

Identification of the sexually dimorphic gastrin-releasing peptide system in the lumbosacral spinal cord that controls male reproductive function in the mouse and Asian house musk shrew (*Suncus murinus*)

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20 Abbreviated title: Sexually dimorphic GRP system in mice and suncus

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Grant sponsor: This work was supported in part by KAKENHI from the Ministry of
Education, Science, Sports, Culture and Technology (MEXT), Japan (to Keiko T;
26870496, 15J40220 and HS; 24680039, 15K15202, 15H05724), by the Naito
Memorial Grant for Natural Science Researches, Japan (to Keiko T and HS), by the
35 Kato Memorial Bioscience Foundation, Japan (to HS), by the Life Science Foundation
of Japan (to HS), and by the Ryobi Teien Memory Foundation, Japan (to HS). This
work was also supported by MEXT KAKENHI (suncus genome resource; 221S0002).
Keiko T and TO are supported by Research Fellowships of JSPS for Young Scientists.

ABSTRACT

Several regions of the brain and spinal cord control male reproductive function. We previously demonstrated that the gastrin-releasing peptide (GRP) system, located in the
5 lumbo-sacral spinal cord of rats, controls spinal centers to promote penile reflexes during male copulatory behavior. However, little information exists on the male-specific spinal GRP system in animals other than rats. The objective of this study was to examine the functional generality of the spinal GRP system in mammals using the Asian house musk shrew (*Suncus murinus*; suncus named as the laboratory strain), a specialized placental
10 mammal model. Mice are also used for a representative model of small laboratory animals. We first isolated complementary DNA encoding GRP in suncus. Phylogenetic analysis revealed that suncus preproGRP was clustered to an independent branch. Reverse transcription-PCR showed that GRP and its receptor mRNAs were both expressed in the lumbar spinal cord of suncus and mice. Immunohistochemistry for
15 GRP demonstrated that the sexually dimorphic GRP system and male-specific expression/distribution patterns of GRP in the lumbo-sacral spinal cord in suncus are similar to those of mice. In suncus, we further found that most GRP-expressing neurons in males also express androgen receptors, suggesting that this male-dominant system in suncus is also androgen-dependent. Taken together, these results indicate that the
20 sexually dimorphic spinal GRP system exists not only in mice but also in suncus, suggesting that this system is a conserved property in mammals.

(231/250 words)

INDEXING TERMS: gastrin-releasing peptide; spinal cord; sexual dimorphism; male
25 reproductive function; *Suncus murinus* (suncus); RRID: AB_2571636; RRID: AB_626757; RRID: AB_2060157

Several regions of the brain and spinal cord control male reproductive function, such as erection and ejaculation. Lower spinal cord injuries frequently cause sexual dysfunction in men, including erectile dysfunction and ejaculation disorders (Sipski, 1998; Brown et al., 2006), suggesting that important neural centers for male reproductive function are located within the lower spinal cord (Breedlove and Arnold, 1983a; b; Breedlove, 1985; Morris et al., 2004; Matsuda et al., 2008; Sakamoto, 2012). Previously, we demonstrated that a population of gastrin-releasing peptide (GRP)-expressing neurons in the lumbosacral spinal cord plays a key role in erection and ejaculation in rats (Sakamoto et al., 2008; Sakamoto et al., 2014). This system of GRP neurons is sexually dimorphic, being prominent in male rats but vestigial or absent in female rats, and controls male reproductive function through an androgen receptor (AR)-mediated mechanism (Sakamoto et al., 2008; Sakamoto et al., 2009b; Sakamoto et al., 2014). Therefore, it has been demonstrated that the male-dominant spinal GRP and its specific receptor (GRP receptor; GRPR) are important in the regulation of penile reflexes during copulatory behavior in rats (Sakamoto and Kawata, 2009; Sakamoto, 2011).

However, little information exists on the spinal GRP system controlling male reproductive function in animals other than rats. The Asian house musk shrew, *Suncus murinus* (suncus named as the laboratory strain), is of the order Eulipotyphla (formerly Insectivora) and thought to resemble specialized placental mammals (eutherians) (Vogel, 2005). Although many aspects of sexual differentiation and reproductive physiology in suncus are unique, the role of reduced androgens in behavioral masculinization is similar to primates than to mice and rats (rodents) (Ewton et al., 2010). In addition, suncus is a reflex ovulator (Inoue et al., 2011); pregnant suncus ovariectomized on days 6–15 of a 30-day (average gestation period in suncus) pregnancy can maintain pregnancy in the absence of ovaries (Hasler and Nalbandov, 1978). Genetic evidence also indicates that the suncus is more closely related to humans than to other common animal models (Hoyle et al., 2003), which may bridge to the primate study. In this study, therefore, we examined the functional generality of the spinal GRP system in eutherians using two different mammalian species: suncus as a model for specialized placental mammals and mice as a representative model for small laboratory rodents. The molecular evolution of the sexually dimorphic spinal GRP system is also discussed.

MATERIALS AND METHODS

Animals

5 Male and female adult suncus (12–52 weeks old) of an outbred KAT strain, established from a wild population in Kathmandu, Nepal, were used in this study. Suncus were maintained according to the established procedure previously described (Goswami et al., 2015). Male and female adult mice of the BALB/c strain (10–12 weeks old) were used in this study. Mice were maintained in an established manner. All experimental procedures were approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Okayama University (Okayama, Japan) and by Okayama University of Science (Okayama, Japan). All efforts were made to minimize animal suffering and reduce the number of animals used in this study.

15 RNA extraction and molecular cloning of suncus *Grp* and *Grpr* genes

Adult male suncus ($n = 2$) were deeply anesthetized with intraperitoneal

injections of 50 mg/kg body weight sodium pentobarbital and sacrificed by blood loss.

Immediately, dissected tissues [hypothalamus (ventral part of the diencephalon), lumbar spinal cord (identified by the lumbar enlargement as an anatomical reference), and stomach (pyloric part)] from male suncus were fixed with RNALater solution (Ambion,

5 Austin, TX) and stored at -80°C until RNA extraction. Total RNA was extracted from samples using Sepasol-RNA I Super G kit (Nacalai Tesque, Kyoto, Japan) according to the manufacture's protocol. The concentration of total RNA was measured using Qubit RNA assay kit (Thermo Fisher Scientific, Waltham, MA). First-strand complementary DNA (cDNA) was synthesized from 200 ng of total RNA with oligo-dT primers using
10 Omniscript RT Kit (QIAGEN, Hilden, Germany).

The predicted suncus *Grp* and *Grpr* sequences were obtained from an unpublished suncus genome resource. To confirm the predicted suncus *Grp* and *Grpr* sequences, reverse transcription (RT)-PCR was performed. The sequences of primers for RT-PCR analysis were designed based on the genome resource (see Table 1). The
15 resulting PCR amplicons (full open reading frame sequence for *Grp* or partial sequence for *Grpr*) were subcloned into the pGEM-T easy vector (Promega, Madison, WI)

followed by transfection into *Escherichia coli* DH5 α competent cells (Takara Bio, Shiga, Japan). Positive clones were identified by blue-white screening and at least three positive clones were sequenced by the Sanger method.

5 **Phylogenetic analysis**

Alignment of the amino acid sequences of the GRP precursor protein from different species was performed using the ClustalW sequence alignment program. Phylogenetic trees were constructed using the neighbor-joining method and viewed with TreeView (Version 1.6.6). Details of the program settings are given in the legend for

10 Figure 1.

RT-PCR of *Grp* and *Grpr* using the lumbar spinal cord and stomach in suncus and mice

To determine the expression of *Grp* and *Grpr* mRNA in the lumbar spinal
15 cord and stomach of suncus ($n = 4$ of each sex) and mice ($n = 4$ of each sex), RT-PCR analysis was performed. Total RNA was extracted from these tissues, using the method

described above. First-strand cDNA was synthesized from 200 ng of total RNA in a 20 μ L reaction volume using oligo-dT primers and Omniscript RT (QIAGEN). The RT-PCR conditions for target genes and β -actin as the internal control are shown in Table 1; resultant PCR amplicons were electrophoresed on 2% agarose gels. RT-PCR studies were repeated four times using independently extracted RNA samples from different animals. Consistent results were obtained from each run.

Immunohistochemistry (IHC) and immunofluorescence

Suncus ($n = 5$ of each sex) and mice ($n = 5$ of each sex) were transcardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) under deep pentobarbital anesthesia (see above). Lumbosacral spinal cords were quickly removed and immersed in the same fixative for 4 hours at room temperature with gentle agitation. After immersion in 25% sucrose in 0.1 M PB at 4°C for cryoprotection until they sank, the preparations were quickly frozen using powdered dry ice and cut into 30 μ m cross or horizontal sections using a cryostat (CM3050 S, Leica, Nussloch, Germany). We performed IHC analysis according to our

established methods (Sakamoto et al., 2008; Sakamoto et al., 2010; Sakamoto et al., 2014). In brief, endogenous peroxidase activity was eliminated from the sections by incubation in a 1% H₂O₂ absolute methanol solution for 30 minutes followed by three 5-minute rinses with phosphate buffered saline (PBS) (pH 7.4). These processes were omitted in the immunofluorescence method. After blocking nonspecific binding with 1% normal goat serum and 1% BSA in PBS containing 0.3% Triton X-100 for 1 hour at room temperature, sections were incubated with primary rabbit antiserum against mouse GRP₂₀₋₂₉ (1:2,000 dilution), a 10-amino acid-peptide called neuromedin C (NMC) or GRP-10 (AssayPro, St. Charles, MO) for 48 hours at 4°C. The GRP antiserum used in this study has previously been shown to be specific for rodent GRP in the spinal cord (Takanami et al., 2014; Satoh et al., 2015b; c). Immunoreactive (ir)-products were detected with a streptavidin-biotin kit (Nichirei, Tokyo, Japan), followed by diaminobenzidine (DAB) development according to our previous method (Sakamoto et al., 2008). GRP-expressing cells in the spinal cord were localized using an Olympus (Tokyo, Japan) Optical BH-2 microscope. To determine the projection site of GRP-ir axons, double-immunofluorescence staining for GRP (1:1,000 dilution) and neuronal

nitric oxide synthase (1:5,000 dilution) (nNOS; A-11; mouse monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA), a marker protein for neurons in the sacral parasympathetic nucleus (SPN), was performed as described previously (Sakamoto et al., 2008; Sakamoto et al., 2014). Alexa Fluor 546-linked anti-mouse IgG (Molecular Probes, Eugene, OR) and Alexa Fluor 488-linked anti-rabbit IgG, both raised in goats (Molecular Probes), were used at a 1:1,000 dilution for detection. Immunostained sections were imaged with a confocal laser scanning microscope (FluoView 1000, Olympus, Tokyo, Japan).

Finally, to characterize the GRP neurons in the male lumbar spinal cord, AR/GRP-double IHC was performed using a DAB-nickel/*NovaRED* (Vector Laboratories, Burlingame, CA) method in suncus ($n = 3$) and mice ($n = 4$). After blocking nonspecific binding, spinal sections were incubated with rabbit monoclonal AR antibody (Epitomics/abcam, Burlingame, CA) at a dilution of 1:1,000 for 4 days at 4°C. Sections were processed using the same immunoperoxidase procedure, enhancing the DAB reaction with 0.02% nickel chloride to produce a blue-black reaction product. After AR-IHC, sections were then reacted with 1% H₂O₂ in absolute methanol for 30

minutes at room temperature, followed by GRP-IHC with the second chromogen reaction performed using *NovaRED* to produce a light red-brown reaction product. In addition, another double staining both in the dark (for GRP) and bright (for AR) fields was also conducted in suncus ($n = 1$) and mice ($n = 3$). After AR-IHC with a DAB-nickel method, GRP-IHC was subsequently performed as an immunofluorescence. Alexa Fluor 488-linked goat anti-rabbit IgG was used. Immunostained sections were imaged with a confocal laser scanning microscope. The bright field images were captured with a differential interference contrast mode. Using the Adobe Photoshop[®] (Adobe Systems, San Jose, CA) software package, the bright field image was inverted to a dark field (inversion) image, and pseudo-colored in magenta. Because GRP-ir cell bodies were easily identified in mice, the GRP/AR-double positive ratio was analyzed using horizontal sections (only in mice).

Antibody characterization

Full details of all the antisera and antibodies used in this study are shown in Table 2. The rabbit polyclonal GRP antiserum (11081-05015; AssayPro) was raised

against a peptide consisting of amino acids 20–29 of mouse GRP. This antiserum produced identical patterns of labeling in a population of GRP neurons in the lumbosacral spinal cord as that achieved by IHC using the same (Takanami et al., 2014; Satoh et al., 2015b) or other antisera (Sakamoto et al., 2008) in rats. Control procedures

5 for the DAB method were performed using pre-absorption of the working dilution (1:1,000) of the primary antiserum with saturating concentration of suncus GRP₁₇₋₂₇ antigen peptide, Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met [50 µg/mL: ²[Asn]-NMC (or GRP-10), produced in AnaSpec; San Jose, CA] or mouse GRP₂₀₋₂₉ antigen peptide, Gly-Ser-His-Trp-Ala-Val-Gly-His-Leu-Met (50 µg/mL: ²[Ser]-NMC, produced in

10 AnaSpec) overnight at 4°C before use. The GRP-ir cells were detected according to the above protocol for peroxidase IHC. Light intensity and exposure time were the same for sections stained with the GRP and pre-absorbed antisera.

nNOS is a well-characterized marker protein for a population of preganglionic parasympathetic neurons in the spinal cord as reported previously (Vizzard et al., 1995;

15 Studeny and Vizzard, 2005). The mouse monoclonal antibody for nNOS (sc-3502; Santa Cruz Biotechnology) was raised against amino acids 2–300 of human nNOS. This

antibody was enrolled in *The Journal of Comparative Neurology*'s online antibody database V14. This antibody has been used to label the spinal neurons in the superficial laminae and the neurons and fibers in the intermediolateral cell column of lumbar spinal cord in pigs (Russo et al., 2013). This antibody produced identical patterns of the labeling in the neurons and fibers in the sacral parasympathetic nucleus in suncus and mice in our study (Fig. 6). This antibody weighs approximately 160 kDa (theoretical molecular weight) on Western blots of porcine dorsal root ganglion tissue (Russo et al., 2013). The rabbit monoclonal antibody for AR (EPR1535(2); Epitomics/abcam) was raised against a synthetic peptide corresponding to human AR (ab191380; abcam).

10 Other registered antibodies for AR in *The Journal Comparative Neurology*'s online antibody database V14 revealed the predominant immunolabeling of nuclei in the rat hypothalamus (Bingham et al., 2006; Wu et al., 2009) and brainstem (Normandin and Murphy, 2008) and recognizes a band approximately 110 kDa on the Western blots of rat and mouse lumbar spinal cord tissues (Sakamoto et al., 2009a; Renier et al., 2014).

15 This antibody showed the nuclei specific identical staining in the rat hypothalamus neurons in our study (data not shown), in the mouse brainstem neurons (Mukudai et al.,

2016), and in the spinal nucleus of the bulbocavernosus motoneurons in the mouse (Smith et al., 2012). In this study, we checked the specificity of the mouse monoclonal antibody for nNOS and the rabbit monoclonal antibody for AR using Western blotting both in the diencephalon and spinal cord of the mouse as well as suncus. Western blotting was conducted according to our previously described methods (Sato et al., 2015a). In brief, adult male suncus ($n = 3$) and mice ($n = 2$) were sacrificed by blood loss under deep pentobarbital anesthesia (see above). Brains and spinal cords were quickly removed and placed on ice, and the diencephali and lumbar spinal cords were carefully dissected under a dissecting microscope (Olympus, Tokyo, Japan). Samples were snap-frozen immediately in liquid nitrogen, and used for Western analysis. The preparations from suncus for nNOS (25 μ g diencephali and 100 μ g lumbar spinal cords protein/lane) and AR (50 μ g diencephali and 100 μ g lumbar spinal cords protein/lane), and from mice for nNOS (25 μ g diencephali and 100 μ g lumbar spinal cords protein/lane) and AR (100 μ g diencephali and lumbar spinal cords protein/lane) were boiled in 10 μ L sample buffer containing 62.5 mM trishydroxymethyl-aminomethane-HCl (Tris-HCl; pH 6.8), 2% SDS, 25% glycerol,

10% 2-mercaptoethanol, and a small amount of bromophenol blue. Samples were run on a 4-20% SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA) using a semidry blotting apparatus (Bio-Rad Laboratories). Membranes were blocked with PVDF Blocking Reagent for
5 Can Get Signal (TOYOBO, Tokyo, Japan) for 30 minutes at room temperature and incubated for 1 hour at room temperature in Can Get Signal Solution 1 (TOYOBO) with a 1:5,000 dilution of mouse monoclonal antibody against nNOS, or with a 1:1,000 dilution of rabbit monoclonal antibody against AR. Blotted membranes were washed three times with 3% BSA and 0.05% Tween 20 in Tris-HCl buffered saline (TBST) and
10 incubated with horseradish peroxidase-conjugated goat polyclonal antibody against mouse IgG (Bio-Rad Laboratories) at 1:10,000 dilution, or with horseradish peroxidase-conjugated goat polyclonal antibody against rabbit IgG (Bio-Rad Laboratories) at 1:10,000 dilution in Can Get Signal Solution 2 (TOYOBO) for 1 hour at room temperature. After washing for three times with TBST, blots were visualized by
15 Immun-Star WesternC Chemiluminescence Kit (Bio-Rad Laboratories). Images of the different immunoblots were slightly adjusted in brightness and contrast to provide a

uniform background.

Morphological analysis

Morphological analysis of GRP expression (GRP-ir) in neurons of the anterior lumbar spinal cord (L3–L4 level) was performed using horizontal sections (30 μm). We first counted GRP neurons in the lumbar spinal cord in mice according to our established methods ($n = 5$ of each sex) (Sakamoto et al., 2008; Sakamoto et al., 2014; Katayama et al., 2016; Oti et al., 2016). Briefly, we counted the number of GRP-ir cell bodies at 200 \times magnification in all sections and analyzed a 600 μm^2 area (approximately ten–fifteen 30- μm -thick sections per animal). GRP neurons were identified by the 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 15–30 μm), and clear round nuclei (diameters approximately 15 μm). To avoid overestimating cell number, only GRP-ir neurons that contained a round, transected nucleus were counted. Because the mean diameter of nuclei in GRP neurons is much smaller than the

30 μm sections, this analysis reduced overestimation of the number of neurons.

In suncus, because it is hard to identify round nuclei of GRP-positive somata, we performed a semi-quantitative analysis of GRP expression level ($n = 5$ suncus for each sex). To determine the density of GRP-ir in the lumbar spinal cord (L3–L4 level),
5 the unit area ($312 \times 312 \mu\text{m}^2$) localized to the midline at the center was analyzed. The optical density of GRP staining was determined using black-and-white images that were converted from micrographs using ImageJ software (ImageJ 1.44p; National Institutes of Health, Bethesda, MD). At least twelve 30 μm thick sections per suncus were analyzed. The optical density of background labeling was estimated by comparisons
10 with similar areas of control sections reacted with the anti-GRP antiserum that was incubated first with an excess of peptide antigen (50 $\mu\text{g}/\text{mL}$), as described above. GRP expression was undetectable in these sections. Each threshold optical density was determined by normalizing the data to those of the preabsorbed sections. The GRP-ir-fiber pixel density was semi-quantitated as the average pixel density in the
15 spinal cord of each animal, and the data were expressed as the ratio to that of females.

The number of GRP neurons in mice and the optical density of GRP-ir in

suncus were expressed as the mean \pm standard error of the mean (S.E.M.) in each sex, respectively, and were analyzed by a Student's *t*-test. $P < 0.05$ was considered statistically significant. 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. All tissues (male and female) were processed simultaneously and under identical conditions to permit conclusions to be made between genders of different species.

10 RESULTS

Cloning and sequence analysis of suncus *Grp* cDNA

We first cloned cDNA encoding GRP in suncus (Fig. 1). This sequence is available in GenBank (accession number: suncus *Grp*; LC138361). The deduced amino acid sequence of suncus preproGRP started with a signal peptide, followed by the bioactive GRP₁₋₂₇ (mature GRP) including the NMC motif at the C terminus of mature GRP, which is highly conserved in mammals, and a carboxyl-terminal extension

peptide termed pro-GRP₃₁₋₁₁₆ in suncus (pro-GRP₃₃₋₁₂₃ in mice; pro-GRP₃₃₋₁₂₄ in rats; pro-GRP₃₁₋₁₂₅ in humans) (Fig. 1A). The mature GRP in suncus shared high similarity, particularly at the identical NMC (GRP-10) region, to human but not to rodents (Fig. 1A). To investigate the evolutionary relationships of suncus GRP with other mammalian species, a phylogenetic tree based on the deduced amino acid sequence of preproGRP was constructed using the neighbor-joining method (Fig. 1B). According to the tree, suncus preproGRP was separated with other mammalian species and clustered to an independent branch that was proximal to the tree (Fig. 1B).

10 **RT-PCR of *Grp* and *Grpr* mRNA in the lumbar spinal cord in suncus and mice**

We examined the expression of *Grp* and *Grpr* mRNA in the lumbar spinal cord of suncus and mice by RT-PCR. To analyze the expression of *Grpr* mRNA by RT-PCR, we partially isolated *Grpr* cDNA from the suncus hypothalamus. This partial sequence was identical to the genome information (accession number in GenBank: suncus *Grpr*; LC149855). Total RNA from lumbar spinal cord and stomach (as a positive control) of adult male and female suncus ($n = 4$ of each sex) and mice ($n = 4$ of

each sex) were reverse-transcribed. The resultant cDNA mixture was used for PCR amplification with specific primers for the *Grp* or *Grpr* gene. Bands were detected at the expected sizes for *Grp* and *Grpr* genes (Fig. 2), indicating that *Grp* and *Grpr* mRNAs were expressed in adult male lumbar spinal cord of suncus as well as mice.

5 RT-PCR for β -actin was performed as the internal control in suncus and mice. Nearly equivalent amounts of β -actin cDNA were amplified from RNA preparations among these tissues, which confirmed no significant RNA degradation occurred and a proper RT was obtained (Fig. 2A, B; *each bottom panel*). No significant difference in the expression of *Grp* mRNA between sexes could be detected in the lumbar spinal cord of

10 suncus (Fig. 2A). In contrast, a male specific expression of *Grp* mRNA in the lumbar spinal cord was observed in mice (Fig. 2B). No sex differences were detected in the expression level of *Grpr* mRNA in the lumbar spinal cord or stomach of either suncus or mice (Fig. 2). Controls in which the RT treatment was omitted as the negative control showed a complete absence of bands in each experiment (Fig. 2). In addition, the

15 expressions of both *Grp* and *Grpr* mRNAs were detected in the suncus hypothalamus as the positive control (data not shown).

Antibody specificity and the distribution of GRP in the lumbosacral spinal cord of suncus and mice

The expression of GRP was next localized in the lumbosacral spinal cord of suncus and mice (Fig. 3). IHC analysis for GRP in male suncus and mice revealed that GRP-containing fibers in the lumbosacral spinal cord (L5–S1 level) were present in the spinal dorsal horn (DH), dorsal gray commissure (DGC), and SPN both in suncus (Fig. 3A) and in mice (Fig. 3C), similar to those of rats. The specificity of the GRP antiserum reactivity was also confirmed by control absorption experiments in which the primary rabbit antiserum against mouse GRP₂₀₋₂₉ was preabsorbed with an excess amount of suncus GRP₁₈₋₂₇ antigen peptide (²[Asn]-NMC); these experiments showed a complete absence of GRP-ir in the lumbosacral spinal cords of suncus ($n = 3$) (Fig. 3B). Similar results were also obtained in mice, absorbing with mouse GRP₂₀₋₂₉ antigen peptide (²[Ser]-NMC in mice) ($n = 3$) (Fig. 3D). IHC studies were repeated independently three times using different animals and produced similar results.

Antibody specificity and the expression of nNOS and AR proteins in the lumbar spinal cord of suncus and mice

Western blot analysis showed that the nNOS antibody labeled a single band at approximately the expected molecular weight of 155 kDa on the blots of suncus ($n = 3$) and mouse ($n = 2$) diencephalon and spinal cord (Fig. 4A). The AR antibody also labeled a band at approximately the expected molecular weight of 98 kDa on the blots of suncus ($n = 3$) and mouse ($n = 2$) diencephalon and spinal cord (Fig. 4B). We demonstrated in suncus and mouse diencephalon and spinal cord that the antibodies used in this study were specific for nNOS and AR. Moreover, we confirmed the expression of nNOS and AR in the diencephalon and spinal cord in suncus at the protein level.

Male-specific expression of GRP in the lumbosacral spinal cord in suncus and mice

Using immunofluorescence for GRP, we next examined the GRP-expressing neurons located in the lumbar spinal cord (L3–L4 level; possibly containing somata and neuropiles of GRP neurons) of male and female suncus (Fig. 5A, B). Because

GRP-positive neuron somata were not clearly visible in suncus, we compared the intensity of GRP-ir in the lumbar spinal cord of suncus. Semi-quantification analysis of GRP-ir confirmed that the intensity of GRP-ir in the lumbar spinal cord (L3–L4 level) of suncus was greater in males than in females ($n = 5$ of each sex, $P < 0.05$) (Fig. 5C).

5 Then, we examined the number of GRP-ir neurons located in the lumbar spinal cord (L3–L4 level) of male and female mice (Fig. 5D, E). The number of GRP-ir neurons was significantly fewer in females than in males in the lumbar spinal cord (L3–L4 level) of mice ($n = 5$ of each sex, $P < 0.01$), similarly to that of rats (Fig. 5F).

Subsequently, we examined the sexually dimorphic projection of
10 GRP-containing fibers to the SPN in the lumbosacral spinal cord (L5–L6 and S1 level; possibly containing GRP neuronal axon terminals) of suncus and mice because SPN provides autonomic preganglionic fibers to the genitalia (Morgan et al., 1981). Double immunofluorescence for GRP and nNOS, a marker for autonomic preganglionic neurons (Vizzard et al., 1995), clearly showed that GRP-containing fibers projected into
15 the SPN and possibly attached to the SPN neuronal neuropile in suncus (Fig. 6A–H) as well as mice (Fig. 6I–P). Consistent with the difference in the GRP soma area (L3–L4

level), double immunofluorescence for GRP and nNOS further revealed that the intensity of GRP-containing fibers was greater in males than in females in the autonomic center SPN and its intermediate region DGC in both suncus and mice (Fig. 6). We found no sex difference in GRP fibers in the spinal DH of suncus and mice (Fig. 6), which presumably processes non-autonomic sensory stimuli such as itch; these results are consistent with previous reports in mice (Sun and Chen, 2007) and rats (Takanami et al., 2014).

AR expression in GRP neurons in suncus and mice

10 To characterize the spinal GRP neurons in the lumbosacral spinal cord, AR/GRP-double IHC was further conducted in suncus and mice (Fig. 7). In suncus, we found that most GRP-expressing neurons in males also express AR ($n = 3$) (Fig. 7A, C). Similar results were observed in male mice. Nearly every GRP-positive neuron also contained AR in mice ($88.1 \pm 2.3\%$; $n = 4$) (Fig. 7B, D). Another double staining was
15 also conducted using a DAB/immunofluorescence combined method in suncus ($n = 1$) and mice ($n = 3$), showing a similar result (Fig. 7E, F). The intensity of GRP-expressing

somata in suncus was relatively weaker than that of mice. Thus, it is difficult to quantitatively detect GRP-positive but AR-negative neurons in suncus (Fig. 7).

5 DISCUSSION

This study demonstrates that the spinal GRP system shows a male-dominant sexual dimorphism in two different mammalian species; (1) suncus, which belongs to the order Eulipotyphla and (2) mouse, a representative rodent model. To the best of our knowledge, this is the first demonstration of the sexually dimorphic GRP system in the
10 lumbosacral spinal cord of mammals other than rodents.

Neuroanatomical generality of the spinal GRP system controlling male reproductive function in mammals remains elusive. In this study, we demonstrated that GRP-containing neurons in the lumbar spinal cord (L3–L4 level) of males express AR not only in mice but also in suncus. These GRP-containing neurons in the lumbosacral
15 spinal cord of males also express AR but do not express the estrogen receptor alpha subtype in rats (Sakamoto et al., 2008) or in mice (our unpublished observations). A

large body of literature suggests that a considerable amount of testosterone is transiently secreted from the testis (the androgen surge) masculinizes the rodent brain and spinal cord during the perinatal critical period (Morris et al., 2004; Matsuda et al., 2008; McCarthy, 2008). In accordance with the results from rats, the results of double IHC for

5 AR and GRP suggest that the sexually dimorphic spinal GRP system is also androgen-dependent in suncus and mice. In addition, the androgen surge during perinatal life and after puberty from the testis may contribute to establishing and maintaining the male-specific GRP system (Katayama et al., 2016; Oti et al., 2016). Sexually dimorphic motoneuron pools have also been identified in suncus that are

10 analogous to the spinal nucleus of the bulbocavernosus in rats and mice (Polak and Freeman, 2010); it is homologous to Onuf's nucleus in humans in that it innervates the striated perineal muscles attached to the base of the penis (Breedlove and Arnold, 1980; Sengelaub and Forger, 2008). Future study is needed to show the relationship between the spinal GRP system and the innervation of the penile muscles in suncus.

15 Although the expression and bioactivity of GRP in the mammalian nervous system has been reported (Battey and Wada, 1991; Ohki-Hamazaki et al., 2005; Roesler

et al., 2012; Takanami and Sakamoto, 2014), its molecular evolution remains unknown.

We have used bioinformatics to identify possible ortholog genes for GRP and GRPR in amphibians [*Silurana (Xenopus) tropicalis*] as well as teleosts [medaka fish (*Oryzias*

latipes), our unpublished observations]. In mammals, GRP is believed to be the

5 mammalian counterpart to the amphibian peptide, bombesin, which was isolated as a

potential anti-microbial peptide from frog skin (Anastasi et al., 1971). Further

understanding of the spinal GRP system in other vertebrates may provide new

approaches for the molecular and evolutionary origin of neural control mechanisms

underlying male reproductive behavior in vertebrates. In this regard, the question

10 remains of whether the spinal GRP system exists similarly in the lower spinal cord in

humans. Thus, future studies should include an emphasis on comparative neurology for

the spinal GRP system in other mammals, particularly humans and/or other primates.

Further understanding of the spinal GRP system in primates may provide new avenues

for therapeutic approaches to sexual problems in men.

15 The Asian house musk shrew (*Suncus murinus*) belongs to the order

Eulipotyphla and is believed to resemble one of the specialized placental mammals

(Vogel, 2005). In Eulipotyphla, we have found for the first time that the sexually dimorphic GRP system and the expression/distribution patterns of GRP are similar to those of rodents in the lumbosacral spinal cord (Sakamoto et al., 2008; Sakamoto, 2012; 2014; Takanami and Sakamoto, 2014). In addition, we found variability in *Grp* mRNA
5 expression in suncus male vs. female samples in this study. Because RT-PCR analysis may not be suitable for the quantification in suncus but just for the expression analysis, real-time quantitative PCR analysis may be required to draw a firm conclusion. Phylogenetic analysis revealed that suncus GRP was possibly separated with other mammalian species and clustered to an independent branch that was proximal to the
10 phylogenetic tree. In addition, GRP₁₈₋₂₇ in humans and suncus (or GRP₂₀₋₂₉ in mice and rats) is a possible C-terminal-fragment of mature GRP, which was isolated from porcine spinal cord and originally named NMC (Minamino et al., 1984). It is recommended that a more appropriate name may be GRP-10. It has been reported in mice and rats that the second amino acid of GRP-10 is a serine but an asparagine exists in this location in
15 most other mammals [*e.g.*, humans (Spindel et al., 1984), cattle (Lemaire et al., 1989), pigs (McDonald et al., 1979), sheep (Fraser et al., 1994), dogs (Reeve et al., 1983), and

guinea pigs (Shaw et al., 1987)]. In this study, we found that the deduced amino acid sequence of GRP-10 in suncus is identical to those of many mammals other than mice and rats. In contrast, it has been reported that the second amino acid of GRP-10 is a serine not only in mice and rats but also in other non-mammalian vertebrates, such as

5 chicks (McDonald et al., 1980; Ohki-Hamazaki et al., 2005), alligators (Wang and Conlon, 1993), frogs (Kim et al., 2001), and possibly cartilaginous fishes (Conlon et al., 1987), but, interestingly, not teleost fishes (²[Asn]) (Jensen and Conlon, 1992). Nevertheless, it is noteworthy that the GRP-10 sequence (²[Ser] or ²[Asn]) is well conserved among vertebrates (mostly ²[Asn] in mammals) including suncus, suggesting

10 that the C-terminal region of GRP (*i.e.*, the GRP-10 sequence) plays an important role in its biological activity. We postulate that this GRP-10 sequence may be critical for specificity of GRP-GRPR binding in mammals, given the molecular co-evolution for the ligand-receptor affinity. In fact, ²[Ser]-GRP-10 is widely used as a specific agonist for GRPR in neuro-pharmacological research in mice and rats [for reviews, see

15 references (Roesler et al., 2012; Takanami and Sakamoto, 2014)]. In this study, we also observed the expression of *Grpr* mRNA not only in the suncus diencephalon and spinal

cord but also in the stomach. Because GRP was first discovered as a gastrin-releasing factor in the porcine gastrointestinal tract (McDonald et al., 1979), this functional property of the brain-gut peptide also appears to be a conserved property in mammals.

While the expression sites of GRPR in the suncus nervous system have never been reported, future attention should be focused on the expression and biological action of GRPR in the lumbosacral spinal cord controlling male reproductive function in suncus.

We found GRP-positive fibers in the spinal DH in suncus as well as in mice.

The expression of GRP in the rodent spinal DH is not sexually dimorphic and is thought to be involved in the transmission of the itch sensation (Sun and Chen, 2007; Sun et al.,

2009; Sakamoto et al., 2014). Our present results in suncus are consistent with previous reports in mice (Sun and Chen, 2007; Sun et al., 2009) and rats (Sakamoto et al., 2009b;

Sakamoto et al., 2014; Takanami et al., 2014). It has been demonstrated in mice that the somatosensory GRP system in the spinal cord contributes to the regulation of itch specific transmission independently of pain transmission (Sun and Chen, 2007; Sun et

al., 2009). Taken together, these results indicate that the spinal GRP system conveying the itch transmission may exist in suncus. Moreover, it is interesting that two different

GRP systems within the spinal cord are separately related to not only male reproductive function but also the itch sensation, using the same peptide GRP hormone as a neuromodulator within the mammalian spinal cord.

5 **Conclusions**

In summary, we have demonstrated, using suncus as a specialized placental mammal, that the GRP system in the lumbosacral spinal cord shows male-specific sexual dimorphism, not only in rodents but also in Eulipotyphla. Our results indicate that the sexually dimorphic spinal GRP system is general in mammals.

10

ACKNOWLEDGMENTS

We thank Prof. Takafumi Sakai and Dr. Ichiro Sakata of Saitama University for their encouragement and critical discussion of this study. The authors would like to thank Enago (www.enago.jp) for the English language review.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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ROLE OF AUTHORS

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. Study concept and design: TS and HS. Acquisition of data: Kei T, YK, AH, Keiko T, TO, and HS.

10 Analysis and interpretation of data: Kei T, YK, AH, Keiko T, TO, and HS. Supply for experimental animals (suncus), advice, anatomy, and equipment: TJ and SO. Obtained funding: TS, Keiko T, and HS. Wrote the paper: YK, Keiko T, and HS. Study supervision: HS.

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Figure Legends

Figure 1. Sequence and phylogenetic analysis of the gastrin-releasing peptide (GRP)

precursor protein. **A:** Sequence alignment of preproGRP. Boxed sequences marked by

5 *asterisks* (*) indicate neuromedin C (NMC or GRP-10). Amino acids identical to those

in other species are indicated by *stars* (*). *Colon* (:) indicates conserved substitution

and *period* (.) indicates semi-conserved substitution. **B:** A phylogenetic tree comprising

preproGRP was constructed using the neighbor-joining method. Branch lengths are

proportional to the number of amino acid changes on the branch. The values represent

10 bootstrap scores for 1,000 trials, indicating the credibility of each branch. Horizontal bar

indicates the genetic distance. The GenBank accession numbers of GRP are: suncus

(LC138361), mouse (EDL09679), rat (AAA41197), human (AAH04488), sheep

(NP_001009321), cattle (NP_001094709), horse (XP_001489353), and opossum

(XP_007487573).

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Figure 2. Reverse transcription (RT)-PCR analysis of gastrin-releasing peptide (*Grp*)

and its receptor (*Grpr*) mRNA expression in the lumbar spinal cord and stomach of

suncus (A) and mice (B) (RT+). A: In suncus, *Grp* and *Grpr* mRNA expression were detected in the lumbar spinal cord (SC) and stomach (Stm). In the mouse lumbar spinal cord, *Grp* mRNA expression was higher in males than in females. RT-PCR for β -actin (*Actb*) was performed as the internal control in suncus (A) and mice (B). Controls in which the RT treatment was omitted as the negative control showed a complete absence of bands in each experiment (RT-).

Figure 3. Immunohistochemistry (IHC) analysis of gastrin-releasing peptide (GRP) in the lumbosacral spinal cords of adult suncus (A, B) and mice (C, D). GRP-containing fibers in the lumbosacral spinal cord (L5–S1 level) were present in the spinal dorsal horn (DH), dorsal gray commissure (DGC), and sacral parasympathetic nucleus (SPN) both in suncus (A) and mice (B). GRP-containing fibers projected into the SPN (*arrowheads*). Preabsorbing the working dilution (1:1,000) of the primary GRP antiserum with a saturating concentration of GRP antigen peptide [neuromedin C (NMC); 50 μ g/mL] overnight at 4°C before use eliminates the staining in suncus (B) and mice (D). *Asterisk* (*) indicates the location of the central canal in mice (C, D).

Note that the central canal is not patent at the lumbar spinal cord level in suncus (A, B) as reported previously (Isomura et al., 1986). *Scale bars* = 100 μm .

Figure 4. Specificity of the neuronal nitric oxide synthase (nNOS) (A) and the androgen receptor (AR) antibody (B). A: The numerical values on the left side indicate the molecular weight. Western blot analysis of nNOS expression in the suncus and mouse diencephalon (D) and lumbar spinal cord (SC) probed with the antibody against nNOS. The monoclonal antibody recognized a single band at the expected molecular weight of nNOS in mice (155 kDa). (B) Western blot analysis of AR expression in the suncus and mouse diencephalon (D) and lumbar spinal cord (SC) probed with antibody against AR. The monoclonal antibody recognized an expected molecular weight of AR in mice (98 kDa).

Figure 5. Immunohistochemistry (IHC) analysis for gastrin-releasing peptide (GRP) in the lumbar spinal cord (L3–L4 level) of suncus (A–C) and mice (D–F). Sexually dimorphic expression for GRP was observed both in suncus (C; $n = 5$, $*P < 0.05$) and

mice (F; $n = 5$, $**P < 0.01$). *Arrowheads* indicate possible GRP-expressing neurons. A possible GRP-positive neuron enclosed in *box* (A) is enlarged. *Dashed lines* indicate the level of the mid-line. *Scale bars* = 50 μm (lower magnification); 10 μm (higher magnification).

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Figure 6. Distribution of gastrin-releasing peptide (GRP)-containing fibers in the lumbosacral spinal cord (L5–L6 and S1 level) of suncus (A–H) and mice (I–P). In the sacral parasympathetic nucleus (SPN), GRP-containing fibers (green) were observed both in suncus (A–H) and mice (I–P). The neuronal nitric oxide synthase (nNOS) serves as a marker for SPN neurons, and the immunoreactivity for nNOS in the SPN neurons (magenta) showed no sex difference. Double immunofluorescence for GRP and nNOS revealed closely appositions of GRP-containing fibers of suncus (C, D, G, H) and mice (K, O, L, P) with the cell bodies and proximal dendrites of nNOS-positive neurons in the SPN. The SPN areas enclosed in *orange boxes* (in merge images) are enlarged in each right panel. *Asterisk* (*) indicates the location of the central canal in mice (K, O). Note that the central canal is not patent in the lumbar spinal cord level in suncus (A–C,

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E–G) as reported previously (Isomura et al., 1986). *Scale bars* = 200 μm (lower magnification); 50 μm (higher magnification).

Figure 7. Double immunohistochemistry analysis for gastrin-releasing peptide (GRP)

5 and androgen receptor (AR) in the lumbar spinal cords of suncus (**A, C**) and mice (**B,**

D). *Arrowheads* in A, B indicate possible AR/GRP-double positive neurons. The areas enclosed in *yellow boxes* in A, B are enlarged in each lower panel. *Dashed lines* in A, B

indicate the level of the mid-line. Another double staining for GRP (green) and AR (magenta) was also conducted in suncus (**E**) and mice (**F**), showing a similar result.

10 *Asterisks* (*) indicate the location of the neuronal nuclei expressing GRP and AR. *Scale bars* = 20 μm (upper panels); 10 μm (lower panels).

Abbreviations

	DAB	diaminobenzidine
	DGC	dorsal gray commissure
5	DH	dorsal horn
	GRP	gastrin-releasing peptide
	GRPR	gastrin-releasing peptide receptor
	IF	immunofluorescence histochemistry
	IHC	immunohistochemistry
10	ir	immunoreactive
	NMC	neuromedin C
	nNOS	neuronal nitric oxide synthase
	PB	phosphate buffer
	PBS	phosphate buffered saline
15	PVDF	polyvinylidene difluoride
	RT	reverse transcription
	S.E.M.	standard error of the mean
	SPN	sacral parasympathetic nucleus
	Tris-HCl	trishydroxymethyl-aminomethane-HCl
20	TBS	trishydroxymethyl-aminomethane-HCl buffered saline
	TBST	0.05% Tween 20 in trishydroxymethyl-aminomethane-HCl buffered saline
	WB	Western blotting

A

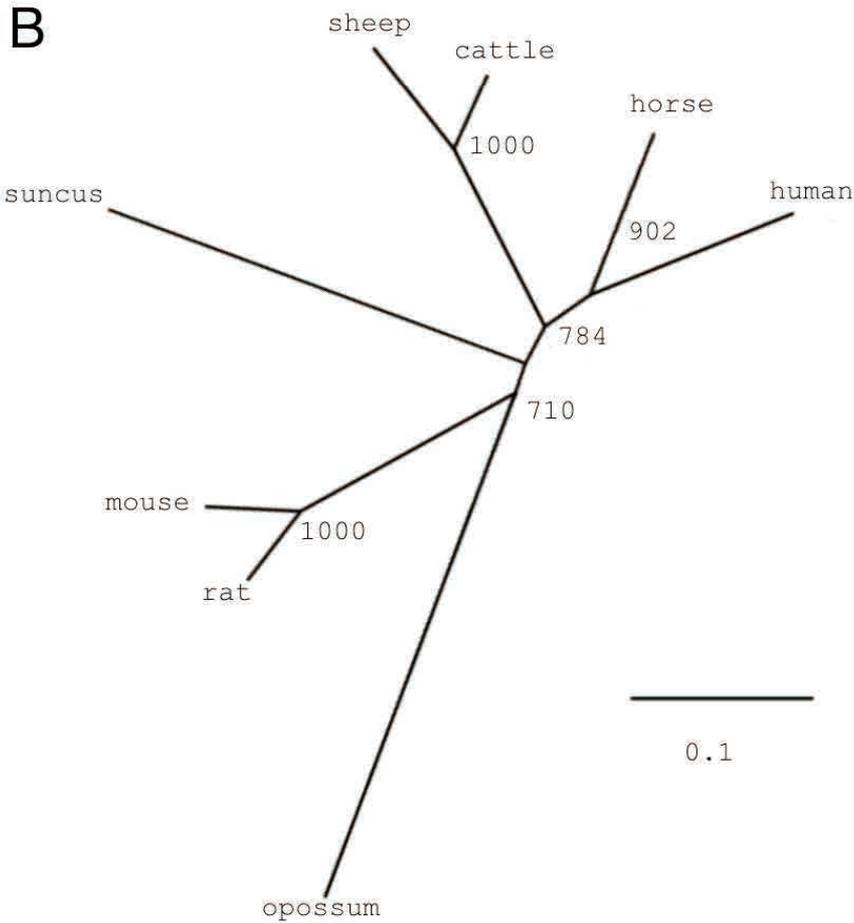
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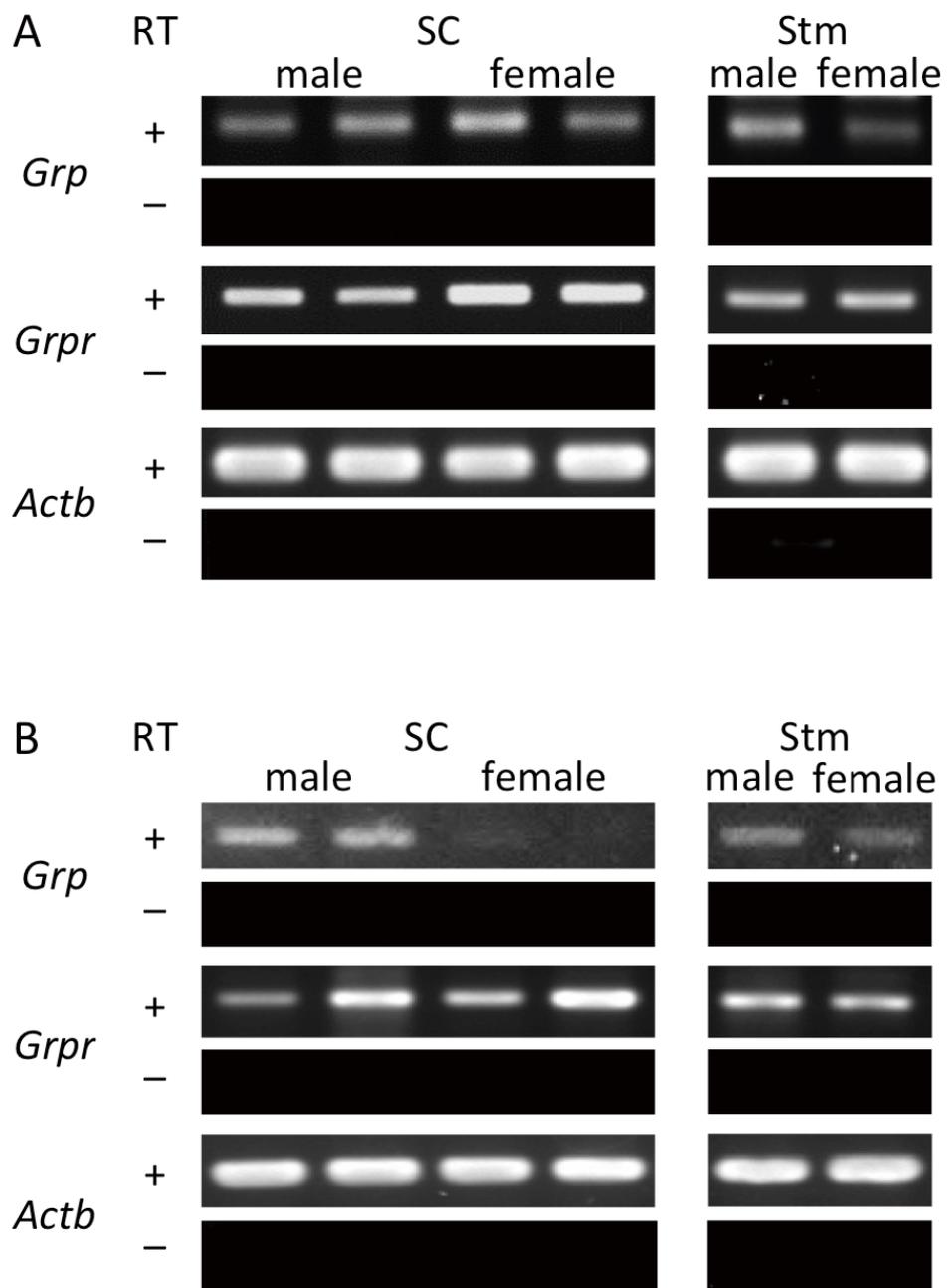
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rat MRGSELSLLLLALVLCQAPRGPAAPVSTGAGGGTVLAKMYPRGSHWAVGHLMGKKSTDEL 60
human MRGRELPLVLLALVLCCLAPRGRAVPLP--AGGGTVLTKMYPRGNHWAVGHLMGKKSTGES 58
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N M C
( GRP-10)

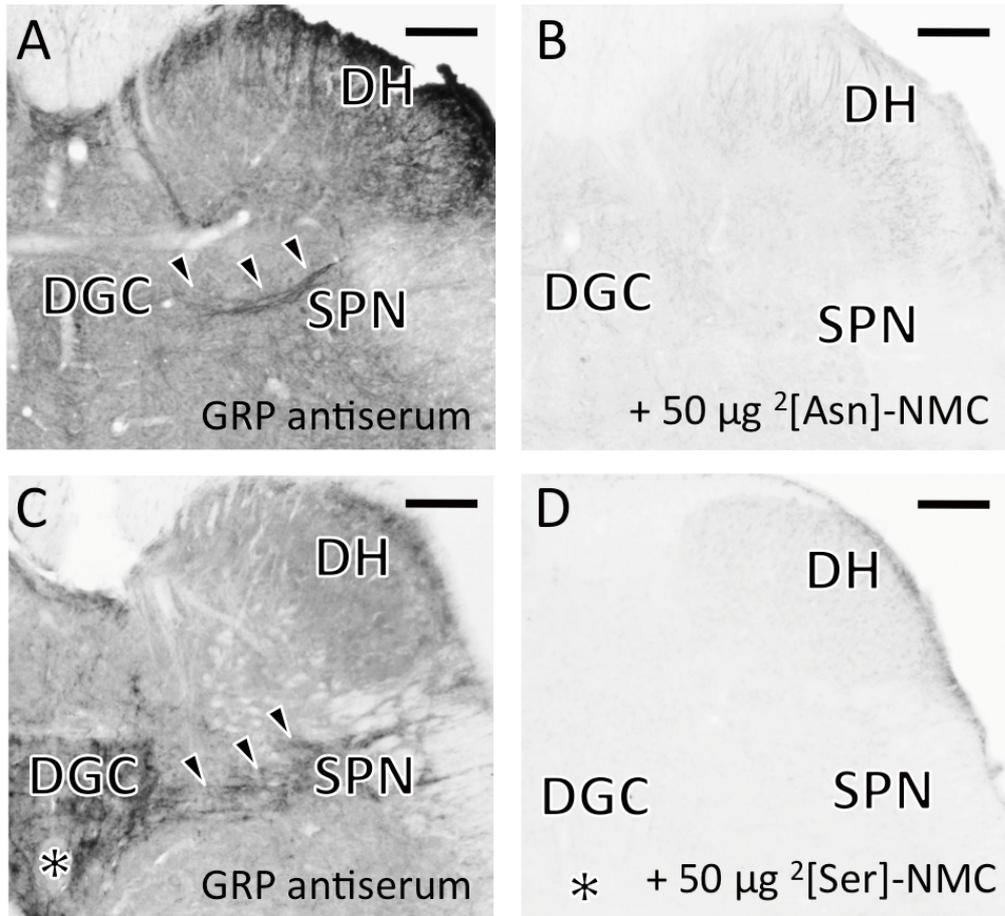
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human SS--VSEKSLKQQLREYIRWEEAARNLLGLIEAKENRNHQPQPQKALGNQQPS-WDSED 115
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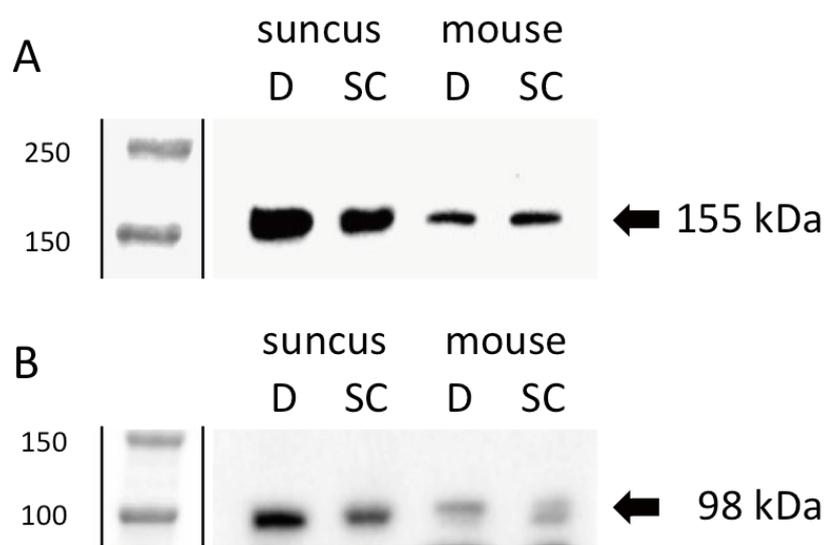
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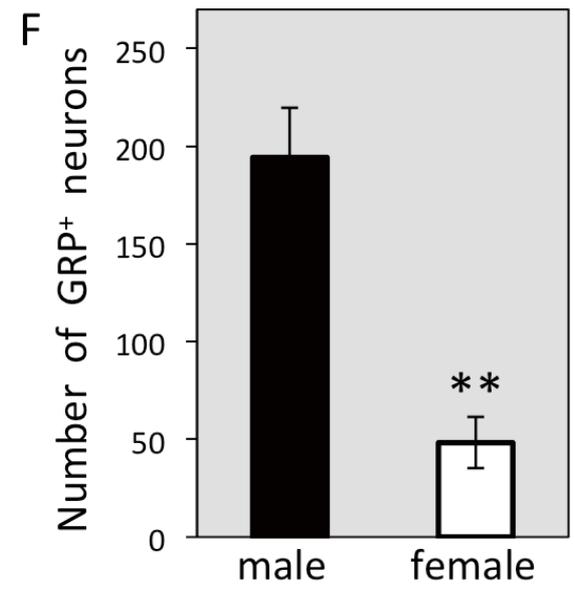
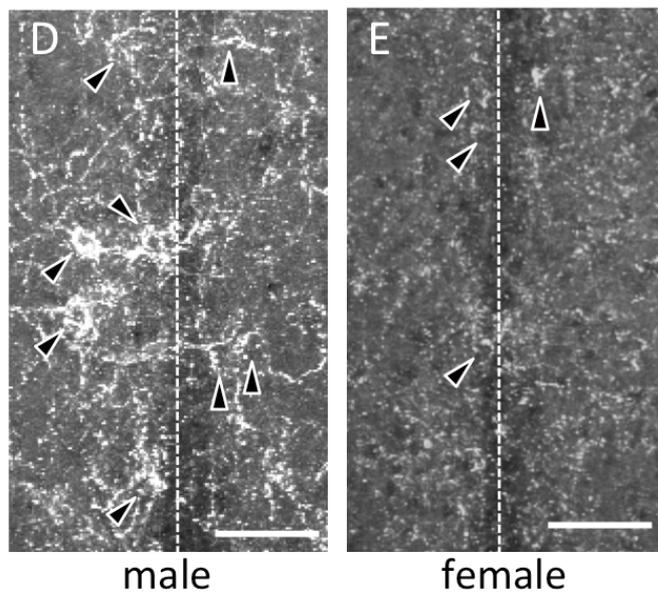
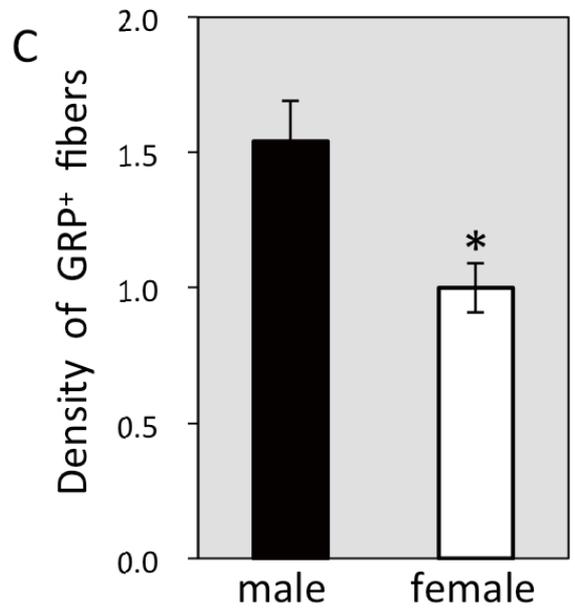
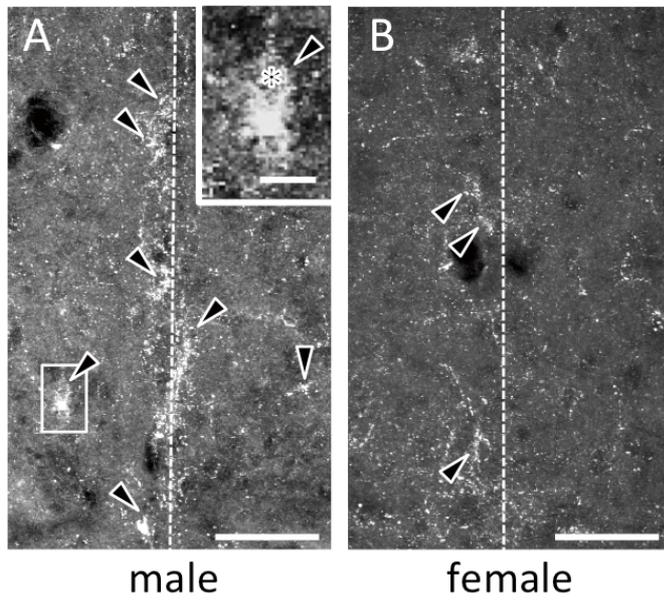
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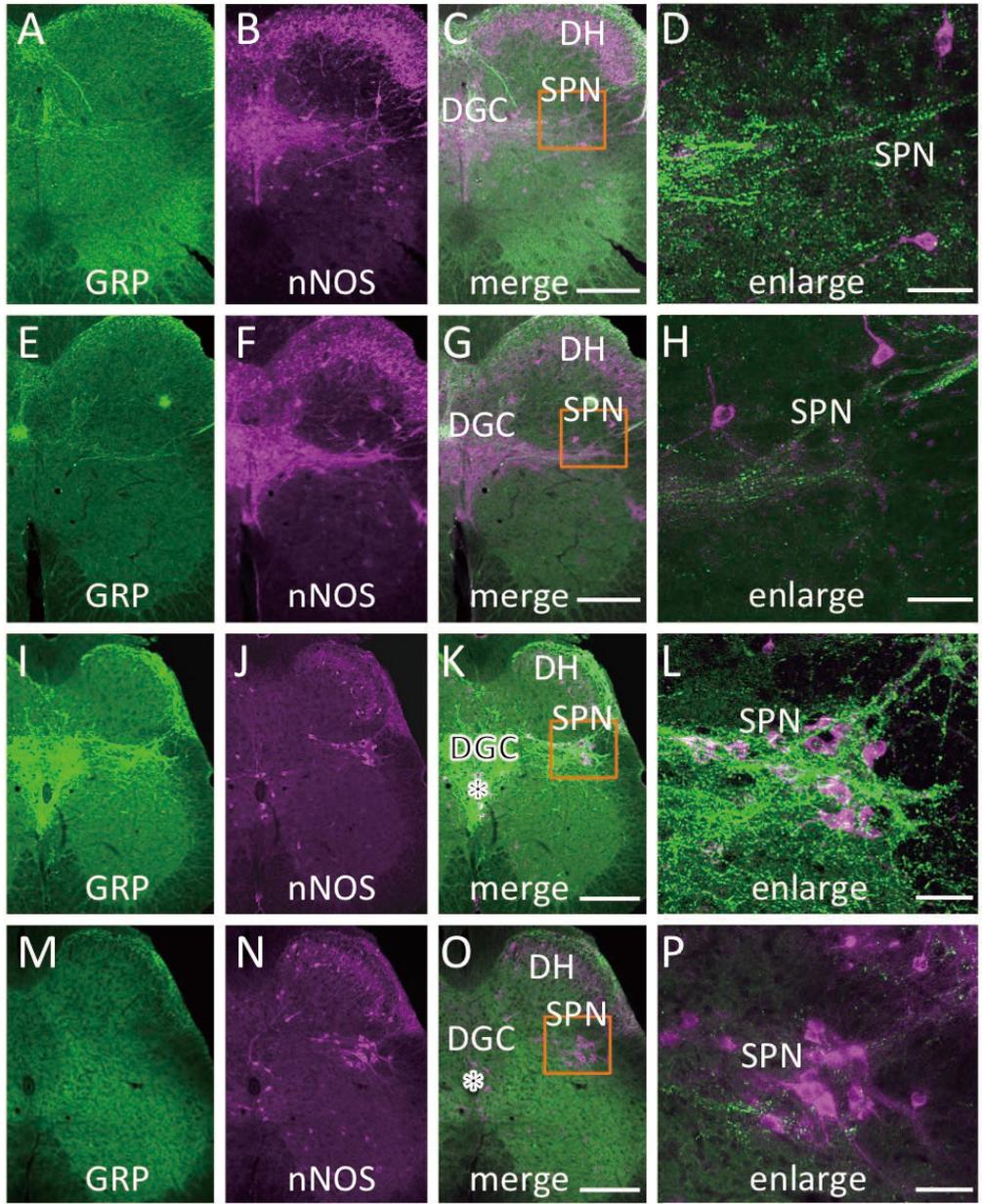


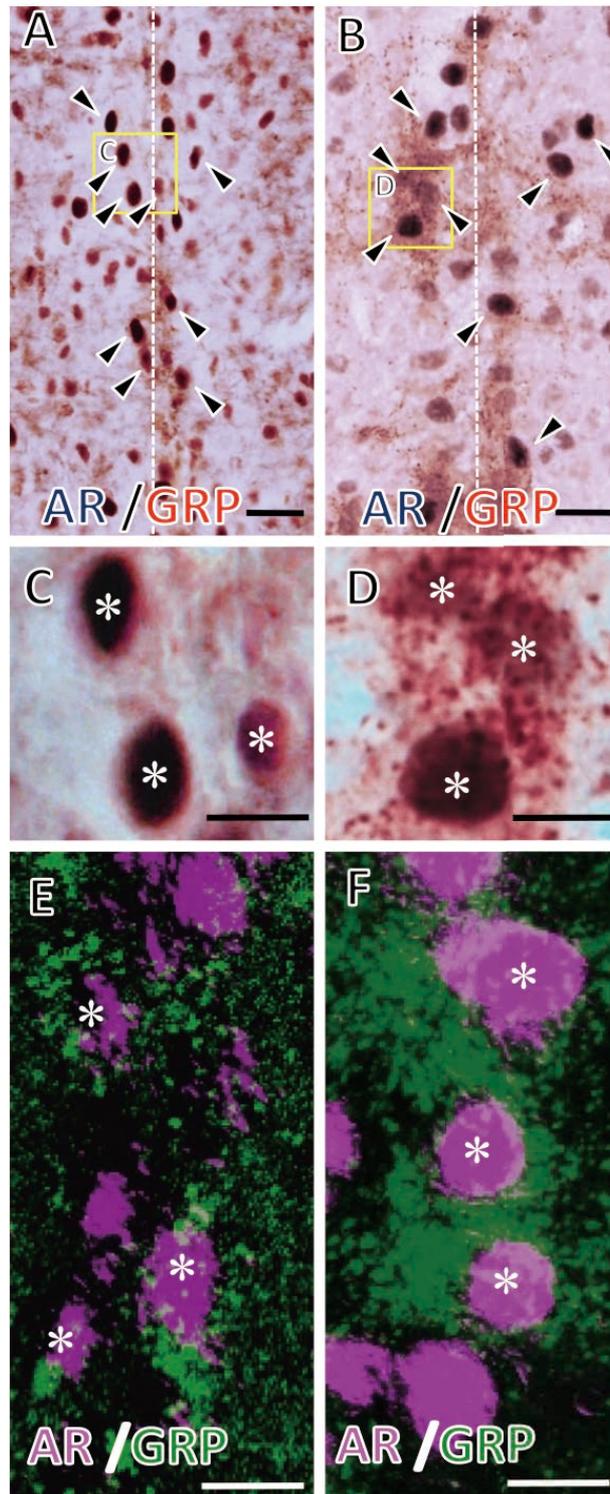


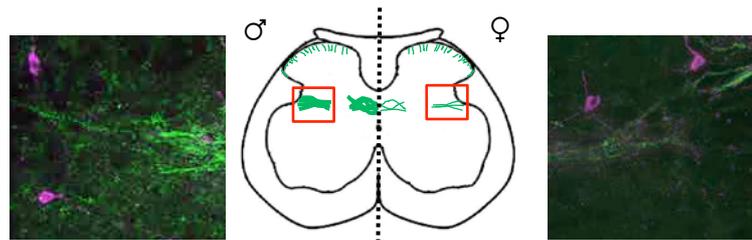












The authors show that the sexually dimorphic gastrin-releasing peptide (GRP) system in the lumbosacral spinal cord exists not only in mice but also in Asian house musk shrews (*Suncus murinus*). This male-specific system controlling male reproductive function may be a conserved property in mammals.