

Postnatal development of the gastrin-releasing peptide system in the lumbosacral spinal cord controlling male reproductive function in rats

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Abstract: A sexually dimorphic spinal gastrin-releasing peptide (GRP) system in the lumbosacral spinal cord, which projects to the lower spinal centers, controls erection and ejaculation in rats. However, little is known about the postnatal development of this system. In this study, we therefore examined the postnatal development of the male-dominant spinal GRP system and its sexual differentiation in rats using immunohistochemistry. Our results show that male-dominant expression of GRP is prominent from the onset of puberty and that sexually dimorphism persists into adulthood. These results suggest that androgen surge during male puberty plays an important role in the development and maintenance of the male-specific GRP function in the rat spinal cord.

Keywords: androgens, gastrin-releasing peptide, sexual differentiation, male sexual behavior, spinal cord, puberty

Introduction

During perinatal life in mammals, androgens such as testosterone (T) induce external and internal genitalia to develop into a masculine form and also masculinize the developing brain and spinal cord. The spinal nucleus of the bulbocavernosus (SNB) is a male-specific, sexually dimorphic nucleus in the lumbosacral spinal cord (L5–S1 level) that innervates perineal striated muscles attached to the base of the penis.^{1)–3)} The sexual dimorphism is caused by sex differences in perinatal androgen exposure.^{1)–3)} Recent research has demonstrated that pubertal sex hormones also organize sex differentiation in brain

functions for sexual behavior in adulthood.⁴⁾ These results in a permanent masculinization of neural populations and synaptic connections underlying male sexual behavior.^{5)–7)} In adulthood, T activates these neural areas including several brain areas and spinal nuclei. Thus, T influences the brain and spinal cord *via* organizational (perinatal) and activational (adulthood) effects.⁸⁾ In the SNB neuromuscular system, sexual dimorphism in the spinal cord is organized through an androgen receptor-mediated mechanism.⁵⁾ Neurons located dorsolateral to the central canal in lamina X within the third and fourth lumbar spinal cord project to the thalamic region of the brain.^{9),10)} These so-called lumbar spinothalamic neurons are sexually dimorphic, with males possessing greater number than females.^{9),10)}

Truitt and Coolen¹¹⁾ demonstrated that a population of the lumbar spinothalamic neurons acts as a “spinal ejaculation generator” because specific toxin treatments that selectively lesion these galanin-positive neurons completely eliminate ejaculation in rats. Independently, we demonstrated that the gastrin-releasing peptide (GRP) system in the lumbosacral segments controls spinal centers promoting penile erection and ejaculation during copulatory behavior in male rats.¹²⁾ It has also been demonstrated that GRP neurons in the lumbar spinal

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Abbreviations: ANOVA: analysis of variance; DGC: dorsal gray commissure; DH: dorsal horn; GRP: gastrin-releasing peptide; PND: postnatal day; SNB: spinal nucleus of the bulbocavernosus; SPN: sacral parasympathetic nucleus; T: testosterone.

region (L3–L4 level) are more numerous in males than in females, revealing a novel sexual dimorphism in the spinal cord.¹²⁾ GRP neurons project axons into the more caudal lumbosacral spinal cord (L5–S1 level), forming synaptic contacts on the sacral parasympathetic nucleus (SPN) and SNB.^{13)–15)} These nuclei are also known to control erection and ejaculation in an androgen-dependent manner in adulthood.^{11),16),17)} Considering these findings, it appears likely that a population of the lumbar spinothalamic neurons, at least partly, expresses both galanin and GRP.¹²⁾ Moreover, GRP is a possible candidate for neurotransmitters involved in the modulation of lumbosacral spinal activity, generating ejaculation at the moment of male orgasm.¹²⁾

Little is known about the postnatal development of this sexually dimorphic spinal GRP system in relation to puberty. In this study, using immunohistochemistry, we examined the postnatal development of the male-dominant GRP system in the lumbosacral spinal cord controlling male reproductive function and its sexual differentiation around puberty in rats.

Materials and methods

Animals. Male and female rats of the Wistar strain (Charles River Japan, Yokohama, Japan) were used on postnatal day (PND) 16, 23, 30, 37, 44, or 70 ($n = 5$, of each sex, respectively). All rats were maintained on a 12-h light/dark cycle and provided with unlimited access to water and rodent chow. All experimental procedures have been authorized by the Committee for Animal Research, Okayama University, Japan.

Tissue preparation. Rats were overdosed with sodium pentobarbital (100 mg/kg body weight) and perfused *via* the left ventricle with 10–100 ml of physiological saline followed by 20–200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The amount of fixative and its duration were determined in proportion to body size. Subsequently, spinal cords were immediately removed and post-fixed in the same fixative for 3 h at room temperature.

Immunohistochemistry. Immunohistochemistry for GRP was performed as previously described.^{12),18),19)} In brief, cryosections (30 μm in thickness) were prepared using a cryostat (CM3050 S, Leica, Nussloch, Germany). After blocking nonspecific binding, the sections were incubated with the primary rabbit antiserum against GRP (1:2,000 dilution) (11081; AssayPro, St. Charles, MO, USA). Immunoreactive products were detected

with a streptavidin-biotin kit (Nichirei, Tokyo, Japan), followed by 3,3'-diaminobenzidine (Dojindo, Kumamoto, Japan) development. To determine the intensity of GRP-immunoreactivity in fibers in the lower lumbosacral spinal cord (L5–S1 level), at least 10 cross sections (30- μm thick) per animal were randomly selected, and the digital images of three regions [the SPN, dorsal gray commissure (DGC), and dorsal horn (DH)] were prepared (magnification, $\times 200$ per section). The unit area ($343 \times 469 \mu\text{m}^2$) was analyzed to localize the nuclei at the center of each area. The optical density of GRP staining was analyzed using ImageJ software (ImageJ 1.44p; National Institutes of Health, Bethesda, MD, USA) (see Fig. 1) according to our established methods.^{12),18),20)} Briefly, each threshold optical density was determined by normalizing the data to those of the (preabsorbed negative) control sections. The GRP-positive-fiber pixel density was quantitated as the average pixel density in the SPN, DGC, and DH of each animal, and the data were expressed as the ratio of each to the density of the DH in males on PND 70. All micrographs were coded and evaluated without the knowledge of sexes and PNDs, and the code was not broken until the analysis was complete.

Immunofluorescence staining for GRP in the upper lumbosacral spinal cord (L3–L4 level) was performed as described above using horizontal sections (at least $n = 5$ on each sex and PND) to count the number of GRP-positive neurons per animal as described previously.^{12),18),20)} In brief, digital microphotographs were processed with Adobe Photoshop computer software (Adobe Systems, San Jose, CA, USA) at 300 dots per inch resolution. We counted the number of GRP-positive cell bodies at $\times 200$ magnification in all sections and analyzed a 600- μm^2 area (see Fig. 1). GRP neurons were identified by their following characteristics: densely immunostained, anatomical localization (mainly dorsal, dorsolateral, or both to the central canal in lamina X of lumbar segments III–IV), relatively large cell bodies (diameters approximately 20–30 μm), and clear round nuclei (diameters approximately 10–15 μm). To avoid overestimating cell number, only GRP-positive neurons that contained a round, transected nucleus were counted. Because the mean diameter of the nuclei in the GRP neurons is much smaller than the 30- μm thick sections, this analysis reduced the overestimation of the number of neurons. All micrographs were coded and evaluated without the knowledge of sexes and PNDs, and the code was

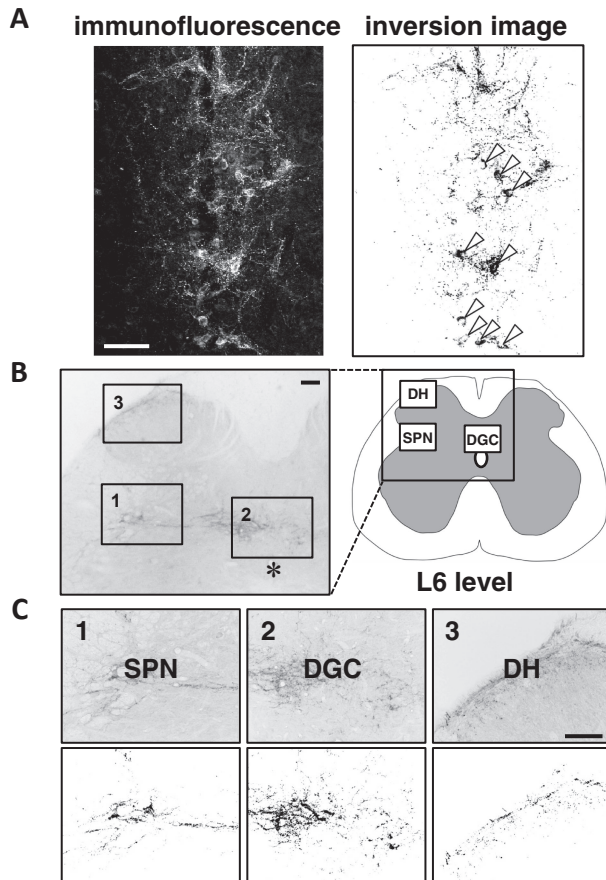


Fig. 1. Quantification analysis of the number of gastrin-releasing peptide (GRP)-positive neurons and the intensity of GRP-immunoreactivity in fibers in the spinal cord. (A) A representative image of immunofluorescence for GRP (left panel). Using the Adobe Photoshop computer software, the image was inverted to a bright field (inversion) image (right panel). (B) A schematic drawing of the lower lumbar spinal cord, indicating the intraspinal location of the sacral parasympathetic nucleus (SPN) (B1), dorsal gray commissure (DGC) (B2), and the dorsal horn (DH) (B3) at the lumbar spinal cord level (L6). The black squares (B1–3) are enlarged in (C). These higher magnification images show the distribution of GRP-immunoreactive fibers in the SPN (C1), DGC (C2), and DH (C3). The lower panels in (C) show the black-and-white images with a set threshold level corresponding to each upper panel. Scale bars = 100 μm (applies to all). *Asterisk marks the central canal.

not broken until the analysis was complete. Alexa Fluor 488-linked anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) was used for detection at a 1:1,000 dilution. Immunostained sections were imaged with a confocal laser scanning microscope (FluoView 1000, Olympus, Tokyo, Japan).

Statistics. The number of GRP-positive neurons in the lumbar spinal cord was reported as the mean \pm SEM in each group of animals. The

differences between sexes and PNDs were assessed statistically using a two-way analysis of variance (ANOVA) (expressed as mean \pm SEM). Statistical analyses of the number of GRP-positive neurons and the optical density of GRP-positive fibers (expressed as mean \pm SEM) were performed using a one-way ANOVA. When significant main effects were found using an ANOVA, *post hoc* Tukey's tests were performed.

Results

Figure 1 shows the analytical protocol for the morphological quantification used in this study. Using immunohistochemistry for GRP, we first examined developmental changes in the number of GRP-positive neurons in the upper lumbar spinal cord (L3–L4 level) at different ages, especially before and around puberty (PND 16, 23, 30, 37, 44, and 70) (Fig. 2). Most GRP-positive somata and arborizations were greater in males relative to females in all experimental periods examined (Fig. 2). Interestingly, on PND 30 and 37, GRP-positive neurons were hardly detected in females, and very few were detected in females on PND 44 (Fig. 2).

Quantification analysis showed that, on PND 16, 30, 37, 44, and 70, the number of GRP-positive neurons was significantly greater in males than in females [$F(1,48) = 793.3$, $P < 0.001$] (Fig. 3). In contrast, on PND 23, no significant difference was found in the number of GRP-positive neurons between sexes (Fig. 3). In males, the number of GRP-positive neurons was much greater on PND 70 than on PND 16, although the number of GRP-positive neurons was fewer on PND 30 than on PND 16 [$F(5,48) = 38.19$, $P < 0.05$] (Fig. 3). In females, the number of GRP-positive neurons was greater on PND 23 than on PND 16 ($P < 0.01$) (Fig. 3).

In the lumbosacral spinal cord (L5–S1 level) of males, the immunohistochemistry for GRP demonstrated that the intensities of GRP-positive fibers both in the SPN [$F(5,24) = 24.55$] and DGC [$F(5,24) = 17.42$] were significantly higher on PND 44 and 70 than on PND 16 ($P < 0.05$) (Fig. 4A). However, in the somatic sensory layers of the DH, a slightly lower intensity of GRP-positive fibers was observed on PND 37 relative to PND 16 [$F(5,24) = 3.770$, $P < 0.05$] (Fig. 4A). In females, the intensities of GRP-positive fibers in the SPN were greater on PND 23, 44, and 70 than on PND 16 [$F(5,24) = 7.921$, $P < 0.05$] (Fig. 4B). In the DGC, the intensity of GRP-positive fibers was greater on PND 23, 44, and 70 than on PND 16 [$F(5,24) = 15.62$, $P < 0.05$]

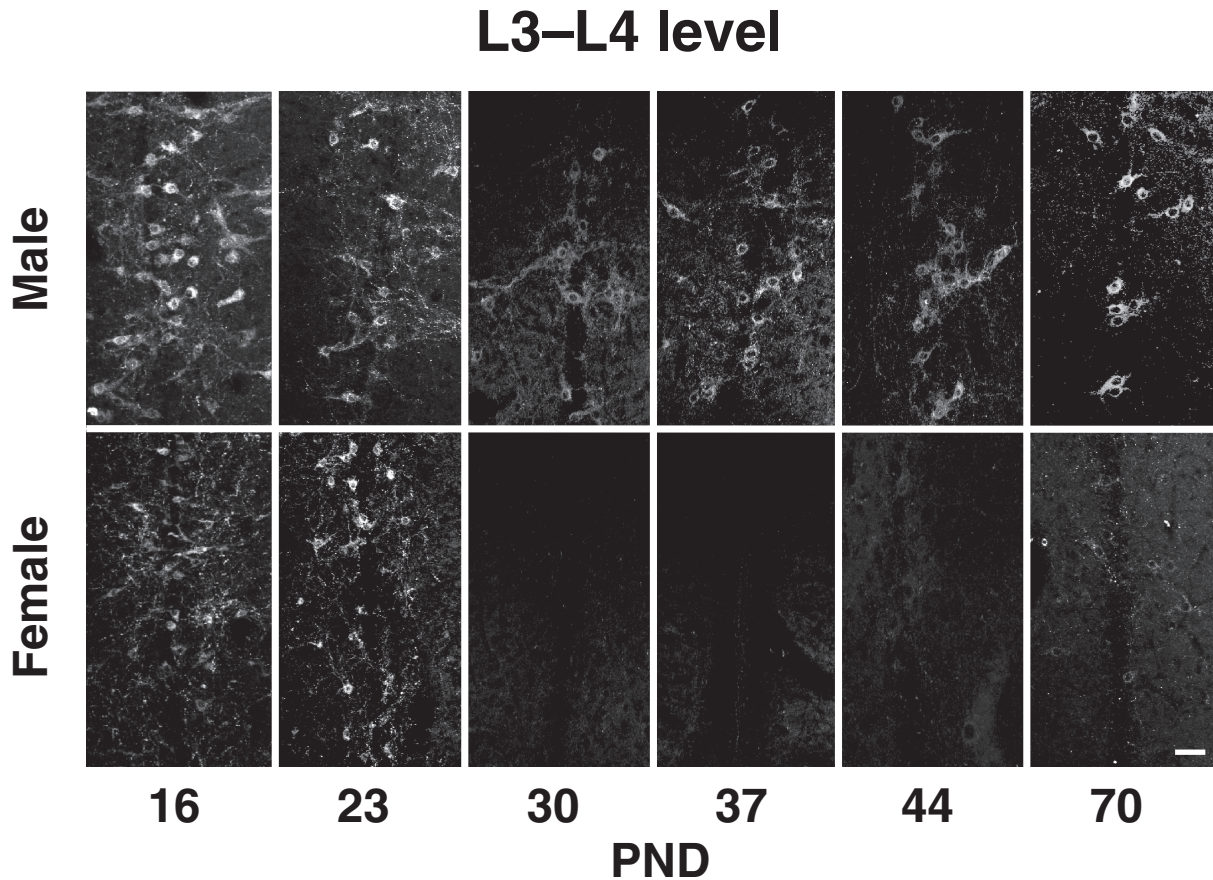


Fig. 2. Representative photomicrographs of developmental changes and sex differences in gastrin-releasing peptide (GRP)-immunoreactivity analysis in the upper lumbosacral spinal cord (L3–L4 level) on postnatal day (PND) 16, 23, 30, 37, 44, and 70. Scale bar = 50 μm (applies to all).

(Fig. 4B). In the DH, no significant difference was detected in the intensity of GRP-positive fibers in females at any time point [$F(5,24) = 2.484$] (Fig. 4B). Collectively, our findings showed that expression levels of GRP were lower in females than in males at all time-points in the SPN and DCG, but were comparable in the DH (Fig. 4). These expression patterns and the intensity of GRP-positive fibers in the SPN, DGC, and DH are in keeping with previous studies in adult male and female rats.^{12),18)}

Discussion

While we previously demonstrated the male-dominant sexual dimorphism of the spinal GRP system which plays an important role in the male reproductive function in adult rats,¹²⁾ little is known about the developmental changes in the spinal center during ontogeny. In this study, we investigated this system at several stages from pre-puberty to post-

puberty (sexual maturation) to assess the effects of the escalated circulation sex steroids during puberty, and compared the development of the spinal GRP system between sexes. Only on PND 23, which is considered to be pre-puberty, did we not find a sex difference in the number of GRP neurons. These results suggest that male-dominant expression of GRP is prominent from the onset of puberty, and that this sexual dimorphism persists into adulthood (PND 70). The PND 60 male rats (post-puberty) displayed significantly higher levels of plasma T than PND 30 male rats (pre-puberty),²¹⁾ suggesting that androgens begin to rise during puberty. Thus, androgens appear to be a likely contributor to changes in the expression of GRP during puberty.

The GRP system in the spinal cord shows sexual dimorphism, even in PND 16, suggesting that the GRP system is organized by sex steroids during the perinatal critical period, similar to the SNB neuro-

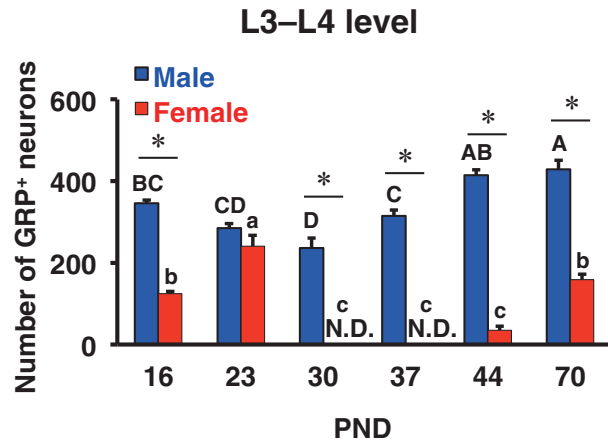


Fig. 3. Quantitative analyses of developmental changes in the number of gastrin-releasing peptide (GRP) neurons in the upper lumbar spinal cord (L3-L4 level) of male and female rats. *Asterisks indicate the sex differences in each postnatal day (PND) ($P < 0.001$). Letters denote differences among means ($P < 0.05$); means with the same letter (capital letters in males; small letters in females) are not significantly different. N.D.: not detectable.

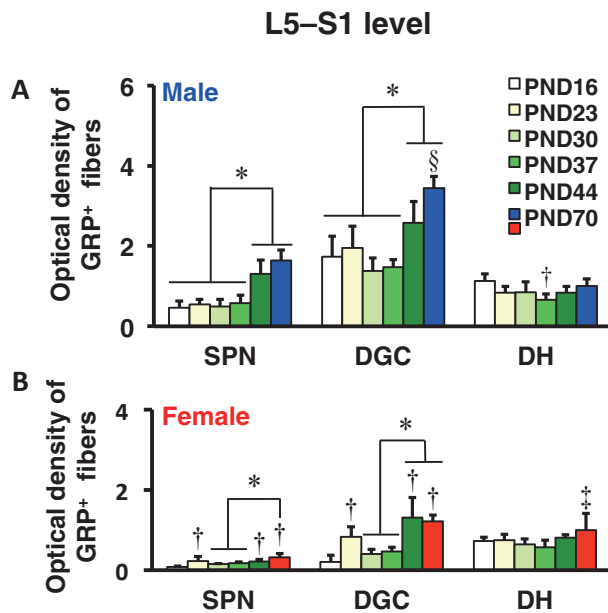


Fig. 4. Quantitative analyses of developmental changes in the intensity of gastrin-releasing peptide (GRP)-positive fibers in the sacral parasympathetic nucleus (SPN), dorsal gray commissure (DGC), and dorsal horn (DH) in the lower lumbar and upper sacral spinal cord (L5-S1 level) of male (A) and female (B) rats. * $P < 0.05$, between postnatal days (PNDs); † $P < 0.05$, vs. PND 16; ‡ $P < 0.05$, vs. PND 37; § $P < 0.05$, vs. PND 44.

muscular system.¹⁾⁻³⁾ The number of GRP-positive neurons in females increased from PND 16 to 23, and no difference in the number of GRP neurons between

sexes was found at PND 23. Levels then rose again by PND 70 after having dropped to non-detectable levels in females. It appears as though the number of GRP-immunoreactive neurons decreased in males compared with PND 16, whereas it increased in females. These results suggest two different possibilities: the change in numbers was due to changes in number of cells or in the expression of GRP in the cells. We further found that the number of GRP neurons in the lumbar spinal cord markedly increased from PND 30 onward. Because sex-related endocrine changes take place during puberty in rats,²¹⁾ PND 30 might be the approximate age of puberty onset in male rats. Circulating T levels also significantly increase in post-pubertal (~PND 60) compared with those in pre-pubertal (~PND 30) male rats.²¹⁾ Our results demonstrate for the first time that the spinal GRP system shows a remarkable male-dominant sexual dimorphism during puberty at the level of the spinal neural circuit, when plasma T levels are dramatically increased. This suggests that the T increase during puberty establishes the male-specific spinal GRP system by upregulating the expression of GRP in cells in the lumbar spinal cord.

From PND 30 onward in females, we observed that few GRP-positive cell bodies were detected and that GRP staining was not intense. Vaginal opening occurs around PND 33 in rats,²²⁾ suggesting that this is the age of puberty onset in females. Circulating estrogen levels markedly increase around the time of vaginal opening in female rats.²¹⁾ Interestingly, our present results showed that, in females, GRP-positive neurons are barely detected on PND 30 and 37 when circulating estrogen levels are high. While circulating T levels during male puberty significantly increased the GRP expression in the lumbar spinal cord, in females, the higher circulating estrogen levels during puberty might attenuate GRP expression. Thus, estrogens may play an important role in the feminization of the male-specific spinal GRP system, possibly by inhibiting the expression of GRP in the lumbar spinal cord during female puberty. On the other hand, there is evidence that circulating estrogen levels are very high in both male and female rats (and do not differ) up until about 6 weeks of age,²³⁾ so the observed decrease in females and increase in males during the same period are unlikely to be estrogen-mediated. Notwithstanding such a discrepancy, GRP neurons in the spinal cord of female rats can again be detected in adulthood, although the number is still lower than that in males.

Therefore, it is suggested that the initiation of the estrous cycle in females might influence the expression of GRP in the rat spinal cord. Further studies examining the effects of estrogens (*e.g.*, estradiol-17 β) are needed to draw a firm conclusion.

The expression of GRP in the spinal DH is considered to be involved in the transmission of the itch sensation.^{19),24)–26)} In this study, the expression of GRP in the DH did not significantly change during ontogeny. We further found that the two GRP systems in the spinal cord, sexual and somatosensory, differ in their sensitivity to androgens, the former being sensitive to androgens and the latter being insensitive. A possible explanation may be the presence of androgen receptors in the GRP neurons of the sexual system with an absence of such receptors in the somatosensory system.

Competing interests

The authors declare that they have no competing interests.

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