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Expression of G protein-coupled receptor 30 in the spinal somatosensory system

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Abstract

Estrogens were originally identified as the primary sex steroid hormones in females and regulators of reproductive function and sexual behavior, but it has long been suggested that estrogens also have local effects on the somatosensory system at the spinal cord level. It is well known that the effects of estrogens are mediated by nuclear estrogen receptors (ERs) through genomic action, but recently a membrane-bound G protein-coupled receptor, GPR30, was identified as a non-genomic estrogen receptor. In this study we investigated the presence and localization of GPR30 in the rat spinal cord and dorsal root ganglion (DRG) in comparison with ER α . Using immunohistochemistry and *in situ* hybridization, we showed the expression of GPR30 in DRG neurons in male and female rats at mRNA and protein levels without specific sexual difference. A dense accumulation of GPR30 immunoreactivity was observed in the outer layer of the spinal dorsal horn, and selective spinal dorsal rhizotomy revealed that GPR30 was transported from the DRG to terminals located in the spinal dorsal horn. GPR30 expression was downregulated in DRG neurons of ovariectomized female rats. The spinal somatosensory system might be modulated by estradiol *via* putative membrane ER, GPR30-mediated mechanism.

Keywords

GPR30; Estrogen receptor α ; Estrogen; Spinal cord; Dorsal root ganglion; Somatosensory neuron system

1. Introduction

Besides their crucial control in reproductive regulation and sexual differentiation, estrogens regulate a host of non-reproductive functions in the nervous system, including protection

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The authors have nothing to disclose.

Appendix A. Supplementary data

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against lesion-induced trauma, learning and memory, stress, mood, cognition, somatosensory perception, and pain sensitivity. The broad spectrum of estrogen action is mediated by two types of estrogen receptor (ER), α and β , through genomic regulation by the modulation of gene transcription (Jensen and DeSombre, 1973; Kuiper et al., 1996; Paech et al., 1997). In addition to these actions, there is increasing evidence of rapid non-genomic effects of estrogen *via* membrane-associated receptors, G protein-coupled receptors (GPCRs) (Hall et al., 2001; Ho and Liao, 2002; Kelly and Levin, 2001; Levin, 2002; Razandi et al., 2003). A membrane-bound GPCR, GPR30, was initially identified as an orphan receptor using screening of a differential cDNA library (Carmeci et al., 1997). Subsequently, two groups independently proposed that GPR30 is a non-genomic ER (Revankar et al., 2005; Thomas et al., 2005). These results suggested that estrogen binds to a GPCR superfamily member directly and that GPR30 is a putative membrane ER. Several studies have shown GPR30 expression in the rat nervous system, reflecting the central and peripheral actions of estrogens (Brailoiu et al., 2007; Dun et al., 2009; Funakoshi et al., 2006; Liverman et al., 2009; Matsuda et al., 2008; Prossnitz et al., 2007, 2008; Sakamoto et al., 2007).

There is substantial evidence of a sex difference in pain perception in humans and animal models (Hapidou and Rollman, 1998; Riley et al., 1999; Wiesenfeld-Hallin, 2005). A higher prevalence of chronic pain states and greater pain sensitivity in women has long been suggested, especially from puberty to menopause and beyond. It has also been reported that estrogens influence the sensitivity, threshold and tolerance to pain in females during the menstrual cycle in humans and estrus cycle in rodents (Hapidou and Rollman, 1998; Riley et al., 1999; Wiesenfeld-Hallin, 2005). It has been reported that the classical nuclear ERs, ER α and ER β , are expressed in the spinal dorsal root ganglion (DRG) in both male and female rats (Papka and Storey-Workley, 2002; Shughrue et al., 1997; Simerly et al., 1990; Taleghany et al., 1999; Williams and Papka, 1996), but these nuclear ERs do not explain all aspects of estrogen action in the somatosensory system. Estrogens are believed to play a neuromodulatory role in the conveyance of sensory sensations, but their functional role in nociception is not fully understood.

The purpose of the present study was to determine the distribution of GPR30 in the spinal cord and DRG in both sexes in comparison with ER α and to examine the effects of ovariectomy (OVX) and estradiol (E₂) replacement in female ganglion cells. Immunohistochemistry (IHC) and *in situ* hybridization revealed that GPR30 appeared to be synthesized in the DRG and transported to the spinal dorsal horn. Expression of GPR30 was downregulated by OVX in DRG neurons. These results provide the basis for understanding new roles of GPR30 in somatosensory modulation of estrogen action in the spinal cord and DRG.

2. Results

2.1. Localization of GPR30 immunoreactivities in the male and female spinal cord and DRG

IHC analysis for GPR30 ($n = 3$ females) revealed that GPR30 immunoreactivities (ir) was present in several other somal size neurons (small: $d < 30 \mu\text{m}$; medium: $30 \leq d < 40 \mu\text{m}$; and

large: 40 μm $\times$ 4; categorized in somal diameters in the section) (Fig. 1A) and somata and fibers in the lumbar spinal dorsal horn (Fig. 1C). Control absorption experiments were performed to confirm the specificity of the GPR30 antibody. Controls, in which anti-GPR30 serum was preabsorbed with an excess amount of antigen peptide, showed a complete absence of GPR30-ir in the lumbar DRG (L4–6) (Figs. 1A, B) and the lumbar spinal dorsal horn (L4–6) (Figs. 1C, D).

Next, we examined the localization of GPR30-ir in the male and female rats spinal cord and DRG through the cervical (C) to the sacral (S) levels using IHC analysis ($n = 6$ for each sex). GPR30-ir was observed at the cervical (C4–5), thoracic (T5–6), lumbar (L4–5) and sacral (S1–2) levels in the spinal cord in male (Fig. 2A) and female (Fig. 2B) rats. GPR30-ir was also observed in the spinal dorsal horn and ventral horn (ventromedial and dorsolateral nuclei) of the gray matter and in the white matter of both sexes. In the spinal gray matter, GPR30-ir was observed in the neuronal soma, dendritic/axonal processes, and/or their terminals, and was also observed in glial cells in the white matter (see Supplementary Fig. 1). Strikingly, dense accumulation of GPR30-ir was seen in the outer layer of the spinal dorsal horn through the cervical to sacral levels (Figs. 2A, B). GPR30-ir was also observed in many DRG neurons (Figs. 2C, D). A similar GPR30-ir pattern was observed in the DRG of all spinal segments through cervical to sacral level in the both sexes (males: Fig. 2C, females: Fig. 2D).

2.2. Double IHC analysis for GPR30- and ER α -ir in the lumbar DRG

Double IHC for GPR30- and ER α -ir was performed in the L4–6 DRG (Fig. 3). GPR30-ir (brown) was observed in the cytoplasm of small, medium, and large neurons in the DRG. In contrast, ER α -ir (violet) was mainly localized in the nuclei of small and medium neurons. The number of GPR30-ir and ER α -ir double-negative cells was not detected. In the present study, the sum of the immunopositive cells for GPR30, ER α , and GPR30 + ER α , was defined as the total cell numbers (100%). The percentage of GPR30-positive cells was significantly higher than ER α -positive cells and GPR30/ER α -double-positive cells ($F(2,35) = 1700.11$, $P < 0.01$) in both sexes (Table 1). The percentage of ER α -positive cells was significantly lower than GPR30/ER α -double-positive cells ($F(2,35) = 1700.11$, $P < 0.01$) in males only, but a similar tendency was observed in females. There was no significant difference between males and females ($F(1,35) = 0.082$, $P = 0.78$) for each of the three cell types (Table 1).

2.3. In situ hybridization of GPR30 mRNA in the lumbar spinal dorsal horn and DRG

In situ hybridization histochemistry was performed in the spinal cord and DRG at the L4–6 levels in adult rats ($n = 3$ for each sex) using RNA probes for rat GPR30 mRNA. Strong expression of GPR30 mRNA was observed in several somal-sized neurons in the lumbar DRG (Fig. 4A), and the distribution of cells containing GPR30 mRNA in the DRG was similar to that of GPR30 protein. In contrast to IHC results, only a slight expression of GPR30 mRNA was observed in the lumbar spinal dorsal horn (Fig. 4C) and the outer layer of the lumbar spinal dorsal horn showed much lower expression levels (Fig. 4C). The patterns of GPR30 mRNA expression in the DRG and spinal cord were similar in both sexes.

Hybridization specificity was demonstrated by the absence of labeling by sense RNA probes in the DRG (Fig. 4B) and the spinal dorsal horn (Fig. 4D).

2.4. Changes of GPR30-ir in the lumbar spinal dorsal horn by rhizotomy

We detected intense GPR30-ir in the spinal dorsal horn, and especially in the outer layer of the dorsal horn (Figs. 1A, B). To examine the origin of this observation in the lumbar spinal dorsal horn (L4–6), unilateral specific dorsal rhizotomy was performed in adult females ($n=4$). Fourteen days after surgery, GPR30-ir in the lumbar spinal cord (L4–6) was analyzed by IHC. GPR30-ir was significantly reduced on the ipsilateral side of the dorsal horn after unilateral dorsal rhizotomy, especially on the ipsilateral side of the superficial layer, compared with the contralateral side ($P<0.01$) (Figs. 5A–C). Substance P (SP)-ir and calcitonin gene-related peptide (CGRP)-ir were also examined in these experiments as positive controls, because SP and CGRP are essential for sensory input into the spinal dorsal horn from DRG neurons. SP- and CGRP-ir were both significantly lower on the ipsilateral side of the dorsal horn after rhizotomy compared with the contralateral side (SP, $P<0.01$, Figs. 5D–F; CGRP, $P<0.01$, Figs. 5G–I). These results suggest that GPR30 appeared to be synthesized in the DRG neuron somata and transported to terminals located in the spinal dorsal horn.

2.5. Modulation of GPR30-ir expression by OVX and OVX+E₂

We further investigated the effects of OVX and E₂ replacement on the expression level of GPR30 in the lumbar DRG (L4–6). The number and percentage of GPR30-ir neurons in the DRG were analyzed 14 days after OVX by comparison with KDEL (Lys-Asp-Glu-Leu)-ir neurons, since KDEL stains the whole soma outline of DRG neurons. Cells positive or negative for GPR30-ir were selected based on our criteria. Representative results of double IHC for GPR30 (green) and KDEL (magenta) are shown in Fig. 6A. Four groups were subjected to this procedure: sham-operated males that underwent similar surgery without castration (male; $n=5$), sham-operated females that underwent similar surgery without OVX (sham; $n=4$), bilateral OVX females with empty capsule implantation (OVX; $n=6$), and bilateral OVX females with E₂ replacement (OVX+E₂, $n=6$). OVX females had a significantly lower percentage of GPR30-ir neurons in the L4–6 DRG than sham-operated males and females ($F(3,17)=23.53$, $P<0.01$ vs. sham, $P<0.05$ vs. male). The reduction in GPR30-ir was recovered by estrogen replacement ($F(3,17)=23.53$, $P<0.01$ vs. OVX) (Fig. 6B) and the percentage of GPR30-ir neurons in the OVX+E₂ group was significantly higher than in sham-operated males ($F(3,17)=23.53$, $P<0.05$ vs. male).

2.6. Cell-type specific analysis of GPR30-ir expression by OVX and E₂ replacement

We examined which cell type among DRG neurons responded to OVX and E₂ replacement. The classification of cell size in the DRG is generally based on the different physiology of DRG neurons (Harper and Lawson, 1985a,b; Study and Kral, 1996; Tu et al., 2004). The sensations, slow pain, itch, and temperature are transmitted by the unmyelinated small-sized C-fiber neurons, fast pain by the myelinated medium-sized A δ -fiber neurons, and touch and pressure by the myelinated large-sized A β -fiber neurons (Dale Purves et al., 2004). And detailed analysis was performed for three groups of DRG neurons established based on the soma diameter (d): small ($d<30\text{ }\mu\text{m}$), medium ($30\text{ }\mu\text{m}\leq d<40\text{ }\mu\text{m}$), and large ($40\text{ }\mu\text{m}\leq d$) cells

((Harper and Lawson, 1985a,b; Study and Kral, 1996; Tu et al., 2004)). Defining the total DRG neurons as 100%, the percentages of GPR30-ir-positive cells were male 39.2 ± 5.3 % and female 43.8 ± 8.7 % in small neurons, male 21.1 ± 1.7 % and female 19.2 ± 2.5 % in medium neurons, and male 27.4 ± 6.1 % and female 28.7 ± 6.5 % in large neurons in the L4–6 DRG. Defining the each type of neurons as 100%, the percentages of GPR30-ir-positive cells were male 83.6 ± 6.2 % and female 87.6 ± 6.6 % in small neurons, male 91.3 ± 5.3 % and female 93.7 ± 5.0 % in medium neurons, and male 93.0 ± 6.1 % and female 94.6 ± 2.7 % in large neurons. No significant difference in the percentages of GPR30-ir-positive cells in the lumbar DRG was detected among the groups or sexes (Fig. 7).

3. Discussion

The present paper presents the details of GPR30 expression in the somatosensory system of male and female rats at the mRNA and protein levels. We detected a dense accumulation of GPR30-ir in the outer layer of the spinal dorsal horn, and selective spinal dorsal rhizotomy indicated that GPR30 protein was synthesized in the DRG and then transported via the dorsal root to the terminals of nociceptive afferents in laminae I and II of the spinal dorsal horn. The expression level of GPR30 was modulated by OVX in DRG neurons. Taken together, these results suggest that GPR30 is a membrane ER that might be associated with estrogen-mediated signal transduction mechanisms in the somatosensory system.

3.1. Expression and physiological significance of GPR30 in DRG neurons

Several studies have indicated sex differences in the sensory threshold. In rodents, females are more sensitive to noxious stimuli and show variable pain sensitivity during the estrus cycle (Hapidou and Rollman, 1998; Riley et al., 1999; Wiesenfeld-Hallin, 2005). There are two possible mechanisms of modulating sensations by estrogens: direct estrogen modulation, and indirect modulation by alteration of the expression levels of ERs, including nuclear and membrane ERs. The indirect mechanism may be consistent with the results in the current study, because GPR30 expression was upregulated by estrogens in the female rat lumbar DRG, an effect opposite to those for nuclear ER α at the protein and mRNA levels (Taleghany et al., 1999; Yang et al., 1998), but the effect same to that for nuclear ER β at the mRNA level (Taleghany et al., 1999). We found the difference localization of GPR30 with ER α in the DRGs. ER α was localized in the nucleus and GPR30 in cytoplasm as shown in Fig. 3. Previous reports have demonstrated recently that GPR30 has a tubuloreticular distribution in culture cell lines, hypothalamic neurons, and hippocampal neurons *in vivo* in which it is colocalized with the Golgi apparatus and the endoplasmic reticulum (Matsuda et al., 2008; Revankar et al., 2005; Sakamoto et al., 2007) while others did not (Funakoshi et al., 2006; Thomas et al., 2005), these studies used different antisera. In the present study, we also found the cytoplasmic distribution of GPR30 in the DRG neurons using double-immunofluorescence labeling for GPR30 and TGN38, a specific marker of the Golgi apparatus (data are not shown). We also found differences in the expression patterns of GPR30 and ER α in the DRG, with a significantly higher number of GPR30-ir cells than ER α -positive cells. In addition, GPR30-ir was observed in all cell types (small, medium, and large neurons), consistent with the results of the previous paper (Dun et al., 2009), but ER α has been shown to be localized mainly in small neurons in the DRG (Papka and Storey-

Workley, 2002; Taleghany et al., 1999). In contrast, Liverman et al. (2009) reported that GPR30 was observed in small neurons and ER α was observed in all cell types in the trigeminal ganglion. These differences might arise from different regions and functions between the spinal DRG and cranial trigeminal ganglion. Small, medium, and large neurons extend C-fibers, A δ -fibers, and A α /A β -fibers, respectively (Harper and Lawson, 1985a,b; Study and Kral, 1996; Tu et al., 2004). Using IHC analysis, it has been reported that the expression patterns of ER α and ER β proteins in the DRG are similar, and these receptors are mainly observed in small and medium DRG neurons in rats (Papka and Storey-Workley, 2002). Some spinal DRG neurons in female rats in the lower lumbar and upper sacral spinal cord show immunoreactivity for ER α (approximately 17%), ER β (approximately 23%), and both ER α and ER β (approximately 5%), over half of all DRG neurons do not express nuclear ERs (Papka et al., 2001; Papka and Storey-Workley, 2002); therefore, different sensations might be diversely activated by, or interact with, different types of neurons *via* GPR30- and ER α -mediated mechanisms. Using *in situ* hybridization analysis, however, it has been reported the contradictory results between expression levels for protein and mRNA that nearly all DRG neurons are ER β expressing in both males and females (and a qualitative sex difference in ER α) at the mRNA level (Taleghany et al., 1999). These findings also raise the possibility that the potential for overlap of GPR30 expression with, at least, the expression of ER β mRNA is likely to be high. Further studies are needed to draw a firm conclusion. The colocalization of ER α with GPR30 in the same neurons in a certain population of DRG neurons suggests that ER β might regulate the expression of GPR30 through genomic actions. In addition, rapid signal transduction in the sensory neurons may be mediated by non-genomic actions of estrogens *via* GPR30, while gene transcription in the sensory neuron may be mediated by genomic actions of estrogens *via* ER α . Taken together, our results raise the possibility that estrogen-mediated signal transduction is diversified by interactions between GPR30 and ER α in sensory neurons. We also noted that a higher percentage of GPR30/ER α double-positive neurons was observed in males, and that almost all DRG neurons expressed GPR30, but a lower number of neurons expressed ER α alone. Thus, it is suggested that estrogen action through GPR30 might be independent of ER α . These differences in the distributions of GPR30 and ER α indicate that the expression of functional receptors may depend on differences in the level of circulating estrogens between sexes.

The sexual dimorphism of GPR30 mRNA expression has been reported in various brain areas of the hamster, a hibernating rodent (Canonaco et al., 2008). While it has been reported that the percentage of nuclear ER-ir neurons in the male lumbar DRG was lower than in female rats (Yang et al., 1998), no sexual difference in the number of GPR30-ir cells in the lumbar DRG was observed in this study. The differences in GPR30-ir in OVX females vs. intact females or males are relatively small, suggesting that the effects of estrogenic regulation on GPR30 expression in the DRGs might be unconvincing, or only low levels should be needed to maintain GPR30 expression in the DRGs.

3.2. Localization of GPR30 in the spinal dorsal horn

We found a dense accumulation of GPR30 in the spinal dorsal horn throughout the cervical to sacral segments, especially in the outer layer, and detected GPR30 expression in all

segmental levels of the DRG in both sexes. Selective spinal dorsal rhizotomy showed significantly reduced GPR30-ir on the ipsilateral side after surgery compared to the contralateral side. Taking these results with the *in situ* hybridization study suggests that GPR30 appeared to be synthesized in the DRG neuron somata and transported to terminals located in the spinal dorsal horn to function as a modulator for the somatosensory system. On the other hand, GPR30-ir expression in the ipsilateral spinal dorsal horn did not completely disappear, and a low degree of GPR30 mRNA expression was observed in the outer layer of the spinal dorsal horn in the present *in situ* hybridization study. It remains to be determined whether GPR30 is expressed in the spinal interneurons and glial cells in the dorsal horn, or transsegmental innervation from upper or lower segment from L4–6.

The distribution pattern of GPR30 is similar to that of opioid receptors, and modulation of sensations is mediated by a variety of endogenous opioid systems (Corchero et al., 2004; Horvath and Kekesi, 2006; Kanjhan, 1995). The superficial layer of the dorsal horn (laminae I and II) is an important region for receiving input associated with nociception from primary sensory neurons through C-fiber and A δ -fiber, and has signal transduction components in secondary sensory neurons. The opioid receptors, which are members of the GPCR family, may serve as antinociceptive mediators and are located on both sides of the synaptic terminals of primary sensory neurons in laminae I and II of the spinal dorsal horn (Kline and Wiley, 2008), in which they show GPCR modulatory activity.

Given the similar localization of GPR30 and opioid receptors, GPR30 may interact with the opioid system in the spinal dorsal horn. It has previously been suggested that opioid systems are profoundly modulated by sex steroid hormones (Amandusson et al., 1999; Flores et al., 2003; Mannino et al., 2007; Stoffel et al., 2005), and that OVX induces hyperalgesia in female mice that is relieved by E₂ replacement (Mannino et al., 2007). It has also been shown that estrogens may modulate μ -, κ - and δ -opioid receptor-mediated antinociceptive actions in male and female rats using specific agonists to each receptor (Stoffel et al., 2005), and the administration of estrogens results in a rapid increase in the expression of enkephalin, an endogenous ligand for δ -opioid receptors (Amandusson et al., 1999). Moreover, some studies have indicated that neurotransmitters (e.g., CGRP) related to nociception are regulated by estrogens in female rats (Yang et al., 1998). A recent pharmacological approach showed that estrogen acts acutely on GPR30 to produce mechanical hyperalgesia *in vitro* and *in vivo* in male rats using the selective GPR30 agonist, G1 (Kuhn et al., 2008), and G1 treatment increased allodynia after complete Freund's adjuvant into the masseter of OVX female rats (Liverman et al., 2009). Recently, Dun et al. (2009) showed GPR30 expression in the rat spinal cord and autonomic and sensory ganglia, and also demonstrated that G1 depolarized cultured spinal cord neurons in a concentration-dependent manner using voltage-sensitive dye imaging. Taken together, these results and the present findings suggest that estrogen actions *via* GPR30-mediated mechanisms may interact with the endogenous pain modulatory system, with resultant modulation of nociception and antinociception at the spinal level.

3.3. Expression and possible actions of GPR30 in the DRG in males

GPR30 expression in the somatosensory system was detected in female and male rats, with no apparent difference between the sexes in this study. Previous studies have shown that nuclear ERs are expressed in the spinal cord and DRG in both sexes, but that the expression levels of these nuclear ERs are higher in the lumbar DRG of female rats (Taleghany et al., 1999; Yang et al., 1998). Testosterone, an essential male sex steroid hormone, can be aromatized by cytochrome P450 aromatase to an active estrogen, E₂. Although little is known about the expression and enzymatic activity of cytochrome P450 aromatase in the spinal cord in rodents, the expression of aromatase in the DRG neurons in adult male rats has been reported recently (Schaeffer et al., 2009). It is possible that E₂ synthesized locally within the DRG and the spinal dorsal horn as neuroestrogens might influence signal transduction in the somatosensory system in rats *via* a GPR30-mediated mechanism. At the behavioral level, it has also been reported that the specific agonists for GPR30, G-1 resulted in the onset of PKC ϵ (an important intracellular signaling molecule in primary afferent nociceptors)-dependent mechanical hyperalgesia in a dose-dependent manner similarly to the estrogen function, if injected into paws of adult male rats (Hucho et al., 2006; Kuhn et al., 2008).

4. Conclusion

Our results show that GPR30 is expressed in the somatosensory system in male and female rats at mRNA and protein levels. Selective spinal dorsal rhizotomy suggested that GPR30 is transported from the DRG to terminals located in the spinal dorsal horn, and the expression of GPR30 in the female DRG was upregulated by circulating estrogens. We conclude that GPR30 is a membrane ER that might play an important role in the somatosensory system *via* non-genomic signal transduction pathways induced by estrogen.

5. Experimental procedures

5.1. Animals

Male ($n = 26$) and female ($n = 44$) Sprague–Dawley rats aged 10–12 weeks old were purchased from Shimizu Laboratory Supplies Co. Ltd., Japan and housed in plastic cages with standard bedding and continuous access to food and water. The temperature was maintained at 22 °C with a 12-h light: dark cycle (06:00 lights on). All experimental procedures were authorized by the Committee for Animal Research, Kyoto Prefectural University of Medicine.

5.2. Tissue preparation

Rats were anesthetized with 100 mg/kg body weight sodium pentobarbital and perfused via the left ventricle with 100 ml physiological saline followed by 200 ml of 4% paraformaldehyde for IHC and *in situ* hybridization in 0.1 M phosphate buffer (PB; pH 7.4). Spinal cords and DRGs were immediately removed and immersed in 4% paraformaldehyde in 0.1 M PB for 24 h (for *in situ* hybridization) or 3 h (for IHC) followed by immersion in 25% sucrose in 0.1 M PB for 48 h at 4 °C. For preparation of cryosections, spinal cords and DRGs were quickly frozen using powdered dry ice and cut into 20- μ m (for IHC) or 14- μ m

(for *in situ* hybridization) transverse sections of the spinal cord and long axis of the ganglion using a cryostat (CM3050 S; Leica, Nussloch, Germany). These sections were washed several times with PBS for 5 min each.

5.3. IHC: immunoperoxidase histochemistry

Endogenous peroxidase activity was eliminated from the sections by incubation with 2% H₂O₂ in absolute methanol for 30 min, and the sections were then rinsed with PBS three times for 5 min each. After blocking nonspecific binding components with 1% normal goat serum and 1% BSA in PBS containing 0.1% Triton X-100 for 1 h at room temperature, the sections were incubated with primary rabbit antiserum against GPR30 (Revankar et al., 2005) at a dilution of 1:8,000 overnight at 4 °C. Several concentrations of antiserum from 1:500 to 1:50,000 had been examined previously, and a dilution of 1:8,000 had proved to be most satisfactory for the immunoperoxidase technique (Matsuda et al., 2008; Sakamoto et al., 2007). Immunopositive products were detected with a streptavidin-biotin (SAB) kit (Nichirei, Tokyo, Japan), followed by diaminobenzidine (DAB) development, as described previously (Sakamoto et al., 2007). The preparations were analyzed using an Olympus Optical microscope (Tokyo, Japan).

To examine colocalization of GPR30 with ER α in the lumbar DRG, GPR30/ER α double-IHC was conducted using the DAB-nickel/DAB method ($n=6$ for each sex) (Sakamoto et al., 2008). First, endogenous peroxidase activity was eliminated as described above. After blocking nonspecific binding, DRG sections from L4–6 levels were incubated with polyclonal anti-ER α antiserum raised in rabbit (MC-20; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:4000 overnight at 4 °C. Sections were processed using the SAB immunoperoxidase procedure described above, with enhancement of the DAB reaction with 0.02% nickel chloride to produce a blue-black reaction product. After ER α immunostaining, sections were blocked with 2% H₂O₂ in absolute methanol for 30 min at room temperature, followed by GPR30 IHC with the second chromogen reaction performed without nickel chloride to produce a red-brown reaction product. GPR30-ir was examined at high magnification to determine the percentage of GPR30-ir neurons expressing ER α in the DRG. Digital photomicrographs were processed with Adobe Photoshop at 400 dpi resolution and printed on photo-quality paper, and the numbers of GPR30- and ER α -ir cells with distinct nuclei were counted by an investigator blinded to the group identity. The total number of immunopositive neurons examined in this study was considered to be 100% in each sample. More than 200 cells in at least 5 sections per animal were randomly assigned for analysis.

5.4. IHC: immunofluorescence histochemistry

The number and percentage of GPR30-ir neurons in L4–6 DRGs were analyzed using KDEL immunofluorescence to stain whole somata of DRG neurons. Double-immunofluorescence staining of GPR30 (1:5000 dilution) and KDEL, a marker protein for the endoplasmic reticulum (10C3; Calbiochem, Darmstadt, Germany; 1:1,000 dilution) was performed as described previously (Matsuda et al., 2008; Sakamoto et al., 2007). Alexa Fluor 546-labeled anti-mouse IgG (Molecular Probes, Eugene, OR, USA) and Alexa Fluor 488-labeled anti-rabbit IgG (Molecular Probes) were both used for detection at 1:1,000

dilution. Immunofluorescent sections were viewed using a LSM510META confocal laser-scanning microscope (Carl Zeiss, Jena, Germany). Preparations were then examined at high magnification to determine the percentage of KDEL neurons in the DRG that expressed GPR30-ir and to analyze the diameter of each DRG neuron. This analysis was performed by an investigator blinded to the group identity. Spinal DRG neurons were categorized into three types based on their somal diameter: small, medium, and large (Harper and Lawson, 1985a,b; Study and Kral, 1996; Tu et al., 2004). More than 200 cells in at least 5 sections per animal were randomly assigned for analysis. Digital photomicrographs were processed with Zeiss LSM Image Browser software (Carl Zeiss) and the number of immunopositive cells with clearly visible transected round nuclei was counted.

5.5. IHC: control

Control procedures for the DAB method were performed using pre-absorption of the working dilution (1:8,000) of the primary antiserum with a saturating concentration of GPR30 antigen peptide (100 µg/ml: produced in Prosnitz laboratory) over-night at 4 °C before use, and substituting primary serum for preimmune rabbit antiserum at the same dilution (1:8,000) (data not shown). Alexa Fluor 488-labeled anti-rabbit IgG was used as the control for immunofluorescent methods.

5.6. In situ hybridization

Synthesis of digoxigenin-labeled GPR30 sense and anti-sense probes and hybridization histochemistry has been described elsewhere (Hosokawa et al., 2008; Matsuda et al., 2008; Sakamoto et al., 2007). Briefly, frozen 14-µm sections of the spinal cord and DRG from both sexes ($n=3$ for each sex) on glass slides were pretreated with 10 µg/ml proteinase K and acetylated with 0.25% acetic anhydride. The sections were incubated with 100 µl of 500 ng/ml antisense or sense probe in hybridization buffer for 18 h at 55 °C. After hybridization, the sections were treated with 12.5 µg/ml RNase A to digest the excess hybridization probe and washed in 2× saline sodium citrate (SSC) (150 mM NaCl, 15 mM sodium citrate), 0.2× SSC, and 0.1× SSC. Subsequently, the slides were incubated with anti-digoxigenin conjugated to horseradish peroxidase (21H8; Abcam, Cambridge, UK, 1:1,000 dilution) for 1 h at room temperature. Finally, the sections were developed with tyramide conjugated to FITC using a Tyramide Signal Amplification kit (K1497; Dako, Glostrup, Denmark) for 15 min at room temperature. The sections of spinal cord and DRG were then viewed by confocal laser-scanning microscope.

5.7. Spinal dorsal rhizotomy

Under deep anesthesia with sodium pentobarbital (100 mg/kg i.p.), the lumbar spinal cord of female rats ($n=4$) was exposed by laminectomy at the L4 region and all exposed right dorsal roots were fully transected (Hummel et al., 2008; Sun and Chen, 2007). Rats were perfusion fixed 14 days later and the spinal cord and DRG were used in immunofluorescence studies. To analyze the effect of unilateral dorsal rhizotomy, we used rabbit polyclonal antisera raised against GPR30, SP (20064; Immunostar, Inc., Hudson, WI, USA; 1:2,500 dilution), and CGRP (AB15360; Chemicon International, Temecula, CA, USA; 1:1,000 dilution), with Alexa Fluor 488-linked goat anti-rabbit IgG used for immunofluorescent detection of the level of GPR30-, SP- or CGRP-ir in soma or fibers of the lumbar spinal dorsal horn and the

DRG (L4–6). At least 3 sections per animal were randomly assigned for analysis using Image J software (Image J ver. 1.47) with a defined threshold level. The densities were quantified as the average pixel density on contralateral and ipsilateral sides of rhizotomy in each animal, and were calculated as a ratio to the level on the contralateral side in each analysis. The analysis was performed by an investigator blinded to the group identity.

5.8. Ovariectomy and estrogen replacement

Rats were divided into 4 groups: sham-operated females ($n=4$), OVX females implanted with an empty capsule for 2 weeks ($n=6$), OVX females treated with E_2 (OVX+ E_2) ($n=6$), and sham-operated males ($n=5$). OVX and sham operations were performed under deep anesthesia with sodium pentobarbital (100 mg/kg i.p.). A silicon rubber capsule (inner diameter 1.57 mm; outer diameter 3.18 mm; length 30 mm) containing crystalline E_2 (Sigma) or an empty capsule was implanted subcutaneously into the interscapular region at the time of OVX, as described previously (Anderson et al., 2008; Yang et al., 1998, 2000). E_2 concentration was 45 ± 5 pg/ml in OVX + E_2 replacement female rats and 13 ± 4 pg/ml in OVX female rats using this method (Yang et al., 2000). The animals were perfused 14 days later and samples were prepared for analysis as above.

5.9. Statistical analyses

All values are expressed as the means $\pm$ standard deviation (SD). Multiple comparisons were made using one-way analysis of variance (ANOVA) for GPR30-ir among four groups (sham female, OVX female, OVX+ E_2 female, male) and two-way ANOVA for double IHC analysis for GPR30 and ER α in both sexes (n =number of animals per group). If significance was reached in one-way ANOVA, it was followed by the *post-hoc* Tukey HSD test. The Bonferroni test was used after two-way ANOVA. Student's *t*-test was used to compare paired data from the unilateral dorsal rhizotomy experiment. $P<0.05$ was considered significant for all statistical analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ERs	estrogen receptors
GPR30	G protein-coupled receptor 30
DRG	dorsal root ganglion
ER	estrogen receptor
GPCR	G protein-coupled receptor

OVX	ovariectomy
E₂	estradiol
IHC	immunohistochemistry; irimmunoreactivity
C	cervical
T	thoracic; Llumbar
S	sacral
SP	substance P
CGRP	calcitonin gene-related peptide
d	diameter
PB	phosphate buffer
SAB	streptavidin-biotin
DAB	diaminobenzidine
SSC	saline sodium citrate
SD	standard deviation
ANOVA	analysis of variance

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Fig. 1 –
Specificity of the GPR30 antibody in the rat lumbar DRG and spinal cord (L4–6). Controls in which anti-GPR30 serum was preabsorbed with an excess of antigen peptide (100 μg/ml) showed a complete absence of GPR30-ir in the lumbar DRG (A, B) and lumbar spinal dorsal horn (C, D). Inserts show representative small (arrowhead) and large (arrow) neurons (A). Scale bars=100 μm.

Fig. 2 –.

Immunohistochemical analysis of GPR30 in the rat somatosensory system. GPR30 immunoreactivity (ir) was observed at the cervical (C4–5), thoracic (T5–6), lumbar (L4–5) and sacral (S1–2) levels in the spinal cord in males (A) and females (B); in the spinal dorsal horn and ventral horn (ventromedial and dorsolateral nuclei) of the gray matter; and also in the white matter (A, B). GPR30-ir was seen in every dorsal root ganglion (DRG) neuron in all spinal segments examined in males (C) and females (D). Scale bars=200 μ m (A, B); 100 μ m (C, D).

Fig. 3 –.
Immunohistochemical analysis of GPR30 and ER α . In double-immunohistochemical analysis of GPR30 and ER α in the lumbar DRG (L4–6), GPR30-ir (brown) was observed in the cytoplasm of small, medium, and large-neurons. ER α -ir (violet) was mainly localized in the nuclei of small and medium neurons. The arrow indicates an ER α -positive DRG neuron that showed GPR30-ir, as also shown in the enlarged image in the insert. Scale bars = 50 μ m.

Fig. 4 –.

In situ hybridization analysis of GPR30 mRNA in the rat lumbar DRG and spinal cord (L4–6). Intense expression of GPR30 mRNA was observed in almost all neurons in the lumbar DRG (A). Inserts show representative small (arrowhead) and large (arrow) neurons (A). Slight expression of GPR30 mRNA was observed in the lumbar spinal dorsal horn (C), with much lower levels in the outer layer of the lumbar spinal dorsal horn (C). Hybridization specificity was demonstrated by the absence of labeling by sense RNA probes in the DRG (B) and spinal dorsal horn (D). Scale bars=100 μm.

Fig. 5 –. Selective spinal dorsal rhizotomy of the lumbar spinal cord. The immunoreactivities of GPR30, substance P (SP) and calcitonin gene-related peptide (CGRP) were all significantly reduced on the ipsilateral side (ipsi) of the L4–6 dorsal horn after unilateral selective spinal dorsal rhizotomy compared with the contralateral side (contra); GPR30 (A–C), SP (D–F) and CGRP (G–I). ** $P < 0.01$. Scale bars=100 μm.

Fig. 6 –.
Quantitative analysis of GPR30-positive neurons in the rat lumbar DRG using a castration/estrogen replacement model. (A) Representative result of GPR30/KDEL double-immunofluorescence analysis. (B) Ovariectomized females (OVX) had a significantly lower percentage of GPR30-ir DRG neurons than sham-operated males and females. The reduced percentage of GPR30-ir DRG neurons in OVX females was reversed by estrogen replacement (OVX+ E₂). ** $P < 0.01$; * $P < 0.05$. Scale bar=100 μ m.

Fig. 7 –.

Cell-type specific analysis of GPR30-ir expression in the lumbar DRG. The percentages of GPR30-positive neurons among the three sizes of neurons (small, medium and large) were analyzed separately. No significant difference in the percentages of GPR30-ir-positive cells in the lumbar DRG was detected among the groups or sexes.

Table 1–

Percentages of immunopositive neurons in the DRG.

	GPR30	ERα	GPR30+ERα
Male (<i>n</i> =6)	89.2 $\pm$ 5.6	0.5 $\pm$ 0.7 **	11.9 $\pm$ 7.0 *** ^{††}
Female (<i>n</i> =6)	93.2 $\pm$ 3.1	1.2 $\pm$ 0.9 **	6.0 $\pm$ 3.8 **

Comparative immunohistochemical analysis of GPR30 and ER α in the rat lumbar DRG (L4–6). The sum of the immunopositive cells for GPR30, ER α , and GPR30+ER α , was defined as the total cells numbers (100%). Quantification revealed a significantly higher percentage of GPR30-positive neurons than ER α -positive and GPR30/ER α -double-positive neurons in the lumbar DRG in both sexes. There was no significant difference between males and females ($F(1,35)=0.082$, $P=0.78$) in each of the three groups.

**
 $P<0.01$ vs. GPR30-ir.

^{††}
 $P<0.05$ vs. ER α -ir.