-adult) (Figure 5). CRF-IR in all spinal cord regions examined (LDH, DCM, LCP and SPN) in the lumbosacral spinal cord increased with increasing postnatal age (Figure 2; $R^2 = 0.87-0.98$; $p \leq 0.001$). We then examined the anatomical relationship of CRF-immunoreactive varicosities in the SPN with preganglionic parasympathetic neurons identified by nNOS-IR (Figure 3).

Effect of Spinalization on CRF-IR in the Lumbosacral Spinal Cord

Two to three weeks after complete spinalization at T8-T10, CRF-IR was now absent in the SPN, LCP and DCM regions of the lumbosacral spinal cord of adult rats (Figure 1H,L). Faint CRF-IR in the DH was still observed after complete spinalization (Figure 1H). A few CRF-immunoreactive fibers could still be observed midway between the SPN and DCM but these fibers were only rarely observed after spinalization (Figure 1L).

Numbers of CRF-Immunoreactive Varicosities Apposed to nNOS-Immunoreactive Cells or Neurites in SPN

There was an age dependent increase in the number of CRF-immunoreactive varicosities that were within a 1 μ m perimeter of nNOS-immunoreactive cells ($R^2 = 0.80$; $p \leq 0.05$)(Figure 3 A, B) or proximal neurites ($R^2 = 0.91$; $p \leq 0.01$) in the SPN (Figure 3 C, D). Whereas the number of CRF-immunoreactive varicosities apposed to nNOS-immunoreactive cells plateaued at P27, the numbers of CRF-immunoreactive varicosities apposed to nNOS-immunoreactive neurites continued to increase after P27 and peaked in the adult SPN (32.8 ± 1.2 varicosities)(Figure 6A). The number of CRF-immunoreactive varicosities apposed to nNOS-immunoreactive neurites was significantly greater ($p \leq 0.001$) in adult rats compared to all other postnatal ages examined (Figure 6A). The number of CRF-immunoreactive varicosities apposed to nNOS-immunoreactive cells in the SPN was significantly greater ($p \leq 0.01$) in P27 and adult rats compared to all other postnatal ages examined (Figure 6A).

Overlap of nNOS-Immunoreactive Cell or Neurite and CRF-Immunoreactive Varicosities in the SPN

There was an age dependent increase in the percentage of overlap of the nNOS-immunoreactive cell ($R^2 = 0.97$; $p \leq 0.001$) or neurite ($R^2 = 0.89$; $p \leq 0.02$) with CRF-immunoreactive varicosities in the SPN (Figure 6B). The greatest percentage of overlap between nNOS-immunoreactive cells and CRF-immunoreactive varicosities was $5.5 \pm 0.6\%$ in the adult SPN and this percentage was significantly ($p \leq 0.01$) greater in the adult compared to all other ages examined (P3-P27). The greatest percentage of overlap between nNOS-immunoreactive neurites and CRF-immunoreactive varicosities was $5.3 \pm 2.7\%$ in the adult SPN.

Discussion

We demonstrate several novel findings with respect to CRF-IR in the sacral parasympathetic nucleus (SPN) region of the lumbosacral spinal cord in postnatal and adult rats. CRF-IR was present predominantly in the SPN region although some staining was also observed in the lateral dorsal horn (DH) and dorsal commissure in the lumbosacral spinal cord. CRF-IR in spinal cord regions was age dependent and was largely eliminated by complete spinalization. Double-label immunohistochemistry was combined with quantitative confocal laser scanning microscopy to quantify the number, percentage of preganglionic cell body or proximal neurite occupied and percentage of colocalization between CRF-immunoreactive varicosities and preganglionic cells or proximal neurites in the SPN in postnatal and adult rats. Results demonstrate an age dependent upregulation of CRF-IR in the SPN region and specifically in association with preganglionic parasympathetic neurons identified with neuronal nitric oxide synthase (nNOS)-IR. CRF-immunoreactive varicosities (number, density and colocalization) in close apposition to nNOS-IR cells or proximal neurites also increased with postnatal age. The upregulation of CRF-IR in bulbospinal projections to the SPN may be an anatomical substrate that supports mature voiding reflexes. These data expand upon a preliminary report of a similar upregulation of CRF-IR in the lumbosacral spinal cord in the postnatal rat (Kruse et al., 1990).

In neonates of many species, the neural control of micturition undergoes marked changes during the early postnatal development (de Groat, 1975; de Groat et al., 1993; Maggi et al., 1988; Thor et al., 1990; Thor et al., 1986). In newborn rats and cats, micturition is dependent upon a spinal reflex pathway that is activated when the mother licks the perineal region of the young animal (de Groat and Kruse, 1993; de Groat et al., 1981; Thor et al., 1986). This reflex pathway consists of a somatic afferent limb in the pudendal nerve and a parasympathetic efferent limb in the pelvic nerve. As the central nervous system matures during the first few postnatal weeks, the spinal micturition reflex is gradually replaced by a spinobulbospinal reflex pathway that is responsible for micturition in adult animals (Capek and Jelinek, 1956; de Groat, 1975; de Groat et al., 1993; Thor et al., 1989). The spinobulbospinal micturition reflex is triggered by tension receptor afferents in the bladder and begins to elicit voiding in the rat between P16–18 (Capek and Jelinek, 1956). A study with the transneuronal tracing agent, pseudorabies virus (PRV) (Sugaya et al., 1997), has demonstrated large numbers of PRV-immunoreactive neurons at various brain sites following PRV inoculation of the urinary bladder in P2-P10 rat pups. The most prominent labeling was in the pontine micturition center or Barrington's nucleus. Thus, these studies demonstrate that even in neonatal animals, supraspinal pathways involved in adult micturition reflex patterns exist but may *not* be functional or may be functioning in an *inhibitory* manner prior to the emergence of a functional, adult micturition pattern at P16–18 (Capek and Jelinek, 1956). In this study, we demonstrate that there is an age-dependent upregulation of CRF-IR in the SPN region of the lumbosacral spinal cord that is largely eliminated by complete spinalization. Thus, these data are consistent

with an upregulation of brainstem regulation of spinal micturition pathways that coincides with development of mature voiding reflexes.

Little information exists concerning the developmental switching mechanism(s) that convert the voiding reflex from the neonatal to the adult pattern (Capek and Jelinek, 1956; de Groat, 1975; de Groat et al., 1993; Thor et al., 1989). One hypothesis is that this switching involves a competition between brain and spinal pathways (Araki and de Groat, 1997; de Groat and Araki, 1999; Goodman and Shatz, 1993). Recent electrophysiological studies in spinal cord slice preparations from younger and older neonatal rat pups have demonstrated changes in synaptic efficiency between interneurons located dorsally to the SPN and preganglionic neurons (3, 92, 111) that may contribute to the down-regulation of the perineal-to-bladder reflex during postnatal development. It has been hypothesized (Araki and de Groat, 1997) that these dorsally located interneurons that receive excitatory input from perineal afferents are part of a disynaptic parasympathetic reflex pathway that might be the basis for the perineal-to-bladder reflex in younger neonates. Postnatal maturation decreased the efficiency of dorsal interneuron-to-preganglionic neuron synaptic transmission suggesting that this could account for the down-regulation of the perineal-to-bladder reflex pathway during development (Araki and de Groat, 1997). It has been hypothesized that up-regulation of bulbospinal projections to the SPN region plays a key role in the down-regulation of the perineal-to-bladder reflex due to competition between supraspinal and segmental inputs (de Groat and Araki, 1999). Neuronal connections at developing synapses appear to be refined by competition between multiple synaptic inputs converging on the same target cells (Goodman and Shatz, 1993). Thus, segmental autonomic reflexes may be suppressed by competition from descending bulbospinal projections for synaptic connections to neurons (preganglionic parasympathetic neurons and/or interneurons) in the SPN. The present studies are consistent with an upregulation of brainstem regulation of CRF-IR and expression of CRF-IR in spinal micturition pathways that coincides with development of mature voiding reflexes (Capek and Jelinek, 1956).

Following spinalization rostral to the lumbosacral spinal cord that disrupts descending pathways, elimination reflexes are initially abolished (spinal shock) and then become hyperactive as excitatory bladder reflexes are unmasked in the spinal cord (de Groat et al., 1990; de Groat and Kruse, 1993; de Groat, 1997). This hyperactive response is believed to be due to the reemergence of immature perineal-to-bladder reflex patterns as well as to the facilitation of normally weak or non-functional bladder-to-bladder reflex connections (de Groat et al., 1990; de Groat and Kruse, 1993; de Groat, 1997). The present studies demonstrate that the majority of CRF-IR in the SPN in the adult rat originates from supraspinal sites. In contrast, CRF-IR in the DH was still evident and may indicate that other sources (i.e., afferent cells in the DRG) contribute to the DH staining as well or perhaps, exclusively. The effectiveness of capsaicin treatment in depleting CRF-IR from the DH has been taken as evidence that CRF originates from some primary afferent cells in the DRG (Skofitsch et al., 1984). However, Puder and Pakpa (Puder and Papka, 2001) indicate that no CRF-IR DRG cells have been identified. With the loss of CRF-immunoreactive varicosities in close apposition to preganglionic parasympathetic neurons in the SPN after complete spinalization, the opportunity could then exist for innervation or reinnervation of these cells by alternative sources. One alternative source may include the perineal afferents leading to the upregulation of the perineal-to-bladder reflex and to the facilitation of normally weak or nonfunctional bladder-to-bladder reflex connections after complete spinalization (de Groat et al., 1990; de Groat and Kruse, 1993; de Groat, 1997).

Conflicting roles for CRF in micturition reflex pathways have been described. CRF is prominently expressed in the descending pathway from Barrington's nucleus to the SPN in the lumbosacral spinal cord and prominent CRF-IR is expressed in the SPN of adult rats (Puder and Papka, 2001; Valentino et al., 1996; Valentino et al., 1995). Intrathecal administration of

CRF decreases the amplitude of bladder contractions induced by electrical stimulation of Barrington's nucleus (Pavcovich et al., 1998) suggesting that CRF in descending projections from Barrington's nucleus to the SPN is inhibitory. On the other hand, a preliminary report has now suggested that intrathecal or systemic CRF induces bladder overactivity (Klausner and Steers, 2004). Different putative roles for CRF in these studies may be related to the use of halothane-anesthetized (Pavcovich et al., 1998) or conscious (Klausner and Steers, 2004) rats for functional studies. Klausner and Steers (Klausner and Steers, 2004) suggest that CRF's role in voiding function and dysfunction is related to a central stress response and may underlie bladder overactivity in the clinical syndrome of interstitial cystitis. On the other hand, Pavcovich and Valentino (Pavcovich et al., 1998) suggest that CRF's role in the voiding reflex is in maintaining continence. The present study does nothing to resolve this controversy but the age-dependent upregulation of CRF-IR in the SPN is more consistent with a role in maintaining continence as the mature voiding reflex must now alternate between two modes of operation, voiding and storage. On the other hand, there is gene expression data from the mouse that demonstrates upregulation of CRF in the urinary bladder after lipopolysaccharide, substance P or antigen sensitization (Saban et al., 2002). Clearly more functional studies are needed to define the role of CRF in normal bladder function and dysfunction.

In the present study, CRF-IR was in the greatest density in the SPN region in all postnatal rat ages examined. The CRF-immunoreactive varicosities also demonstrated an age-dependent increase in number, percent area occupied and percent overlap between nNOS-immunoreactive cells or proximal neurites. We interpret these data to reflect the potential for axosomatic and axodendritic synapses between CRF-immunoreactive varicosities and nNOS-immunoreactive cells and neurites in the SPN region. Although we have previously demonstrated that all nNOS-immunoreactive cells in the SPN also exhibited Fluorogold after retrograde transport from the major pelvic ganglion (MPG) (Vizzard et al., 1995a) and are therefore preganglionic neurons, we do not know which preganglionic neurons receive innervation from CRF-immunoreactive varicosities in the present study. There is considerable overlap in the distribution of lower urinary tract-, reproductive- and gastrointestinal-related preganglionic neurons in the SPN of the lumbosacral spinal cord. Thus, preganglionic neurons that innervate postganglionic urinary bladder (Marson, 1997; Nadelhaft and Vera, 1995), colon (Vizzard et al., 2000a), or urethral cells in the MPG are all possibilities as are reproductive related preganglionic neurons (Marson et al., 1993; Puder and Papka, 2001).

The mechanism(s) underlying the changes in CRF expression in the SPN region of the postnatal rat in the present study and the development of mature voiding reflexes are not known. Previous studies have suggested that maturation of peptidergic afferent pathways in the urinary bladder of the neonatal rat may be involved (Maggi et al., 1988). However, it has also been demonstrated that regional noradrenergic and cholinergic neurochemistry of the rat urinary bladder is relatively stable as the rat matures (Johnson et al., 1988). Thus, maturation of peptidergic afferent pathways may play a greater role in the maturation of micturition reflexes compared to adrenergic and cholinergic systems. Maturation of central pathways is also likely to be a factor in the postnatal development of bladder reflexes. Serotonin (Bregman, 1987), enkephalins (Fitzgerald, 1991), somatostatin (Marti et al., 1987), NADPH-diaphorase and nNOS (Vizzard et al., 1994) appear or increase in prominence in the lumbosacral spinal cord during the second to third postnatal week.

In conclusion, the age dependent upregulation of CRF-IR in the SPN region and increase in number, area of overlap and percent colocalization of CRF-immunoreactive varicosities and preganglionic neurons may play a role in the development of mature voiding reflexes during the second to third postnatal weeks. The role of CRF in voiding function and dysfunction remains to be defined.

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Figure 1.

Fluorescence photographs of corticotropin releasing factor (CRF)-immunoreactivity (IR) in lumbar (L6-S1) spinal cord of postnatal (P) (A-F) and adult (G) rats. Expression of CRF-IR at birth (P0)(A) and at P3(B) was not observed in the spinal cord. Dotted line in A, B outlines the edge of the grey matter that is difficult to discern in young, postnatal ages. CRF-IR was present at P3(C) primarily in the region of the sacral parasympathetic nucleus (SPN) although faint CRF-IR was present in the superficial dorsal horn (DH). With increasing postnatal age (P14, P21, P36, adult), increased CRF-IR was observed in the SPN region (E-G, I-K) and in the superficial DH, dorsal commissure (DCM; D,G) and in the lateral collateral pathway of Lissauer (LCP)(J, K; arrows). Higher power photographs of CRF-IR (E-G; regions in white box) in the SPN at P21 (I), P36 (J) and in the adult rat (K) are shown. CRF-IR was observed to make pericellular baskets around cells in the SPN region. Complete spinalization at T8-T10, eliminated the majority of CRF-IR in the SPN region (H, L), some faint CRF-IR fibers were occasionally observed projecting from the SPN to the region of the DCM (L). Faint CRF-IR was still observed in the superficial DH after complete spinalization (H). CC, central canal. Calibration bar represents 80 μ m in A-E, G, H, 100 μ m in F, 40 μ m in I, K, L and 50 μ m in J.

Figure 2. Histogram summarizing changes in CRF staining density (O.D., optical density) in specific regions of the L6-S1 spinal cord with increasing postnatal (P) age (P0-Adult). The spinal cord inset depicts the areas analyzed: lateral dorsal horn (LDH), lateral collateral pathway of Lissauer (LCP), sacral parasympathetic nucleus (SPN), dorsal commissure (DCM). The density of CRF-IR within each region analyzed was correlated with increasing postnatal age ($R^2 = 0.87-0.98$; $p \leq 0.001$). At each age examined, CRF-IR in the SPN was significantly greater than all other regions. *, $p \leq 0.001$.

Figure 3. Pictorial representation of the methods used to quantify CRF-immunoreactive varicosities (red, A, B; yellow, C, D) on neuronal nitric oxide synthase (nNOS)-immunoreactive cells (green, A, B) (presumptive preganglionic neurons) in the region of the sacral parasympathetic nucleus. CRF-immunoreactive varicosities on or within a 1 µm perimeter (A-D, blue dashed lines) of a selected nNOS-immunoreactive cell (A, C) or a nNOS-immunoreactive proximal neurite (B, D) were thresholded (yellow, C, D) and quantified (number, area occupied and colocalization) as detailed in the methods section. Calibration bar represents 5 µm.

Figure 4.

Fluorescence images of CRF-IR surrounding nNOS-IR (presumptive preganglionic parasympathetic neurons) in the sacral parasympathetic nucleus (SPN) in postnatal (P)3 (A, D, G, J), P7 (B, E, H, K) and P14 (C, F, I, L) rat pups. CRF-IR in the SPN region (A-F; CRF-IR, red; nNOS-IR, green) increased with increasing postnatal age and CRF-IR in close apposition to nNOS-immunoreactive cells also increased with increasing postnatal age (thresholded images of CRF-immunoreactive varicosities, G-I, yellow; thresholded images of nNOS-immunoreactive cells, J-L, yellow). Higher power images of the SPN region (D-F) showing increasing CRF-IR (red) in the SPN and in association with nNOS-immunoreactive cells (green). CRF-IR and nNOS-IR was thresholded separately as described in the methods section and only CRF- or nNOS-IR that exceeded threshold (G-L, yellow) was quantified. Only nNOS-immunoreactive preganglionic parasympathetic cells that exceeded threshold (J-L)

were used to determine CRF-immunoreactive varicosity measurements (number, density, colocalization) in association with nNOS-immunoreactive cells (J-L, yellow). Calibration bar represents 40 μm in A-C and 20 μm in D-L.

Figure 5.

Fluorescence images of CRF-IR surrounding nNOS-IR (presumptive preganglionic parasympathetic neurons) in the sacral parasympathetic nucleus (SPN) in postnatal (P)21 (A, D, G, J), P28 (B, E, H, K) rat pups and Adult (C, F, I, L) rat. CRF-IR in the SPN region (A-F; CRF-IR, red; nNOS-IR, green) increased with increasing postnatal age and CRF-IR in close apposition to nNOS-IR cells also increased with increasing postnatal age (thresholded images of CRF-immunoreactive varicosities, G-I, yellow; thresholded images of nNOS-IR cells, J-L, yellow). Higher power images of the SPN region (D-F) showing increasing CRF-IR (red) in the SPN and in association with nNOS-immunoreactive cells (green). CRF-IR and nNOS-IR was thresholded separately as described in the methods section and only CRF- or nNOS-IR that exceeded threshold (G-L, yellow) was quantified. Only nNOS-immunoreactive preganglionic parasympathetic cells that exceeded threshold (J-L) were used to determine

CRF-immunoreactive varicosity measurements (number, density, colocalization) in association with nNOS-immunoreactive cells (J-L, yellow). Calibration bar represents 40 μm in A-C and 20 μm in D-L.

Figure 6.

Summary histogram of the average number of CRF-immunoreactive varicosities on or within a 1 μ m distance from nNOS-immunoreactive cell bodies or proximal neurites in the sacral parasympathetic nucleus (presumptive preganglionic parasympathetic neurons). There was an age dependent increase in the numbers of CRF-immunoreactive varicosities closely apposed to nNOS-immunoreactive cells ($R^2 = 0.80$; $p \leq 0.05$) or proximal neurites ($R^2 = 0.91$; $p \leq 0.01$). The number of CRF-immunoreactive varicosities in close apposition to nNOS-immunoreactive cell bodies at P27 and in the adult rat was significantly ($p \leq 0.01$) greater than other ages examined (P3-P21). The number of CRF-immunoreactive varicosities in close apposition to nNOS-immunoreactive proximal neurites in the adult rat was significantly ($p \leq 0.001$) greater than all other ages examined (P3-P27). #, $p \leq 0.01$; *, $p \leq 0.001$.

Table 1

Table of primary and secondary antibodies, working dilutions and sources used.

Primary Antibody	Source	Working Dilution	Secondary Antibody	Source	Working Dilution
CRF	Phoenix Pharmaceuticals Inc. Belmont, CA	1:3000	Cy3-goat anti-rabbit	Jackson ImmunoResearch Laboratories, Inc., West Grove, PA	1:500
nNOS	Santa Cruz Biotechnology, Inc. Santa Cruz, CA	1:100	Cy2-goat anti-mouse	Jackson ImmunoResearch Laboratories, Inc., West Grove, PA	1:50