

Published in final edited form as:

Nat Neurosci. 2008 June ; 11(6): 634–636. doi:10.1038/nn.2126.

Sexually dimorphic gastrin releasing peptide system in the spinal cord controls male reproductive functions

Hirota¹, Ken-Ichi Matsuda¹, Damian G Zuloaga², Hisayuki Hongu¹, Etsuko Wada³, Keiji Wada³, Cynthia L Jordan², S Marc Breedlove², and Mitsuhiro Kawata¹

¹ Department of Anatomy and Neurobiology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan

² Program in Neuroscience, Departments of Psychology and Zoology, Michigan State University, East Lansing, Michigan 48824-1101, USA

³ Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan

Abstract

Neurons in the upper lumbar spinal cord project axons containing gastrin-releasing peptide (GRP) to innervate lower lumbar regions controlling erection and ejaculation. This system is vestigial in female rats and in males with genetic dysfunction of androgen receptors, but in male rats, pharmacological stimulation of spinal GRP receptors restores penile reflexes and ejaculation after castration. GRP offers new avenues for understanding potential therapeutic approaches to masculine reproductive dysfunction.

GRP, a member of the bombesin-like peptide family¹, is distributed widely in the central nervous system and gastrointestinal tract of mammals^{2,3}. GRP and neuromedin B (NMB), the mammalian counterpart of bombesin, play a role in many physiological processes, including itch⁴, circadian rhythms⁵, food intake⁶ and fear memory consolidation^{7,8}. In mammals, bombesin-like peptides act through a family of at least three G protein-coupled receptors: GRP-preferring receptor (GRP-R), NMB-preferring receptor (NMB-R) and bombesin receptor subtype-3 (BRS-3)⁹.

Using immunocytochemistry (ICC) directed at GRP, we found a group of neurons within a region previously dubbed the ‘spinal ejaculation generator’ because toxins that selectively lesion galanin-containing neurons there virtually eliminate ejaculation in rats¹⁰. These galaninergic neurons project to the thalamus¹⁰, but it had been unclear whether there are also direct connections between this center and the lower spinal cord regions that directly trigger ejaculation¹¹. The separate, GRP-containing neurons that we found within the center projected axons to more caudal spinal regions and were much more prominent in wild-type (WT) males than in WT females (Fig. 1a,b; Supplementary Fig. 1 online) ($n = 5$, $F_{2,12} = 299.9$, $P < 0.001$). Semiquantitative reverse transcription (RT)-PCR confirmed more pre-pro

Correspondence should be addressed to H.S. (hsakamo@koto.kpu-m.ac.jp).

AUTHOR CONTRIBUTIONS

H.S., K.-I.M. and M.K. designed experiments and conducted data analysis. H.S. and H.H. performed histological experiments. H.S. and K.-I.M. performed molecular experiments. H.S. and D.G.Z. performed behavioral experiments. C.L.J., S.M.B., E.W. and K.W. provided Tfm rats, advice, antibodies and equipment. H.S., S.M.B. and M.K. wrote the paper. All authors discussed the results and commented on the manuscript.

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions>

Note: Supplementary information is available on the Nature Neuroscience website.

Grp transcripts in this region of males than of females (Supplementary Fig. 2 online). To test whether androgen receptors direct sexual dimorphism of these neurons, we examined genetically male (XY) Long-Evans rats carrying the testicular feminization mutation (Tfm) of the androgen receptor gene *Ar*. These rats develop testes embryologically and secrete testosterone pre- and postnatally but, because their androgen receptor protein is dysfunctional, develop a wholly feminine exterior, including a clitoris rather than a penis. The spinal cord of Tfm rats was hyperfeminine, having even fewer GRP-positive neurons in this region than did WT females ($P < 0.001$) (Fig. 1c,d). In normal males, GRP-expressing neurons also expressed androgen receptor ($96.1 \pm 1.7\%$; $n = 4$ WT males), but not estrogen receptor alpha (ER α) (Fig. 1e-h). Because androgens such as testosterone augment ejaculation in male rats and humans¹², the presence of androgen receptor in the GRP-positive neurons of the ejaculation center offers a locus for androgenic modulation of ejaculation and other sexual reflexes.

ICC revealed that these GRP neurons in the upper lumbar spinal cord projected their axons to the lower lumbar and upper sacral spinal cord, presenting a sexually dimorphic pattern of fibers innervating autonomic centers that regulate male sexual reflexes¹¹. These fibers were much more prominent in male rats than in females in the sacral parasympathetic nucleus (SPN) ($n = 5$, $F_{2,12} = 42.2$, $P < 0.001$) and the dorsal gray commissure (DGC) surrounding the central canal ($F_{2,12} = 53.9$, $P < 0.001$) (Fig. 1i-l), both of which provide autonomic preganglionic fibers to genitalia¹³. Mirroring the results in the upper spinal cord, the lumbosacral cords of Tfm rats showed an entirely feminine pattern of GRP⁺ fibers in the SPN autonomic nucleus and a hyperfeminine pattern in the DGC ($P < 0.001$) (Fig. 1i-l). Double ICC confirmed that GRP-containing fibers surrounded neurons expressing neuronal nitric oxide synthase (nNOS; Fig. 1m-u), a marker for autonomic preganglionic neurons¹⁴ and an enzyme involved in erection and ejaculation¹². Electron micrography demonstrated GRP-immunoreactive presynaptic boutons innervating nNOS-positive dendrites in these autonomic nuclei of the lumbosacral spinal cord (Fig. 1v,w). In males, GRP-immunoreactive fibers also extended to the spinal nucleus of the bulbocavernosus (Supplementary Fig. 3 online), which innervates striated muscles attached to the penis and is important in copulatory behavior. There was no sex difference in GRP fibers in the dorsal horn ($F_{2,12} = 0.8$) (see Fig. 1l), which presumably processes non-autonomic sensory stimuli such as itch⁴.

Castration of adult male rats reduced the intensity of GRP-immunoreactive fibers in the SPN ($n = 4$, $F_{2,9} = 15.6$, $*P = 0.001$, $\dagger P < 0.001$) and DGC ($F_{2,9} = 10.1$, $*P = 0.003$, $\dagger P = 0.004$) (but not in the dorsal horn; $F_{2,9} = 0.3$) of the lower spinal cord 28 d later, a reduction that was averted by androgen replacement (Supplementary Table 1 online). Ovariectomy had no effect on GRP expression in females, and androgen treatment of ovariectomized females increased GRP-immunoreactive fibers to only a modest degree (Supplementary Table 1). These results suggest that the GRP spinal cord system is masculinized by perinatal androgen yet retains some androgen responsiveness in adulthood.

We probed the presence of GRP-R in the lower spinal cord by binding of GRP, RT-PCR, and double ICC, finding heavy concentration of GRP-R on the nNOS-positive neurons of the lumbosacral autonomic nuclei ($n = 4$ in each experiment) (Fig. 2). Furthermore, the rat homolog of GRP-R agonists (rat GRP₂₀₋₂₉)_{4,6}, which had no effect on pain perception in tail-flick tests ($n = 8$, $F_{4,28} = 0.8$) (Supplementary Fig. 4 online), restored many of the spinal reflexes of the penis that were lost after castration ($n = 10$; simple erections, $F_{4,36} = 34.0$, $P < 0.001$ compared with saline-injected intact males, $P < 0.018$ compared with saline-injected castrated males; dorsal flips of the penis, $F_{4,36} = 8.1$, $P < 0.003$ compared with saline-injected intact males; cup-like flaring erections of the glans, $F_{4,36} = 34.7$, $P < 0.001$ compared with saline-injected intact males; latencies to the first erection, $F_{4,36} = 4.4$, $P < 0.018$ compared with saline-injected intact males, $P = 0.013$ compared with saline-injected

castrated males) (Fig. 3a). Most notably, the agents were particularly effective in restoring ejaculation *per se*, resulting in a greater frequency of ejaculation in treated castrated rats than in gonadally intact control males (Fig. 3b). We administered RC-3095, a specific GRP-R antagonist⁸, intrathecally at the level of the fifth lumbar to first sacral vertebrae (L5–S1) in gonadally intact males ($n = 7$). This treatment suppressed penile reflexes, including erections ($F_{4,24} = 4.4$, $P < 0.013$ compared with uninjected males, $P < 0.026$ compared with vehicle-injected males), flips ($F_{4,24} = 2.8$, $P < 0.008$ compared with uninjected males) and cups ($F_{4,24} = 2.8$, $P = 0.007$ compared with uninjected males, $P = 0.022$ compared with vehicle-injected males), without affecting latencies to the first erection ($F_{4,24} = 0.4$) and also attenuated the spontaneous ejaculation rate in a dose-dependent manner (Fig. 3c,d), indicating that the GRP/GRP-R system regulates masculine reproductive functions within the spinal cord.

Expression of spinal *Grpr* mRNA is also sexually dimorphic (Fig. 2e; Supplementary Fig. 5 online), and GRP-R is localized not only in the SPN but also in the spinal nucleus of the bulbocavernosus, suggesting that GRP simultaneously stimulates autonomic preganglionic neurons (see Fig. 2c) and somatic motor neurons controlling the penis (Supplementary Figs. 6 and 7 online), consequently augmenting penile reflexes and ejaculation (Supplementary Fig. 8 online). Thus, reduced GRP expression in the spinal cord of castrated rats may mediate their reduced male copulatory behavior.

It is unclear how the previously identified galanin-containing neurons interact with these GRP-containing neurons within the ejaculation generator¹⁰. Some neurons may contain both galanin and GRP, but the distributions of somata containing the two peptides differed somewhat (Supplementary Figs. 9 and 10 online). Double ICC showed many GRP-immunoreactive, galanin-negative neurons just dorsal to the central canal (Supplementary Fig. 10a–d). Notably, most GRP-containing neurons lateral to the central canal and in the medial portion of lamina VII also expressed galanin (Supplementary Fig. 10e–h). Indeed, in the lower lumbar spinal cord (L5–6 level), the ventral portion of the GRP-immunoreactive fibers projecting to the SPN also contained galanin, whereas the dorsal portion contained GRP only (Supplementary Fig. 10i–l). Thus, both GRP- and galanin-containing neurons within the ejaculation center project axons into the SPN through DGC (see Supplementary Fig. 8), and these two populations are at least partially independent.

The galanin-containing neurons, which are required for ejaculation¹⁰, project to the thalamus, so either information from the thalamus returns to the ejaculation center to trigger the GRP neurons or the two populations within the center interact more directly¹⁵. We conclude that the sexually dimorphic GRP system in the lumbar spinal cord is crucial in male sexual function, revealing a hitherto unrecognized role for GRP in mammalian reproductive behavior. It is of particular note that GRP replacement alone can restore reproductive functions in the absence of androgen. To our knowledge, this is the first neural pathway known to restore ejaculation in castrated male rats. Because this system relies on a specific peptide, it may offer an avenue for pharmacological interventions to reverse male reproductive disorders.

Supplementary Material

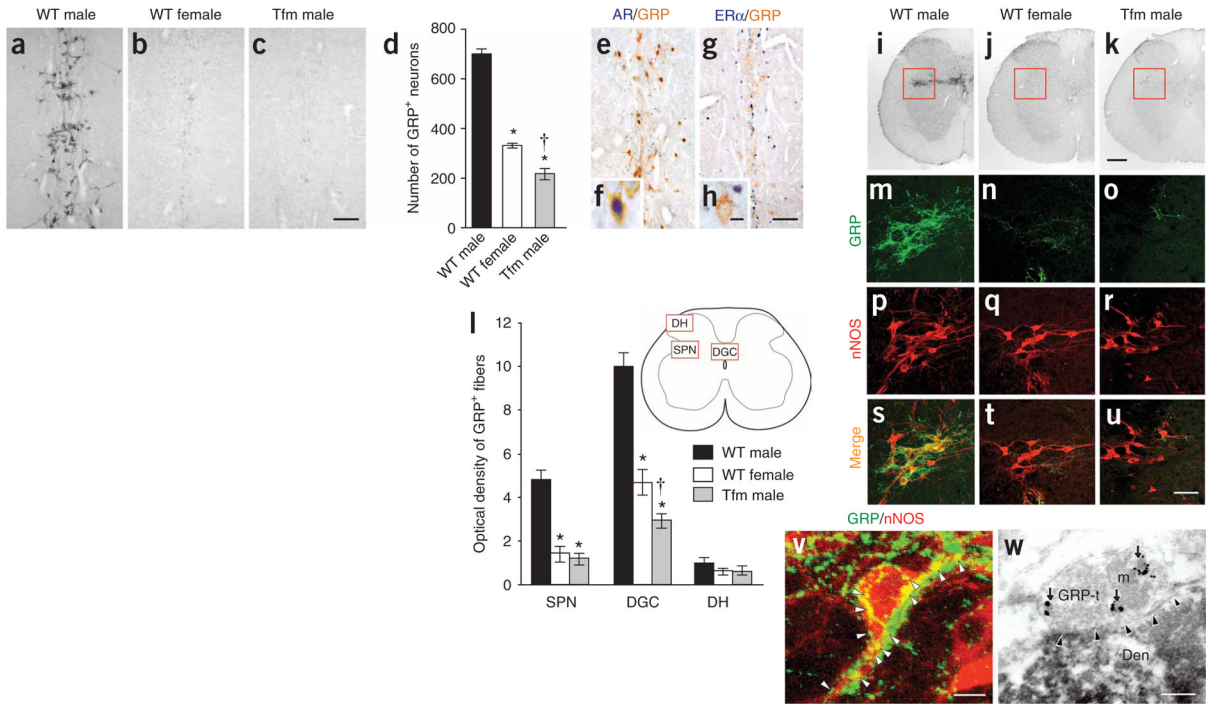
Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank A. Takara for technical assistance. Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology, Japan (H.S., K.-I.M. and M.K.) and US National Institutes of Health grant NS28421 (S.M.B.).

References

1. Anastasi A, Erspamer V, Bucci M. *Experientia*. 1971; 27:166–167. [PubMed: 5544731]
2. Battey J, Wada E. *Trends Neurosci*. 1991; 14:524–528. [PubMed: 1726343]
3. Panula P, Nieminen O, Falkenberg M, Auvinen S. *Ann NY Acad Sci*. 1988; 547:54–69. [PubMed: 3239895]
4. Sun YG, Chen ZF. *Nature*. 2007; 448:700–703. [PubMed: 17653196]
5. Shinohara K, Tominaga K, Isobe Y, Inouye ST. *J Neurosci*. 1993; 13:793–800. [PubMed: 8426236]
6. Ladenheim EE, Taylor JE, Coy DH, Moore KA, Moran TH. *Am J Physiol*. 1996; 271:R180–R184. [PubMed: 8760218]
7. Shumyatsky GP, et al. *Cell*. 2002; 111:905–918. [PubMed: 12526815]
8. Roesler R, et al. *Eur J Neurosci*. 2004; 19:1041–1045. [PubMed: 15009151]
9. Jensen RT, Battey JF, Spindel ER, Benya RV. *Pharmacol Rev*. 2007; 60:1–42. [PubMed: 18055507]
10. Truitt WA, Coolen LM. *Science*. 2002; 297:1566–1569. [PubMed: 12202834]
11. Carro-Juarez M, Rodriguez-Manzo G. *Brain Res Brain Res Rev*. (in the press).
12. Meston CM, Frohlich PF. *Arch Gen Psychiatry*. 2000; 57:1012–1030. [PubMed: 11074867]
13. Morgan C, Nadelhaft I, de Groat WC. *J Comp Neurol*. 1981; 201:415–440. [PubMed: 7276258]
14. Vizzard MA, Erdman SL, de Groat WC. *J Neurosci*. 1995; 15:4033–4045. [PubMed: 7538569]
15. Zoli M, Agnati LF. *Prog Neurobiol*. 1996; 49:363–380. [PubMed: 8888115]

**Figure 1.**

Origin, targets and androgen dependence of GRP neurons in the lumbar spinal cord. (a–d) More GRP-immunoreactive neurons were found in upper lumbar spinal cord of WT males (a) than females (b). Tfm males were feminine in this regard (c), with significantly fewer GRP⁺ neurons in this region than WT females (d; means \pm s.e.m.). (e–h) In WT males, nearly every GRP-immunoreactive neuron also contained androgen receptor (e,f), but these neurons did not contain ER α (g,h). (i–l) In lower lumbar spinal cord autonomic nuclei, males (i) had more GRP-immunoreactive fibers in the SPN (red inset) and the DGC than did females (j; quantitation in l). Androgen receptor-lacking Tfm males were feminine in this regard (k). GRP fibers in the non-autonomic dorsal horn region of the spinal cord (DH) were equivalent across groups (l). (m–u) GRP-containing fibers closely apposed cell bodies and proximal dendrites of nNOS⁺ neurons in the SPN. Left column, WT male; center, WT female; right, Tfm male. (v) Possible synaptic sites (arrowheads). (w) Electron micrographs confirmed GRP-immunoreactive presynaptic boutons innervating nNOS-containing dendrites in the SPN. Arrows, GRP-immunoreactive secretory granules; arrowheads, synapse. GRP-t, GRP terminal; m, mitochondrion; Den, nNOS-immunoreactive dendrite. * $P < 0.001$ compared with WT males; † $P < 0.001$ compared with WT females (one-way (d) or two-way (l) analysis of variance; significant effects followed by *post hoc* Bonferroni tests). Scale bars: c (also applies to a,b) and g (applies to e), 100 μ m; h (applies to f), 10 μ m; k (applies to i,j), 200 μ m; u (applies to m–t), 50 μ m; v, 10 μ m; w, 200 nm. All animal procedures were approved by institutional review boards of Kyoto Prefectural University of Medicine and/or Michigan State University. For methodological details, see Supplementary Methods online.

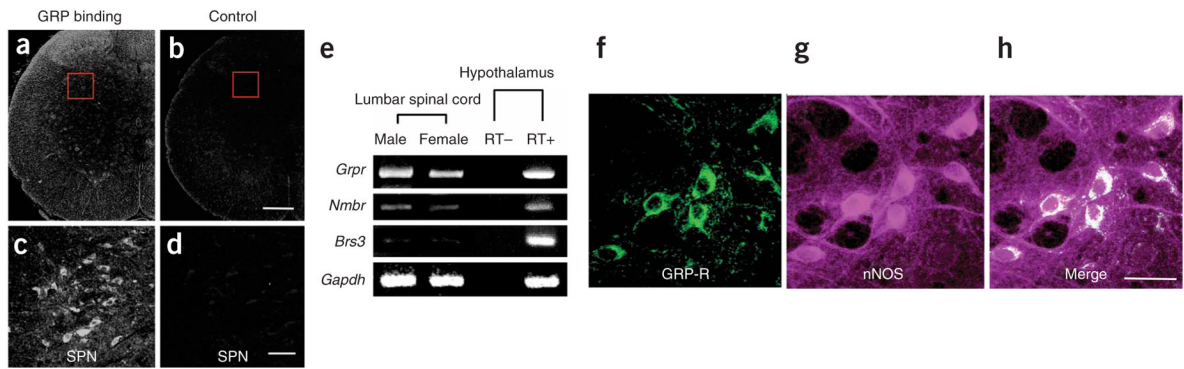
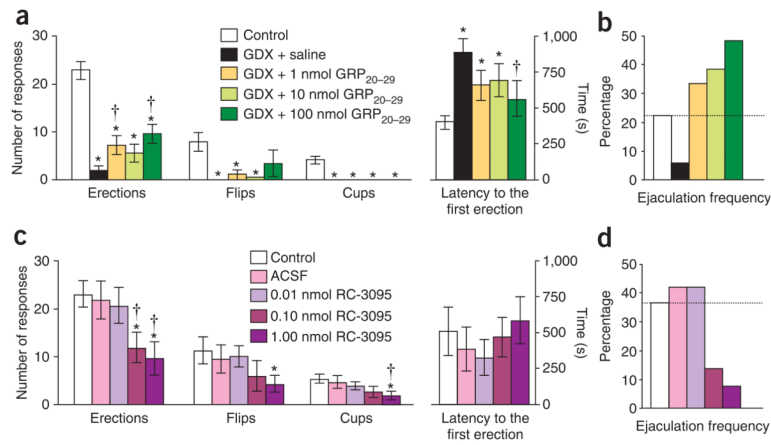


Figure 2.

GRP-Rs in autonomic regions of the lower lumbar spinal cord. (a–d) Incubating L5–6 spinal cord sections with biotinylated GRP yielded accumulation of GRP (a), especially among neurons in the SPN (red inset in a, magnified in c). Control incubations with biotinylated GRP and excess free ligand demonstrated the specificity of this binding (b,d). (e) RT-PCR confirmed the presence of *Grpr* and the related *Nmbr*, but few or no *Brs3* transcripts, in the lumbar spinal cord. All three receptor transcripts were found in hypothalamus, as expected. (f–h) Double ICC localized the spinal GRP-R to cell bodies of nNOS⁺ neurons in the autonomic SPN. Scale bars: b (applies to a), 200 μ m; d (applies to c) and h (applies to f,g), 50 μ m.

**Figure 3.**

Role of spinal cord GRP system in sexual reflexes. **(a)** Gonadectomy (GDX) of male rats reduced spinal reflexes of the penis, including simple erections, dorsal flips of the penis and cup-like flaring erections of the distal glans. Systemic treatment of GDX males with a specific agonist for GRP-R (rat GRP₂₀₋₂₉) restored erections and flips, without affecting cups. * $P < 0.05$ compared with saline-injected intact males (control); † $P < 0.05$ compared with saline-injected castrated males (GDX + saline). **(b)** Spontaneous ejaculations during reflex tests were also reduced after castration, an effect fully reversed, in a dose-dependent manner, by systemic treatment with rat GRP₂₀₋₂₉. **(c)** In gonadally intact males, these penile reflexes were suppressed by intrathecal injection of a specific antagonist for GRP-R (RC-3095). * $P < 0.05$ compared with uninjected males (control); † $P < 0.05$ compared with vehicle-injected males (ACSF). **(d)** Spontaneous ejaculations were attenuated by intrathecal RC-3095 in intact males. Significant effects in analyses of variance were followed by *post hoc* Bonferroni test. Data are means \pm s.e.m. (**a,c**) or percentages (**b,d**).