Effects of Sex Steroids on the Spinal Gastrin-Releasing Peptide System Controlling Male Sexual Function in Rats

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The gastrin-releasing peptide (GRP) system in the lumbosacral spinal cord controls male sexual function in rats. In contrast, in female rats, GRP neurons could scarcely be detected around puberty when circulating ovarian steroid hormones such as estradiol and progesterone levels are increasing. However, little information is available on feminizing or demasculinizing effects of ovarian steroids on the central nervous system in female puberty and adulthood. In this study, to visualize the spinal GRP neurons *in vivo*, we generated a *GRP-promoter-Venus* transgenic (Tg) rat line and studied the effects of the sex steroid hormones on GRP expression in the rat lumbar cord by examining the Venus fluorescence. In these Tg rats, the sexually dimorphic spinal GRP neurons controlling male sexual function were clearly labeled with Venus fluorescence. As expected, Venus fluorescence in the male lumbar cord was markedly decreased after castration and restored by chronic androgen replacement. Furthermore, androgen-induced Venus expression in the spinal cord of adult Tg males was significantly attenuated by chronic treatment with progesterone but not with estradiol. A luciferase assay using a *human GRP-promoter* construct showed that androgens enhance the spinal GRP system, and more strikingly, that progesterone acts to inhibit the GRP system *via* an androgen receptor–mediated mechanism. These results demonstrate that circulating androgens may play an important role in the spinal GRP system controlling male sexual function not only in rats but also in humans and that progesterone could be an important feminizing factor in the spinal GRP system in females during pubertal development. **(*Endocrinology* 159: 1886–1896, 2018)**

Androgens such as testosterone (T) and 5α-dihydrotestosterone (DHT) play an important role in the sexual differentiation of the central nervous system *via* a mechanism that is mediated by signaling through the nuclear androgen receptor (AR) (1–4). A large body of literature shows that, during embryonic and perinatal life, masculinization of the rodent brain depends on estradiol-17β (E_2) converted from T by the enzymatic activities of the cytochrome P450 aromatase: the "aromatization hypothesis" (3–5). Ovarian steroid hormones E_2 and progesterone (P_4) are also mediated through their nuclear receptors, that is, the estrogen receptor and progesterone receptor (PR), respectively, and act to maintain the cellular proliferation and growth of tissues

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Abbreviations: AR, androgen receptor; cDNA, complementary DNA; DHT, 5α-dihydrotestosterone; E_2, estradiol-17β; F-luc, firefly luciferase; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GRP, gastrin-releasing peptide; GRP^+, GRP-positive; hGRP, human-derived gastrin-releasing peptide; IgG, immunoglobulin G; IgY, immunoglobulin Y; i.p., intraperitoneal; mRNA, messenger RNA; ORX, orchiectomy; OVX, ovariectomy; P_4, progesterone; PCR, polymerase chain reaction; PR, progesterone receptor; qPCR, quantitative polymerase chain reaction; rGRP, rat-derived gastrin-releasing peptide; RRID, Research Resource Identifier; RT, reverse transcription; s.c., subcutaneously; SEM, standard error of the mean; T, testosterone; Tg, transgenic; $Venus^+$, Venus-positive; Wt, wild-type.

in the central nervous system as well as sex organs during the estrus cycle and to develop the secondary sex characteristics during female puberty (5). In adulthood, sex steroid hormones also act on the nervous system to regulate behavior: androgens promote aggressive and masculine sexual behavior, whereas ovarian steroids such as E_2 and P_4 facilitate feminine sexual behavior (6). Therefore, sex steroid hormones play an essential role in the expression of sexual dimorphism during pubertal development and also a role in some sociosexual behaviors in adulthood (7).

Gastrin-releasing peptide (GRP) is a neuropeptide expressed and distributed widely in the central nervous system in addition to the gastrointestinal tract of mammals (8). It has been demonstrated that GRP regulates many physiological processes, including anxiety (9), circadian rhythms (10), food intake (11), and itch sensation (12, 13). We have previously demonstrated that the GRP system in the lumbosacral spinal cord controls male sexual activity such as penile erection and ejaculation during male copulatory behavior in rats (14). GRP neurons in the lumbar spinal cord (L3 to L4 level) are greater in number and immunoreactivity in males than in females (14). It has also been reported that GRP neurons in this system coexpress ARs, but not estrogen receptors (14). Taken together, these findings demonstrated that the androgen signaling that plays an important role in the development of the male-specific sexually dimorphic spinal GRP system and in the regulation of GRP expression in the male lumbar cord (14, 15) does not require aromatization. We have also reported that, in male rats, the number of GRP-positive (GRP^+) neurons is greater after than before puberty, corresponding to the increase in circulating T levels in males (16). In contrast, in female rats, GRP^+ neurons could scarcely be detected around puberty when circulating ovarian steroid hormones, such as E_2 and P_4 levels, are increasing (16). Additionally, we recently reported that the male-dominant number of GRP^+ neurons was never observed during the prepubertal postnatal period in female rats (16). Therefore, these results suggest that ovarian steroid hormones might contribute to the feminization of spinal GRP system in females, possibly by inhibiting the expression of GRP in the lumbosacral cord during female puberty. However, little information is currently available on feminizing or demasculinizing effects of ovarian steroids, in females, on the central nervous system during pubertal development.

To visualize the spinal GRP-expressing neurons *in vivo*, we generated a rat-derived GRP (*rGrp*)-*promoter-Venus* transgenic (Tg) line. Because *rGrp-promoter-Venus* Tg rats express Venus under the control of the *rGrp-promoter*, GRP-expressing neurons could be visualized *in vivo* efficiently as Venus fluorescence. Using this newly developed model, we studied the *in vivo* effects of sex steroid hormones on *rGrp* expression in the rat lumbar cord. Subsequently, using HEK293T cells and rat-derived *rGrp-promoter-Venus* construct, we examined the *rGrp-promoter* response to the sex steroid hormones *in vitro*. We further used a human-derived GRP (*hGRP*)-*promoter* construct to study the modulation of *hGRP* gene expression by sex steroid hormones in humans *in vitro*.

Materials and Methods

Animals

Adult male and female *rGrp-promoter-Venus* Tg rats (Wistar strain; age, 2 months old to ~1 year old) bred in the animal facilities of Okayama University were used. Adult male wild-type (Wt) rats of the Wistar strain (age, 2 months old to 3 months old; Charles River Japan, Yokohama, Japan) were also used. All rats were maintained on a 12-hour 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 and the Kyoto Prefectural University of Medicine Animal Care and Use Committee.

Generation of Tg rats

An adult male Wistar rat was deeply anesthetized with intraperitoneal (i.p.) injections of 50 mg/kg body weight sodium pentobarbital and a 5-mm portion of the tail tip was cut off. Genomic DNA was extracted using a NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The *preproGrp* gene including a brain-specific *Grp-promoter* region (17) was cloned by polymerase chain reaction (PCR) analysis: (sense) 5′-TCTGCCTTGGCAGCACACTT-3′, (antisense) 5′-ACCAGAGCCAACAGCAAGAG-3′. The resulting PCR amplicons (rat *preproGrp* gene region) were subcloned into the pGEM-T easy vector (Promega, Madison, WI) followed by transfection into *Escherichia coli* DH5α competent cells (Takara Bio, Shiga, Japan). Positive clones were identified by blue-white screening, and sequence of the clones was confirmed by the Sanger method. A plasmid containing the *rGrp-promoter* gene was cut by *Bam*HI and *Apa*I to remove the *rGrp* gene sequence. A DNA fragment encoding Venus and an SV40 polyadenylation signal digested from a PCS2 plasmid vector were ligated into the *rGrp* gene–deleted pGEM-T easy plasmid. In the resulting transgene construct, Venus complementary DNA (cDNA) and the SV40 polyadenylation signal were fused just upstream of the first ATG of the DNA fragment of the *rGrp* gene. The plasmid was digested by *Not*I. The digested plasmid fragment was microinjected into the pronucleus of fertilized oocytes of Wistar rats (Institute of Immunology, Utsunomiya, Japan). The Tg rats were identified by standard PCR analysis of extracted ear DNA using primers detecting the *Venus* gene: (sense) 5′-CACCATCTTCTTCAAGGACGAC-3′, (antisense) 5′-ATGATATAGACGTTGTGGCTGTTGT-3′.

Plasmids

The *rGrp-promoter-Venus* plasmid was constructed as described previously. A reporter plasmid, *hGRP-promoter-Firefly*

luciferase (*F-luc*), was constructed by inserting the upstream region of the *Grp* gene (–2500 to +1) into the pGL3-promoter vector (Promega).

Cell culture and transfection

The HEK293T cell line was purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle medium (Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum at 37°C under 5% CO_2. Venus expression in HEK293T cells was assessed by Western blotting as described later. Transfection of human *Ar-pcDNA3* plasmid and *rGrp-promoter-Venus* plasmid or *hGRP-promoter-F-luc* plasmid cDNAs into HEK293T cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. To normalize transfection efficiency, pRL-CMV vector (Promega), which expresses the *Renilla luciferase*, or ptdTomato C1 vector (Promega) was also transfected into HEK293T cells.

Western blotting

Whole-cell lysates were extracted with lysis buffer [10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA]. The preparations from HEK293T cells expressing Venus were boiled in 10 μL of sample buffer containing 2% sodium dodecyl sulfate, 25% glycerol, 62.5 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, and a small amount of bromophenol blue. The samples were run on a 4% to 20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) from the gel by a semidry blotting apparatus (Bio-Rad Laboratories). The blotted membranes were blocked with polyvinylidene difluoride blocking reagent from the Can Get Signal kit (Toyobo, Tokyo, Japan) for 30 minutes at room temperature and incubated overnight at 4°C with a 1:1000 dilution of rabbit monoclonal antibody against green fluorescent protein [GFP; Research Resource Identifier (RRID): AB_1196615; Cell Signaling Technology Japan, Tokyo, Japan] or a 1:1000 dilution of chicken polyclonal antibody against GFP (RRID: AB_1537402; 600-901-215; 1:5000 dilution; Rockland, Gilbertsville, PA) in Can Get Signal solution1 (Toyobo). The blotted membranes were washed three times with 0.05% Tween 20 in Tris-buffered saline (pH 7.6) and incubated with horseradish peroxidase–conjugated goat polyclonal antibody against rabbit immunoglobulin G (IgG; Bio-Rad Laboratories) or horseradish peroxidase–conjugated goat polyclonal antibody against chicken immunoglobulin Y (IgY; Abcam, Cambridge, MA) at a 1:10,000 dilution in Can Get Signal solution2 (Toyobo) for 1 hour at room temperature. After washing for three times with 0.05% Tween 20 in Tris-buffered saline, blots were visualized by an Immun-Star WesternC chemiluminescence kit (Bio-Rad Laboratories). A list of antibodies used in this study is shown in the Antibody Table.

RNA extraction and reverse transcription

To confirm the *Venus* and *Grp* messenger RNA (mRNA) expression in the lumbar spinal cord and stomach, *rGrp-promoter-Venus* Tg rats (n = 3 of each sex) were euthanized by decapitation under deep anesthesia with 50 mg/kg body weight with an i.p. sodium pentobarbital injection. Lumbar cords (L3 to L4 level) were quickly removed on ice. Dissected tissue was immediately fixed with RNA*Later* solution (Ambion, Austin, TX) and stored at –80°C until RNA extraction. Total RNA was extracted from these tissues using a Sepasol-RNA I Super G kit (Nacalai Tesque) and an Illustra RNAspin Mini RNA isolation kit (GE Health Care, Buckingham, United Kingdom) according to the manufacturer's protocol. The concentration of total RNA was measured using a Qubit RNA assay kit (Thermo Fisher Scientific, Waltham, MA). First-strand cDNA was synthesized from 100 ng of total RNA in a 20-μL reaction volume using oligo(dT) primers and an Omniscript reverse transcription (RT) kit (Qiagen, Hilden, Germany).

For quantitative PCR (qPCR) analysis, male rats were euthanized by decapitation under deep anesthesia with an i.p. injection of 50 mg/kg body weight sodium pentobarbital. Lumbar spinal cords, which contain the soma region of GRP neurons (L3 to L4 level), were quickly removed and frozen by powdered dry ice. The preparations were mounted in a cryostat (CM3050 S; Leica, Nussloch, Germany) and the lamina X region was dissected along the rostrocaudal axis by use of a stainless steel needle (outer diameter, 1.20 mm; inner diameter, 0.94 mm). The dissected tissue was stored at –80°C until RNA extraction. Total RNA was extracted using an Illustra RNAspin Mini RNA isolation kit (GE Health Care) according to the manufacturer's protocol. The concentration of total RNA was measured using a Qubit RNA assay kit (Thermo Fisher Scientific). First-strand cDNA was synthesized from 250 ng of total RNA with random primers using an Omniscript RT kit (Qiagen).

RT-PCR of *Venus* and *rGrp* in Tg rats

RT-PCR was performed using Takara Ex Taq (Takara Bio) under the following conditions: 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds, 30 cycles (*Venus*); 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, 35 cycles (*Grp*); 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 25 cycles [rat *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*)]. Sequences of primers were as follows: *Venus* (sense) 5′-ATGGTGAGCAAGGGCGAGGAGCTGT-3′, (antisense) 5′-TTGGGGTCTTTGCTCAGGGCGGACT-3′; *Grp* (sense) 5′-GACAACGCACTCTCAGCCTAGT-3′, (antisense) 5′-AGAACCTGGAGCAGAGAGTCTAC-3′; *Gapdh* (sense) 5′-GGTGAAGGTCGGTGTGAACG-3′, (antisense) 5′-CAAAGTTGTCATGGATGACC-3′. After the thermal cycling, the PCR product was electrophoresed on 1.5% agarose gel. In this study, the *Venus* primers give a 638-bp amplified fragment, the *rGrp* primers give a 467-bp amplified fragment, and the *Gapdh* primers give a 497-bp amplified fragment. RT-PCR studies were repeated at least four times using independently extracted RNA samples from different animals. Consistent results were obtained from each run.

Orchiectomy and chronic T treatment of Tg males

rGrp-promoter-Venus Tg rats were anesthetized with i.p. injections of 50 mg/kg body weight sodium pentobarbital and bilaterally orchiectomized and implanted subcutaneously (s.c.) with 50-mm Silastic capsules (inner diameter, 1.59 mm, outer diameter, 3.18 mm; Compagnie de Saint-Gobain, Courbevoie, France) containing crystalline T (Tokyo Chemical, Tokyo, Japan) [orchiectomy (ORX) plus T] (n = 3) or empty control capsules (ORX plus blank) (*n* = 4). Sham surgeries (n = 4) were also performed under deep anesthesia. One to two months after surgery, rats were perfusion fixed under deep anesthesia with sodium pentobarbital.

Ovariectomy and chronic T treatment of Tg females

rGrp-promoter-Venus Tg rats were anesthetized with an i.p. sodium pentobarbital injection, bilaterally ovariectomized, and implanted s.c. with 50-mm Silastic capsules containing crystalline T [ovariectomy (OVX) plus T] (n = 5) or empty control capsule (OVX plus blank) (n = 5). Sham operations ($n = 4$) were also performed under deep anesthesia. One to two months after surgery, rats were perfusion fixed under deep anesthesia with sodium pentobarbital.

Chronic E_2 or P_4 treatment of intact Tg or Wt males

rGrp-promoter-Venus Tg or Wt male rats were anesthetized with an i.p. sodium pentobarbital injection and implanted s.c. with 15-mm Silastic capsules containing crystalline E_2 (Sigma-Aldrich, St. Louis, MO), 40-mm Silastic capsules containing crystalline P_4 (Sigma-Aldrich), or empty control capsule. One to two months after surgery, rats were euthanized by decapitation for qPCR under deep anesthesia with sodium pentobarbital (at least n = 3 of each group), and rats were perfusion fixed under deep anesthesia with sodium pentobarbital for histological analysis (n = 3 of each group).

Tissue preparation

Rats were transcardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep pentobarbital anesthesia. The spinal cords were removed and immersed in the same fixative for 16 hours at 4°C. After cryoprotection by immersion in 25% sucrose in 0.1 M phosphate buffer at 4°C until they sank, the preparations were quickly frozen using powdered dry ice and cut into 30-μm-thick horizontal spinal sections using a cryostat.

Immunofluorescence

We performed immunofluorescence analysis according to our established methods (15, 16, 18). The sections were incubated with a primary rabbit polyclonal antibody against rat $GRP_{20–29}$, a 10–amino acid peptide called neuromedin C (RRID: AB_2571636; 1:1000 dilution; AssayPro, St. Charles, MO), or a primary chicken polyclonal antibody against GFP (1:5000 dilution; Rockland) for ~48 hours at 4°C after blocking nonspecific binding with 1% bovine serum albumin, 1% normal goat serum, and 0.3% Triton X-100 in phosphate-buffered saline (pH 7.4) for 30 minutes at room temperature. To detect PR expression, we used a rabbit monoclonal antibody (SP2; RRID: AB_471063; 1:200 dilution; Abcam). Alexa Fluor 555–linked anti-rabbit IgG raised in goats (Molecular Probes, Eugene, OR) or Alexa Fluor 488–linked anti-chicken IgY raised in goats (Molecular Probes) was used at 1:1000 dilution for detection. The antibodies used in this study are shown in Table 1.

Microscopic imaging and morphological analysis

Sections were imaged with a confocal laser scanning microscope (FluoView 1000; Olympus, Tokyo, Japan) and an Olympus FSX100 fluorescent microscope. We first counted Venus-positive ($Venus^+$) neurons in the lumbar spinal cord. Analysis of Venus expression in neurons of the lumbar cord (L3 to L4 level) was performed as described previously using horizontal spinal sections [~18 to 22 sections (30 μm thick) per animal]. Briefly, we counted the number of $Venus^+$ cell bodies at ×42 magnification in all sections and analyzed a 600-μm^2 area localized to the midline at the center. We acquired 5 to 15 micrographs per section, the number of which depended on the distribution of the $Venus^+$ neurons. These digital micrographs were selected and processed using Adobe PhotoShop (Adobe Systems, San Jose, CA) and printed at 300 dots per inch on photographic paper. $Venus^+$ neurons were identified by the following characteristics: densely positive fluorescence; anatomical localization (mainly dorsal, dorsolateral, or both to the central canal in lamina X of lumbar segments III to IV); relatively large cell bodies (diameters ~20 to 30 μm); and clear round nuclei (diameters ~10 to 15 μm). To avoid overestimating cell number, only $Venus^+$ neuronal profiles 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 $Venus^+$ neurons. All micrographs were coded and evaluated without the knowledge of the experimental group designation, and the code was not broken until the analysis was complete.

We next performed a semiquantitative analysis of Venus expression. To determine the density of $Venus^+$ somata in the lumbar spinal cord (L3 to L4 level), at least 15 horizontal sections (30 μm thick) per animal were captured (magnification, ×42 per section). The optical density of Venus fluorescence was determined using black-and-white images that were converted from micrographs using ImageJ software (ImageJ 1.44p; National Institutes of Health, Bethesda, MD) according to our established methods (15, 16, 19). Briefly, the optical density of the intrinsic fluorescence was estimated by comparison with similar areas of the control sections. The $Venus^+$ soma pixel density was semiquantitated as the total pixel density of each animal, and the data were expressed as the ratio to the density of controls.

Real-time qPCR analysis for *Venus, Grp, Ar, Pr* and *Gapdh*

To assess the *Venus* and *Gapdh* expressions in the lumbar spinal cord of *rGrp-promoter-Venus* Tg rats that were implanted s.c. with empty capsule (blank control; n = 3) or P_4 capsule (n = 5), we demonstrated qPCR using a KAPA SYBR Fast qPCR kit (Kapa Biosystems, Wilmington, MA). Primer pairs were designed using Primer3 software. The following primers were applied for

Table 1. Antibody Table

Name of Antibody	Source, Catalog No.	Species Raised in; Monoclonal or Polyclonal	Dilution Used	RRID
Anti-GFP	Cell Signaling Technology, 2956S	Rabbit; monoclonal	1:1000	AB_1196615
Anti-GFP	RockLand, 600-901-215	Chicken; polyclonal	1:5000	AB_1537402
Anti–neuromedin C (GRP)	AssayPro, 11081-05015	Rabbit; polyclonal	1:1000	AB_2571636
Anti-PR	Abcam, ab27596	Rabbit; monoclonal	1:200	AB_471063

qPCR: *Venus* (sense) 5′-CGACCACTACCAGCAGAACA-3′, (antisense) 5′-GAACTCCAGCAGGACCATGT-3′; *Gapdh* (sense) 5′-TGCCACTCAGAAGACTGTGG-3′, (antisense) 5′-TTCAGCTCTGGGATGACCTT-3′. The PCR cycle conditions were 95°C for 3 minutes and 40 cycles with 95°C for 1 second, 60°C for 20 seconds, and 65°C for 5 minutes according to the manufacturer's protocol. The data of qPCR were analyzed using a CFX96 qPCR detection system (Bio-Rad Laboratories).

To assess the *Grp*, *Ar*, *Pr*, and *Gapdh* expressions in the lumbar cord of Wt Wistar rats that were implanted s.c. with empty capsule (blank control) or P_4 capsule (n = 5, respectively), we used 15-μL reaction volumes consisting of ×1 TaqMan universal PCR master mix (Applied Biosystems, Foster, CA), 300 nM each of forward and reverse primer, and 200 nM TaqMan probe. We used TaqMan qPCR methodology for rat *Grp, Ar, Pr*, and *Gapdh* (gene expression assays nos. Rn00592059, Rn00560747, Rn00674394, and Rn99999916; amplicon lengths 75, 75, 105, and 87, respectively). Amplification was carried out at 95°C for 10 minutes and 40 cycles with 95°C for 15 seconds and 60°C for 1 minute. The data of qPCR were analyzed using the CFX96 qPCR detection system (Bio-Rad Laboratories). The expression in each reaction was normalized by the expression of *Gapdh* as an internal control. Duplicate qPCR analysis was performed for each sample.

Steroid treatments and luciferase assay

HEK293T cells (60% to 70% confluent) were transfected with *rGrp-promoter-Venus* plasmids or *hGRP-promoter-F-luc* plasmids and reporter plasmids using Lipofectamine for 24 hours. As a reference plasmid to normalize transfection efficiency, pRL-CMV plasmid (Promega) was cotransfected. Steroids dissolved in ethanol were added to the cultured medium at the concentration of 10^{-8} M DHT (*rGrp* construct) or 10^{-7} M DHT (*hGRP* construct) or 10^{-7} M E_2/P_4 (*rGrp* construct) or 10^{-6} M E_2/P_4 (*hGRP* construct) for 24 hours before analysis. Cocktail administration of steroids (DHT plus E_2 or DHT plus P_4) was also performed using the same concentrations. Addition of the same amount of vehicle (ethanol) was used as control. At 24 hours after steroids treatments, total RNA from HEK293T cells was isolated using Isogen reagent (Wako, Osaka, Japan). RT was performed using PrimeScript RT master mix (Takara Bio) in accordance with the manufacturer's instructions. cDNAs were quantified by qPCR using SYBR qPCR mix (Toyobo) and a TP800 thermal cycler (Takara Bio). All values are mean ± standard error of the mean (SEM) of at least two independent experiments. Primers were: *F-luc* (sense) 5′-AAGGTTGTGGATCTGGATAC-3′, (antisense) 5′-GATTGTTTACATAACCGGAC-3′; *Renilla* luciferase (sense) 5′-CTTCGTGGAAACCATGTTGCC-3′, (antisense) 5′-CTAACGGGATTTCACGAGGCCATG-3′; *Venus* (sense) 5′- ACCCTGAAGCTGATCTGCAC-3′, (antisense) 5′- GGTCTTGTAGTTGCCGTCGT-3′.

Statistical analysis

All data are reported as mean ± SEM. All statistical analyses were performed using one-way analysis of variance or *t* test. When significant main effects were found using analysis of variance, *post hoc* Tukey tests were performed. A P value <0.05 was considered significant.

Results

Generation and characterization of *rGrp-promoter-Venus* Tg rats

Venus fluorescence was visualized in HEK293T cells transfected with *rGrp-promoter-Venus* plasmids (Fig. 1A). Venus expression at the protein level was examined by Western blotting in HEK293T cells using an anti-GFP antibody raised in the chicken (Fig. 1B). The Venus$^+$ band (~27 kDa) was detected from the lysate of *Venus*-transfected HEK293T cells (Fig. 1B). The Venus$^+$ band (~27 kDa) was also confirmed using another

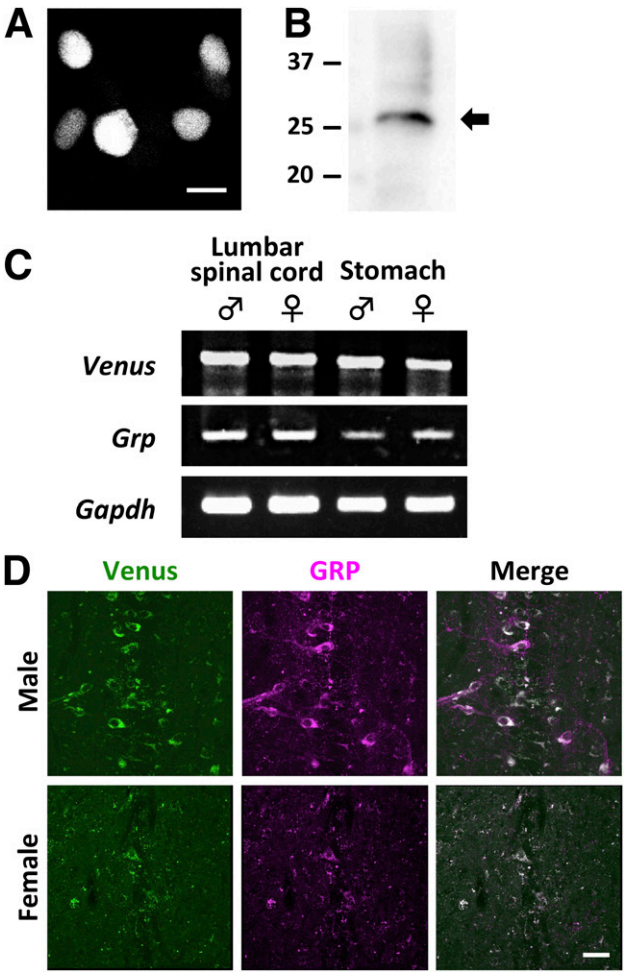

Figure 1. Generation and characterization of *rGrp-promoter-Venus* Tg rats. (A) Venus fluorescence was observed in HEK293T cells transfected with *rGRP-promoter-Venus* plasmids. (B) Venus$^+$ band (arrow) was detected from the lysate of HEK293T cells transfected with *rGrp-promoter-Venus* plasmids. (C) *Venus* mRNA and *Grp* mRNA expression were detected in the lumbar spinal cord and stomach of *rGrp-promoter-Venus* Tg rats. *Gapdh* was performed as the internal control. (D) To demonstrate the colocalization of Venus fluorescence and GRP immunoreactivity in the lumbar spinal cord (L3 to L4 level) of Tg rats, sections were stained with anti-GRP antiserum. Venus fluorescence is shown in green and GRP immunoreactivity is pseudocolored in magenta. Most Venus$^+$ neurons expressed GRP in the lumbar cord of Tg rats (~95% in both sexes). Scale bars, 20 μm in (A), 50 μm in (D).

monoclonal anti-GFP antibody raised in the rabbit (data not shown). Analysis by RT-PCR of *Venus* and *Grp* mRNA in total RNA extracted from the lumbar spinal cord and stomach (as a positive control) of adult *rGrp-promoter-Venus* Tg rats (n = 3 of each sex) showed that both *Venus* mRNA and *Grp* mRNA are expressed in the lumbar cord and stomach (Fig. 1C, upper and middle panels, respectively). RT-PCR for *Gapdh* showed that no significant RNA degradation occurred and a proper RT was obtained (Fig. 1C, bottom panel). Histological localization of Venus and GRP immunoreactivity in the lumbar cord (L3 to L4 level) of *rGrp-promoter-Venus* Tg rats revealed many Venus$^+$ neurons in the lumbar cord. The number and fluorescence intensity of Venus$^+$ and GRP$^+$ neurons were greater in males than in females (Fig. 1D). Quantification confirmed that nearly every Venus$^+$ neuron also contained GRP (Fig. 1D) (95.1% ± 1.1% in males; 99.4% ± 0.4% in females; n = 4 of each sex).

Effect of gonadectomy and chronic androgen treatment on *Grp* expression *in vivo*

Removal of the testes (ORX) in adult *rGrp-promoter-Venus* Tg males and visualization of Venus expression in the lumbar spinal cord showed that ORX for 1 to 2 months resulted in a lower fluorescence intensity of Venus$^+$ somata in the ORX group (n = 4) than in sham control (n = 4) (Fig. 2A). Chronic androgen treatment of ORX males prevented the decrease in Venus expression (Fig. 2A). Quantification confirmed that the number of Venus$^+$ neurons in the lumbar cord was significantly decreased in the ORX plus blank group compared with sham and ORX plus T group (n = 3) ($F_{2,8} = 5.09$; *vs* sham, $P < 0.05$; *vs* ORX, $P < 0.05$) (Fig. 2B). Although the number of Venus$^+$ neurons in the ORX plus T group was not significantly different compared with sham control, T replacement appeared to increase Venus fluorescence intensity (Fig. 2). Removal of the ovaries (OVX plus blank) (n = 5) from Tg females caused an increase in the number of Venus$^+$ neurons in the lumbar cord (Fig. 3A), and the number of Venus$^+$ cells was still further increased when the OVX animals had received chronic androgen treatment (OVX plus T) (n = 5) ($F_{2,11} = 23.6$; *vs* sham, $P < 0.05$; *vs* OVX, $P < 0.05$) (Fig. 3B). Interestingly, the intensity of Venus fluorescence also tended to increase after OVX alone, which would correspond to the increase in the number of detectable Venus$^+$ neurons after OVX (Fig. 3).

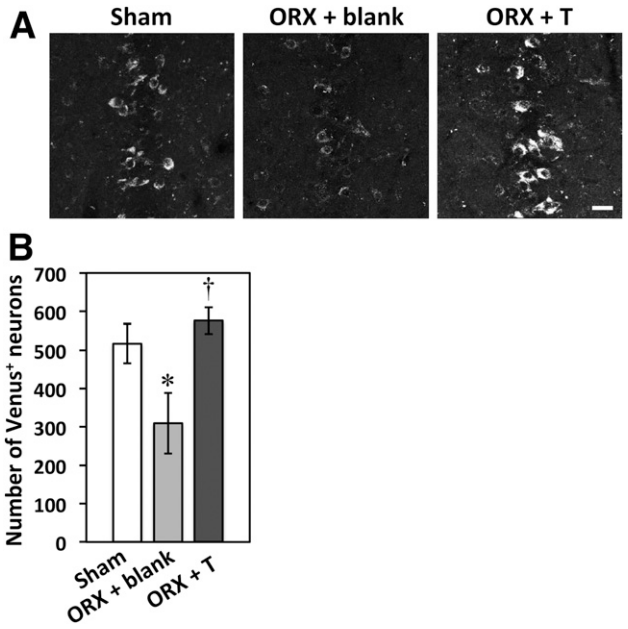

Figure 2. Effects of ORX and androgen replacement on Venus expression in the lumbar spinal cord (L3 to L4 level) of Tg males. (A) Venus$^+$ neurons were decreased by ORX (ORX plus blank) (n = 4) compared with sham-operated males (sham) (n = 4), but were prevented by the T treatment (ORX plus T) (n = 3). (B) Semiquantitative analysis showed that ORX males decreased the number of Venus$^+$ neurons in the lumbar cord. This effect was prevented when the castrates were treated with T (ORX plus T) immediately after castration. Data are presented as mean ± SEM. Scale bar, 50 μm. *$P < 0.05$, *vs* sham; †$P < 0.05$, *vs* ORX plus blank. ORX + blank, orchiectomy plus blank; ORX + T, orchiectomy plus testosterone.

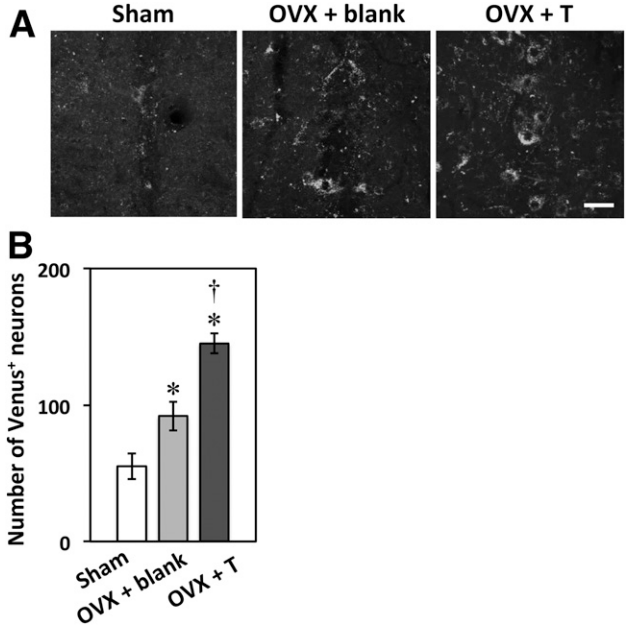

Figure 3. Effects of OVX and androgen replacement on Venus expression in the lumbar spinal cord (L3 to L4 level) of Tg females. (A) OVX females treated with T (OVX plus T) (n = 5) had more Venus$^+$ neurons compared with control (sham) (n = 4) and OVX plus blank females (n = 5). (B) Quantification analysis showed that OVX plus blank females and OVX plus T females increased the number of Venus$^+$ neurons in the lumbar cord compared with sham-operated females (sham). Interestingly, the number of Venus$^+$ neurons was increased by only OVX. Data are presented as mean ± SEM. Scale bar, 50 μm. *$P < 0.05$, *vs* sham; †$P < 0.05$, *vs* OVX plus blank. OVX + blank, ovariectomy plus blank; OVX + T, ovariectomy plus testosterone.

Effect of chronic ovarian steroid hormones on *Grp* expression in males *in vivo*

Implantation of E_2-containing Silastic capsules into sexually mature *rGrp-promoter-Venus* Tg males for 1 to 2 months (n = 3) had no apparent effect on the fluorescence intensity or number of Venus$^+$ neurons compared with controls (blank) (n = 3), but in the P_4-treated group (n = 3) the intensity of Venus$^+$ neurons was significantly decreased compared with both the blank control and E_2-treated group (Fig. 4A–4C; intensity $F_{2,6} = 7.46$; *vs* blank, $P < 0.05$; *vs* E_2, $P < 0.05$), and the number of Venus$^+$ neurons also tended to decrease in the P_4-treated group ($F_{2,6} = 2.27$). Subsequently, to investigate the mechanism of the P_4 action on the spinal GRP neurons, PR immunohistochemistry was performed in adult *rGrp-promoter-Venus* Tg rats (n = 4) (Fig. 4D). Unexpectedly, scarcely any PR/Venus double$^+$ neurons could be detected in the lumbar cord (Fig. 4D).

To confirm the effects of P_4 treatment on *Venus* and *Grp* expressions at the transcription level, we performed qPCR analyses. Figure 5 shows that, whereas *Ar* and *Pr* expressions were clearly unchanged by P_4 treatment in Wt males (n = 5) (*Ar*, $t_8 = 2.30$; *Pr*, $t_8 = 2.50$), *Venus* expression in Tg males was significantly suppressed by P_4 treatment (n = 5) ($t_6 = 6.76$), and *Grp* expression in Wt males also decreased by ~25% in the P_4-treated group ($t_8 = 1.48$) compared with blank controls (Tg males, n = 3; Wt males, n = 5).

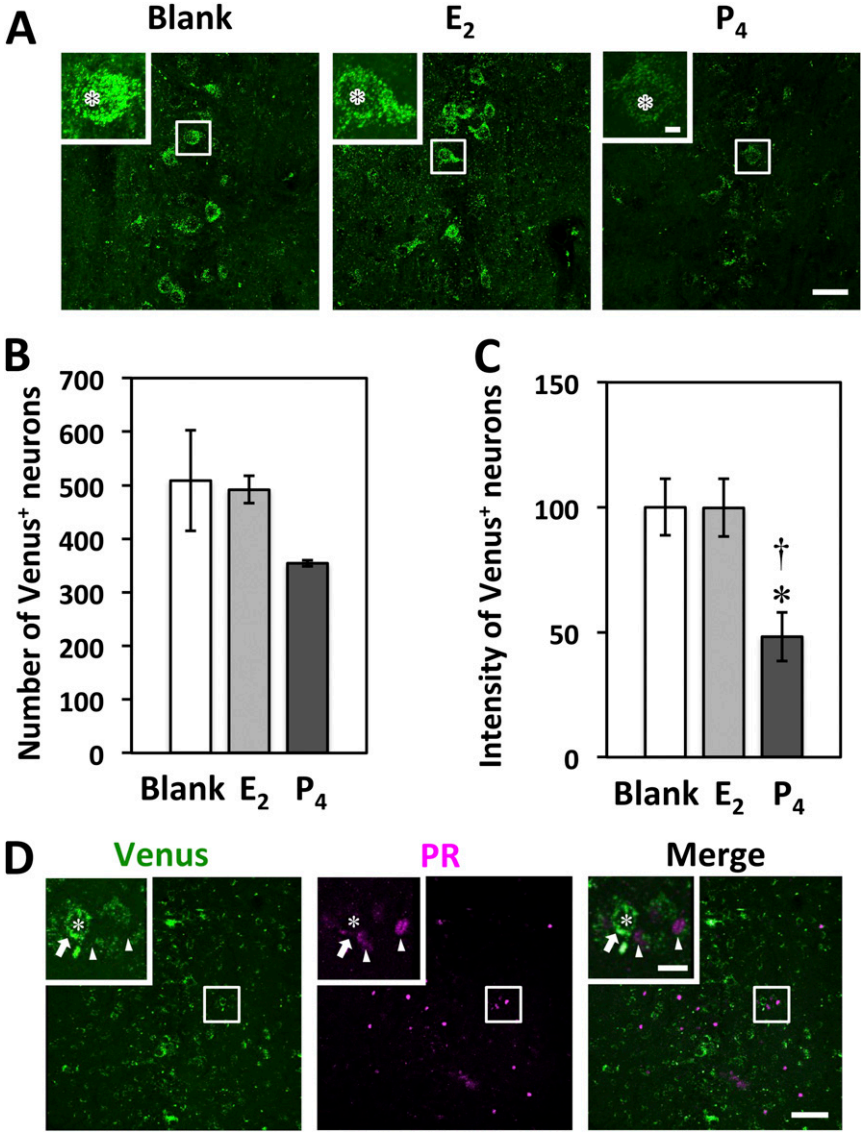

Figure 4. Effects of chronic E_2 or P_4 treatment on Venus expression in the lumbar spinal cord (L3 to L4 level) of testis-intact Tg males. (A) Venus fluorescence was decreased by chronic P_4 treatment (n = 3) compared with control (blank) (*n* = 3) and chronic E_2 treatment (n = 3). (B) Chronic P_4 treatment tended to decrease the number of Venus$^+$ neurons compared with control (blank) and chronic E_2 treatments. (C) The intensity of Venus$^+$ neurons was significantly decreased in P_4-treated males than in control and E_2-treated males. (D) Immunohistochemistry for PR in the lumbar cord of Tg females showed that most Venus$^+$ neurons (green; arrows) did not express PRs (magenta; arrowheads) (n = 4). The blocked areas are enlarged. Asterisks in (A) and (D) indicate the location of the neuronal nuclei expressing Venus. Data are presented as mean ± SEM. Scale bars, 50 μm in the low magnification of (A), 10 μm in the high magnification of (A), 100 μm in the low magnification of (D), 20 μm in the high magnification of (D). $^*P < 0.05$, *vs* blank; $^{\dagger}P < 0.05$, *vs* E_2.

Effect of sex steroid hormones on *GRP* expression *in vitro*

To determine whether the sex steroid hormones have an effect on *rGrp* expression using Venus fluorescence as a reporter, we assessed the reporter activity using HEK293T cells transfected with the AR expression plasmids and *rGrp-promoter-Venus* plasmids. Venus fluorescence was markedly enhanced in the cells treated with DHT alone, but attenuated in cells exposed to DHT plus E_2 and especially after DHT plus P_4 (Fig. 6A). The tdTomato was transfected to normalize the transfection efficiency, and we confirmed that the red fluorescence did not change among all groups (Fig. 6A). To quantify the sex steroid effect, we performed the qPCR for *Venus* mRNA using sex steroid hormone–treated cells. *Venus* mRNA expression was significantly increased by DHT treatment ($F_{5,30} = 52.9$; *vs* control, $P < 0.05$) (Fig. 6B). Moreover, the DHT effect was completely blocked in cells treated with DHT plus P_4 (*vs* DHT, $P < 0.05$) (Fig. 6B). Subsequently, to study the *hGRP-promoter*

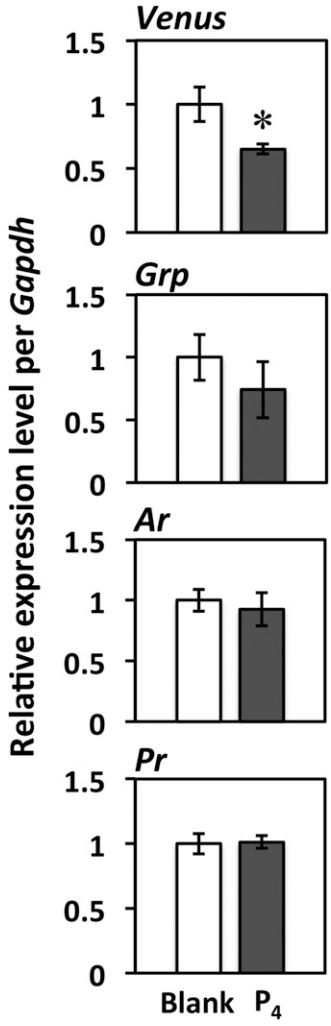

Figure 5. Effects of chronic P_4 treatment on *Venus*, *Grp*, *Ar*, and *Pr* expressions at the transcription level. P_4 treatments (n = 5) significantly decreased *Venus* expression in the lumbar spinal cord of transgenic males compared with blank control males (n = 3). *Grp* expression in Wt males also decreased by ~25% in P_4-treated group (n = 5) compared with blank controls (n = 5). *Ar* and *Pr* expression were clearly unchanged by P_4 treatment in Wt males. Data are presented as mean ± SEM. $*P < 0.05$, *vs* blank.

response to the sex steroid hormones, we performed luciferase assays using HEK293T cells that were transfected with reporter plasmids containing the *hGRP-promoter* fused with the *F-luc* gene (Fig. 6C). In these cells, the *hGRP-promoter* was also activated by DHT treatment, and P_4 treatment again significantly reduced the DHT-induced *GRP* expression in HEK293T cells ($F_{5,30} = 339$; *vs* control, $P < 0.05$; *vs* DHT, $P < 0.05$) (Fig. 6C). Although the *in vitro* effects of DHT and P_4 were consistent between the rat and human GRP promoter, those of E_2 were not. For the rat promoter, although E_2 caused some inhibition of the DHT effect, it increased promoter activity in comparison with the control ($F_{5,30} = 52.9$; *vs* control, $P < 0.05$; *vs* DHT, $P < 0.05$) (Fig. 6B). For the human promoter, E_2 not only more profoundly inhibited the DHT effect but also inhibited promoter activity in comparison with control ($F_{5,30} = 339$; *vs* control, $P < 0.05$; *vs* DHT, $P < 0.05$) (Fig. 6C).

Discussion

This study was designed to reveal the effects of sex steroid hormones on the spinal GRP system controlling male sexual function. To visualize the spinal GRP-expressing neurons *in vivo*, we generated Tg rats expressing Venus under the control of the *rGrp-promoter*. In these Tg rats, the spinal GRP neurons controlling male sexual function were clearly labeled with Venus fluorescence. As expected, Venus fluorescence in the male lumbar cord was markedly reduced after ORX and restored by chronic androgen replacement. In this study, we further found that androgen-induced Venus (and thus *Grp*) expressions were markedly attenuated by chronic treatment with P_4 and less consistently with E_2. To our knowledge, this is the first demonstration that ovarian hormones could inhibit GRP expression in the lumbar cord, suggesting that P_4 and E_2 could be important feminizing factors in the spinal cord of females at least during pubertal development. Therefore, it is also suggested that P_4 attenuates the spinal GRP system controlling male sexual function that is not necessary in females. Of course, both male and female rats will be subject to high levels of ovarian P_4 during their prenatal development. Although the developing gonad does not actively produce steroids before puberty (20), fetuses are exposed to maternal P_4 and E_2 that readily cross the placenta and are presumably present in mother's milk in neonates (21). Thus, ovarian hormones might also contribute to inhibit the spinal GRP expression during neonatal life. Indeed, we recently reported that the male-dominant number of GRP neurons is not observed at 23 days after birth (just before puberty onset) in rats (16). We therefore suggest that GRP expression in the lumbar cord is only initiated at the time of puberty by the increasing androgens in males.

We have previously demonstrated that ORX of adult male rats reduced the expression of *Ar* mRNA in the lumbar spinal cord and attenuated GRP expression (22) and also that an entirely feminine pattern or hyperfeminine appearance of the spinal GRP system exists in two AR-deficient models of genetic males: (1) genetically male (XY) rats carrying a testicular feminization mutation of *Ar* genes, which express a defective AR protein (14, 22), and (2) a mouse line specifically lacking *Ar* genes in the nervous system, that is, the AR^{NesCre} mouse model (19). The present study using HEK293T cells demonstrated that the *rGrp-promoter* was activated by androgens *in vitro*. A possible ARE consensus sequence has been identified at ~726 bp and ~1062/1689 bp upstream

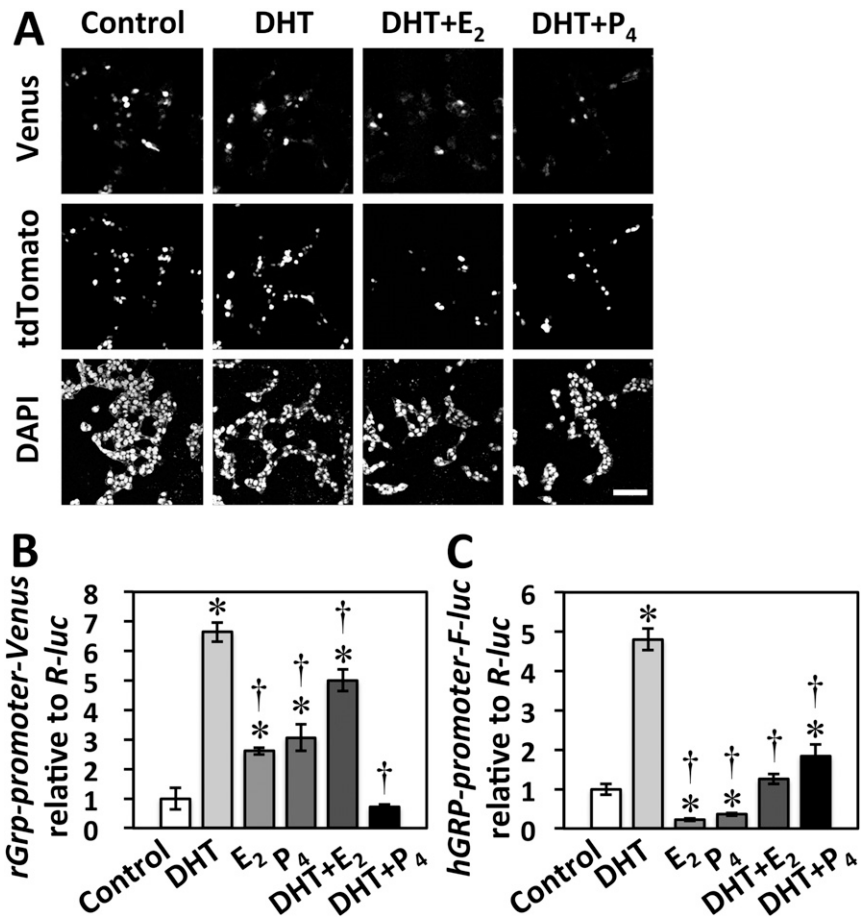

Figure 6. Reporter activity of (A and B) *rGrp-promoter* and (C) the *hGRP-promoter* in HEK293T cells. (A) Venus fluorescence was observed in HEK293T cells transfected with the *rGrp-promoter-Venus* plasmids. DHT treatments increased the Venus fluorescence compared with controls. DHT plus P_4 treatment decreased the Venus fluorescence compared with DHT-treated cells. The tdTomato was transfected to normalize transfection efficiency. (B) Venus mRNA levels in HEK293T cells treated with sex steroid hormones. DHT treatment significantly increased the Venus expression level compared with control. P_4 treatment concurrently with DHT inhibited the increase of Venus expression in DHT treated cells. (C) Reporter activity of *hGRP-promoter* in HEK293T cells. DHT significantly increased the *hGRP-promoter* activities. P_4 treatment concurrently with DHT inhibited the activation of the *hGRP-promoter* by DHT treatments. Transcripts of *F-luc* and *Renilla* luciferase (*R-luc*), which transfected to normalize transfection efficiency, were quantified by qPCR. For the rat promoter, although E_2 caused some inhibition of the DHT effect, it increased promoter activity in comparison with (B) the control. For the human promoter, E_2 not only more profoundly inhibited the DHT effect but also inhibited promoter activity in comparison with (C) the control. Data are presented as mean ± SEM. Scale bar, 100 μm. $^*P < 0.05$, *vs* control; $^\dagger P < 0.05$, *vs* DHT. DHT+ E_2, 5α-dihydrotestosterone plus estradiol-17β; DHT+ P_4, 5α-dihydrotestosterone plus progesterone.

We previously reported that, in female rats, spinal GRP neurons were barely detectable at puberty when circulating E_2 and P_4 levels are rising (16). This indicated that ovarian steroids E_2 and/or P_4 play an important role in the feminization or demasculinization of the male-dominant spinal GRP system during puberty, possibly by inhibiting GRP expression in the female lumbosacral spinal cord. We have also previously demonstrated that the spinal GRP neurons express ARs but not estrogen receptors (14). However, no information was available for the expression of PR in the spinal GRP neurons. Therefore, it was unclear why GRP expression declined during female puberty. We now show that P_4 treatment concurrent with DHT markedly inhibited the upregulation of GRP expression by DHT in HEK293T cells transfected with plasmids containing AR genes but not PR genes. It has been reported that P_4 can antagonize AR activity (23) and treatment with the P_4 antagonist mifepristone (RU486) significantly increased AR expression in monkey and human endometrium (24). However, our qPCR analysis showed that *Ar* or *Pr* mRNA expression in the lumbar cord was not affected in P_4-treated males. Using the *rGrp-promoter-Venus* Tg rats, we showed that the expression of *Venus* mRNA in the spinal cord was decreased by chronic P_4 treatment to testis-intact adult males, but that the Venus$^+$ neurons did not express PRs, again indicating that P_4 may act to inhibit the spinal GRP expression *via* an AR-mediated mechanism. Taken together, these results suggest that elevated circulating P_4 in female puberty inhibits GRP expression in the lumbar cord *via* an AR-mediated mechanism. As a consequence, lumbar spinal GRP neurons are barely detectable during and after puberty in females (16).

of the *rGrp* and *hGRP* open reading frame, respectively (unpublished data). Taken together, these results indicate that androgens activate the *rGrp/hGRP-promoter* activity *via* ARs, resulting in the male-dominant expression of GRP in rats as well as in humans. Although in the present study we show that P_4 can act to profoundly inhibit the spinal GRP system *via* an AR-mediated mechanism, unknown mechanisms regulating the activation or inactivation of ARE on the *rGrp/hGRP-promoter* may develop the sex differences in response to androgens or P_4 in the central nervous system or other organs.

Recently, we reported that the sexually dimorphic spinal GRP system can be identified not only in rodents but also in the Asian house musk shrew (*Suncus murinus*; order of Eulipotyphla, formerly Insectivora) (25) and, in a nonhuman primate, the Japanese macaque monkey (*Macaca fuscata*) (26), suggesting that the sexually

dimorphic spinal GRP system is widespread in mammals. Taken together with our present results, we predict that a spinal GRP system controlling male sexual function is present in the human spinal cord and that circulating androgens could regulate the spinal GRP system in humans. Clinical data show that erectile dysfunction is an increasingly common condition predicted to affect >300 million men worldwide by 2025 (27, 28). The increasing rates may reflect the increasing stress in society and could contribute to the declining birth rate that is becoming a serious social problem in certain societies (28). It has long been established that erectile dysfunction is a multifactorial dysfunction, but most treatments, including sildenafil citrate, have targeted penile vasculopathy (29). Present treatments can have unwanted side effects and, importantly, fail to address the underlying neural control problem. Our findings suggest an avenue for treatments targeting the spinal GRP system to ameliorate sexual problems in men.

It is also well documented that GRP and the GRP receptor are often excessively expressed in cancer cells such as small cell lung cancer, prostate cancer, and gastrointestinal tract cancer (30–33). Pro-GRP is known to be a specific tumor marker for the small cell lung cancer (33), because the half-life of pro-GRP in the circulation is much longer than that of GRP. The GRP receptor is also important in prostate cancer growth and progression (32), and cell growth and progression of LNCaP, a prostate cancer cell line, are induced by the GRP signaling in an androgen-dependent manner (32, 34). Our finding that P_4 can decrease GRP expression could provide a new therapeutic approach to the androgen-dependent development of cancer cells.

In summary, we report in the present study the generation of a *rGrp-promoter-Venus* Tg rat line, which could be useful for investigating the hormonal regulation of *rGrp* expression simply by examining Venus fluorescence without staining *in vivo*. Using this Tg rat model, we found that P_4 but not E_2 is probably an important feminizing factor in the developing female spinal cord. Using the human construct *hGRP-promoter-F-luc*, we further revealed modulations of *hGRP* gene expression by sex steroid hormones in human cells. Consequently, it is likely that circulating androgens also regulate the spinal GRP system controlling sexual function in men. Further understanding of the regulatory mechanisms of the spinal GRP system in humans may provide a new approach to treating sexual problems in men as well as cancers associated with GRP expression. Because the Venus expression in the spinal GRP neurons was highly sensitive to sex steroid manipulations, this Tg rat line appears to be a powerful tool for *in vivo* analyzing the hormonal regulations of the spinal GRP neural circuits underlying male sexual activity.

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Author Contributions: T.O., K.I.M., and H.S. generated the transgenic rats. T.O., K.T., and H.S. performed histological and biochemical experiments. T.O., S.I., and T.U. performed the molecular and *in vitro* experiments. M.K., J.S., O.U., and T.S. interpreted the data and provided the advice and equipment. T.O. and H.S. wrote the paper. H.S. supervised the whole study. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Disclosure Summary: The authors have nothing to disclose.

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