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Expression of estrogen receptor GPR30 in the rat spinal cord, autonomic and sensory ganglia

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Abstract

The G protein-coupled receptor GPR30 has recently been identified as a non nuclear estrogen receptor. RT-PCR revealed expression of GPR30 mRNA in varying quantities in the rat spinal cord, dorsal root ganglia, nodose ganglia, trigeminal ganglia, hippocampus, brain stem and hypothalamus. Immunohistochemical studies using a rabbit polyclonal antiserum against the human GPR30 C-terminus revealed a fine network of GPR30-immunoreactive (irGPR30) cell processes in the superficial layers of the spinal cord; some of which extended into deeper laminae. A population of neurons in the dorsal horn and ventral horn were irGPR30. Dorsal root, nodose and trigeminal ganglionic neurons displayed varying intensities of irGPR30. Positively labeled neurons were detected in the major pelvic ganglion, but not in the superior cervical ganglion. A population of chromaffin cells in the adrenal medulla was irGPR30, so were cells of the zona glomerulosa. Double-labeling the adrenal medulla with GPR30 antiserum and tyrosine hydroxylase (TH) antibody or phenylethanolamine-N-methyltransferase (PNMT) antiserum revealed that irGPR30 is expressed in the majority of TH-positive chromaffin cells. Lastly, some of the myenteric ganglion cells were irGPR30. Tissues processed with pre-immune serum resulted in no staining. Voltage-sensitive dye imaging studies showed that the selective GPR30 agonist G-1 (1, 10 and 100 nM) depolarized cultured spinal neurons in a concentration dependent manner. Collectively, our result provides the first evidence that GPR30 is expressed in neurons of the dorsal and ventral horn as well as in sensory and autonomic neurons, and activation of GPR30 by the selective agonist G-1 depolarizes cultured spinal neurons.

Keywords

adrenal gland; myenteric plexus; nodose ganglia; pelvic ganglia; trigeminal ganglia

Introduction

Much of our current knowledge regarding the physiology and pharmacology of estrogens in various tissues is derived from studies related to the interaction of estrogens with the nuclear receptors ER α and ER β , which serve as transcription factors modifying the activity of target genes (Jensen and DeSombre, 1973; Kuiper et al., 1996; Mosselman et al., 1996). ER α , the first to be identified, is highly expressed in the female reproductive tract, mammary gland, endothelial cells, vascular smooth muscle cells and the hypothalamus; whereas, ER β is more abundantly expressed in the prostate, ovary and lungs, but less in the brain and vasculature (Nilsson et al., 2001).

Recently, two groups independently reported that, in addition to the genomic receptors, 17 β -estradiol (E2) binds to a G protein-coupled receptor, GPR30 (Revankar et al., 2005; Thomas et al., 2005). Accordingly, a high-affinity, displaceable, single binding site specific for E2 was detected, respectively, in membranes of SKBR3 breast cancer cells expressing GPR30 but lacking genomic ERs, and in COS7 cells transfected with GPR30 (Revankar et al., 2005; Thomas et al., 2005). The identification of GPR30 as a putative estrogen receptor (IUPHAR classification: GPER [http://www.iuphar-db.org/GPCR/ReceptorDisplayForward?receptorID=3055]) raises the possibility that steroid hormones may signal through receptors other than classical nuclear receptors.

Earlier immunohistochemical studies showed that GPR30-immunoreactive (irGPR30) cells are present in several regions of the rat brain including the olfactory tubercle, hypothalamic paraventricular and supraoptic nucleus, hippocampus, nucleus of the solitary tract and nucleus ambiguus (Funakoshi et al., 2006; Brailoiu et al., 2007, Sakamoto et al., 2007). Though overlapping with ER α and ER β in several regions of the rat brain (Laflamme et al., 1998), the topographic distribution of irGPR30 is uniquely distinct from that of ER α and ER β (Brailoiu et al., 2007). Expression of ER α and ER β is not limited to the central nervous system; as the rat spinal cord, peripheral autonomic and sensory ganglia are endowed with genomic estrogen receptors (Papka et al., 1997; Zoubina and Smith, 2002; Bennett et al., 2003; Fan et al., 2007). We hypothesized that GPR30, in parallel with the genomic estrogen receptor, is expressed in the spinal cord and peripheral nervous tissues. The present study was undertaken to demonstrate the presence of GPR30 mRNA in the rat spinal cord, autonomic and sensory ganglia by RT-PCR, to detect immunohistochemically the distribution of irGPR30 in these tissues using a previously characterized GPR30 antiserum (Revankar et al., 2005; Brailoiu et al., 2007), and to investigate the functionality of GPR30 in cultured spinal neurons by monitoring membrane potential changes induced by the selective GPR30 agonist G-1 (Bologa et al., 2006; Brailoiu et al., 2007).

Materials and Methods

Immunohistochemistry

Adult male and female Sprague-Dawley rats, weighing 250–300 g (Ace Animals Inc., Boyertown, PA, USA) were used. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee. Rats were anesthetized with urethane (1.2 g/kg, i.p.) and intracardially perfused with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde/0.2% picric acid in PBS. Spinal cord, dorsal root ganglia, nodose ganglia, trigeminal ganglia, superior cervical ganglia, major pelvic ganglia, adrenal glands, and proximal segments of the ileum were removed, post-fixed for 2 h, and stored in 30% sucrose/PBS solution overnight. Spinal cord and ganglia were sectioned to 40 μ m using a cryostat. In preparing the myenteric plexus, layers of smooth muscles were removed and the plexus was processed as a whole mount (Dun et al., 1997).

Tissues were processed for GPR30-immunoreactivity (irGPR30) by the avidin-biotin complex procedure or fluorescent method (Brailoiu et al., 2005b; 2007). Tissues were first treated with 3% H₂O₂ to quench endogenous peroxidase, washed several times, blocked with 10% normal goat serum, and incubated in GPR30 antiserum (1:1000 dilution). GPR30 antiserum was a rabbit polyclonal antibody directed against the human C-terminus GPR30 (CAVIPDSTEQSDVRFSSAV; Revankar et al., 2005). The specificity of GPR30 antiserum has been previously confirmed (Revankar et al., 2005; Brailoiu et al., 2007). After thorough rinsing, sections were incubated in biotinylated anti-rabbit IgG (1:150 dilution, Vector Laboratories, Burlingame, CA, USA) for 2 h, rinsed with PBS, and incubated in avidin-biotin complex solution for 1 h (1:100 dilution, Vector Laboratories). Following washes in Tris-buffered saline, sections were incubated in 0.05% diaminobenzidine/0.001% H₂O₂ solution and washed for at least 2 h with Tris buffered saline. Sections were mounted on slides with 0.25% gel alcohol, air-dried, dehydrated with absolute alcohol followed by xylene, and coverslipped with Permount. For control experiments, sections were processed with pre-immune serum in place of GPR30 antiserum.

In double-labeling and some single staining experiments, the fluorescent method was used. Tissue sections were first blocked with normal goat serum (1:10 in PBS, 0.5% BSA, 0.4% Triton X-100) and then incubated with GPR30 antiserum (1:500 dilution). Following several washes with PBS, sections were incubated with biotinylated anti-rabbit IgG (1:50, Vector Laboratories), washed with PBS, and incubated with avidin-fluorescein isothiocyanate (FITC). After rinsing with PBS, adrenal gland sections were incubated with either tyrosine hydroxylase antibody (TH; 1:700, a monoclonal antibody from Chemicon, Temecula, CA, USA) or phenylethanolamine-N-methyltransferase antiserum (PNMT; 1:300, a guinea pig polyclonal from Chemicon). Tissues were incubated in Texas Red conjugated to appropriate second primary antibodies, washed with PBS, and mounted in Citifluor and coverslipped. Sections of major pelvic ganglion or spinal cord were double-labeled with GPR30 antiserum and choline acetyltransferase antiserum (ChAT; 1:100, a goat polyclonal from Chemicon). Sections were examined under a confocal scanning laser microscope (Leica TCS SL) with excitation/emission wavelengths set to 488/520 nm for FITC and 543/620 nm for Texas Red in the sequential mode.

RT-PCR

Total RNA from various tissues and brain regions of the rats was isolated using Trizol® reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The purity and concentration of RNA were assessed by spectrophotometric analysis. First-strand cDNA was synthesized from 3 µg of total RNA with random hexamer primers and the SuperScript III reverse transcriptase according to the manufacturer's protocol (Invitrogen). 2 µl of 20 µl cDNA product was amplified with the following specific primers based on GPR30 (NM_133573) and β-actin (NM_031144) sequences: GPR30: 5' TCTGTG AC CC TTT CAGCAAGTCCT 3' (upstream), 5' AGAAGGCTG CCAG GT TG ACTATGT 3' (downstream); β-actin: 5' CCATTGAACACGGCAT TG TCACCA 3' (up stream), 5' ACTCCTGCTTGCTGATCCACATCT 3' (downstream). Amplifications were performed using Platinum Taq DNA polymerase (Invitrogen) as follows: GPR30: 38 cycles of 94°C for 60 s, 58°C for 60 s, 72°C for 60 s; β-actin: 29 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 60 s. PCR products were visualized by an ethidium bromide staining under UV light following electrophoresis on 1 % agarose gel.

Spinal cord culture

Spinal cord neurons were dissociated from neonatal (1-3-days old) Sprague Dawley rats as previously described (Brailoiu et al., 2005a, 2008). Spinal cord was removed and quickly immersed in ice-cold Hank's balanced salt solution without calcium and magnesium (HBSS,

Mediatech, Herndon, VA). Tissues were minced and subject to enzymatic digestion with papain for 20 min at 37°C followed by mechanical trituration. After centrifugation at 500x g, fractions enriched in neurons were collected and re-suspended in Neurobasal-A medium (Invitrogen, Carlsbad, CA) containing 2 mM glutamine, 100 units/ml penicillin G, 100 µg/ml streptomycin and 10% fetal bovine serum. Cells were plated on round glass coverslips in 24-well plates. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂. The mitotic inhibitor cytosine β-arabino furanoside (1 µM) (Sigma-Aldrich, St. Louis, MO) was added to the culture to inhibit glial cell proliferation (Schoniger et al., 2001). Cultures were maintained by changing the media every 2–3 days.

Optical imaging using DiSBAC₄(3)

DiSBAC₄(3), bis-[1,3-dibutylbarbituric acid] trimethineoxonol, a slow-responding voltage-sensitive fluorescent dye, was used to detect relative changes in membrane potential of single cells in response to G-1 (PubChem ID 5322399 [<http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=5322399>]). Optical imaging using voltage sensor probes is a reliable approach in monitoring membrane potential changes in neurons (Ebner and Chen, 1995; Kunkler et al., 2005; Brailoiu et al., 2008). Briefly, the cells were incubated for 30 min in HBSS containing 0.5 mM DiSBAC₄(3). The fluorescence (excitation wavelength = 480 nm, emission wavelength = 540 nm) was continuously recorded at a rate of 10 points min⁻¹. Background values (windows of identical area placed beside the cells) were always subtracted. The dye partition between the cell membrane and the cytosol is a function of membrane potential. Depolarization of the membrane leads to a sequestration of the dye into cytosol and is associated with an increase in the fluorescence intensity (Brauner et al., 1984); whereas, the dye concentrates in the cell membrane during membrane hyperpolarization, leading to a decrease of cytoplasmic fluorescence intensity.

Calibration of DiSBAC₄(3)

Calibration of DiSBAC₄(3) fluorescence was performed using the Na⁺-K⁺ ionophore gramicidin in Na⁺-free physiological solution (Brauner et al., 1984). The osmolarity was maintained constant by addition of N-methylglucamine. In the presence of gramicidin (1 µM), the Na⁺ concentration gradient is zero, and the membrane potential is approximately equal to K⁺ equilibrium potential which is determined by the Nernst equation. The intracellular K⁺ and Na⁺ concentrations were assumed to be 130 mM and 10 mM, respectively. The addition of gramicidin with various concentrations of K⁺ to the cultured neurons alters the cell membrane potential, thereby, altering fluorescence. According to the calibration measurement, a change in DiSBAC₄(3) fluorescence intensity by 1.092 are equivalent to a change in membrane potential of 1 mV.

Statistics

Paired *t*-test followed by one way ANOVA was used to evaluate significant differences between controls and G-1 treated neurons in optical imaging experiments.

Results

RT-PCR

RT-PCR results show that GPR30 mRNA was abundantly expressed in the rat spinal cord, brain stem, and trigeminal ganglia (Fig.1). The level appeared to be lower in nodose and dorsal root ganglia, and barely detectable in superior cervical ganglia (Fig. 1). Consistent with an earlier report that irGPR30 is strongly expressed in the rat hippocampus and

hypothalamus (Brailoiu et al., 2007), GPR30 mRNA was abundantly expressed in these tissues (Fig. 1).

GPR30-immunoreactivity in the spinal cord

Preliminary results showed that the distribution profile of irGPR30 in the spinal cord, adrenal glands, autonomic and sensory ganglia was similar in the adult male (n=4) and female (n=3) rats. Immunohistochemical results described below were from adult male rats.

Immunoreactivity was conspicuously present in two regions of the cervical, thoracic, lumbar and sacral segments of the spinal cord; namely, the superficial and deeper layers of the dorsal horn and ventral horn (Fig. 2A, C–D). Fine irGPR30 cell processes or terminals were detected in the superficial layers; with fewer cell processes in the deeper laminae (Fig. 2A). In addition, small diameter irGPR30 cells occurred in the superficial as well as deeper layers (Fig. 2A). With respect to the ventral horn, few strongly labeled and many moderately labeled neurons were seen in each spinal section (Fig. 2C & D). Unlike the genomic estrogen receptor, irGPR30 was detected in the cytoplasm (Fig. 2D). Double-labeling the spinal cord sections with GPR30 antiserum and choline acetyltransferase (ChAT)-antiserum revealed that irGPR30 is present in ChAT-positive cells (Fig. 5. J–L). For negative controls, spinal sections processed with pre-immune serum resulted in no labeling (Fig. 2B).

Sensory and sympathetic ganglia

Immunoreactivity of varying intensities was detected in neurons of the trigeminal ganglia (Fig. 3A), nodose ganglia (Fig. 3B) and dorsal root ganglia (Fig. 3C). In all three ganglia, immunoreactivity was detected in large as well as small diameter cells (Fig. 3A–C). On the other hand, superior cervical ganglia, which are taken as representatives of sympathetic ganglia, expressed little or no irGPR30 (Fig. 3D).

Major pelvic ganglia, myenteric plexus and adrenal glands

Within the major pelvic ganglia, irGPR30 was detected in numerous ganglion cells (Fig. 4A). With respect to the myenteric plexus, irGPR30 was present in clusters of ganglion cells and cell processes (Fig. 4B). In the case of adrenal glands, cells of the zona glomerulosa (arrows), which lie immediately beneath the capsule, expressed irGPR30 (Fig. 4C). Within the medulla, groups of irGPR30 cells intermixed with clusters of non labeled or lightly labeled chromaffin cells (Fig. 4D).

Adrenal chromaffin cells are either epinephrine- or norepinephrine-containing. Double-labeling the sections with GPR30 antiserum and tyrosine hydroxylase (TH) antibody or phenylethanolamine-N-methyltransferase (PNMT) antiserum revealed that irGPR30 is expressed in the majority of TH-positive cells (Fig. 5A–C), and is not expressed in PNMT-positive cells (Fig. 5D–F). Rat major pelvic ganglion cells express synthetic enzymes for either norepinephrine or acetylcholine (Keast et al., 1995). Double-labeling the major pelvic ganglion sections with GPR30- and choline acetyltransferase (ChAT)-antiserum revealed that irGPR30 is present in ChAT-positive cells (Fig. 5G–I, solid arrows). By inference, irGPR30 ganglion cells that do not express ChAT are presumably noradrenergic (Fig. 5G–I; open arrows); the latter were often encircled by a network of ChAT-positive fibers.

G-1 and resting membrane potential

The mean resting membrane potential of cultured spinal motoneurons as monitored by changes in DiSBAC₄(3) fluorescence was -63 ± 5 mV, which was comparable to that recorded by intracellular microelectrodes from motoneurons in neonatal rat spinal cord slices (Jiang et al., 1990; Wang and Dun, 1990). G-1 is a selective agonist of GPR30, lacking affinity for the genomic ER α and ER β estrogen receptors (Bologa et al., 2006).

Administration of G-1 (100 nM) produced a depolarization of spinal motoneurons with an amplitude of 8.6 ± 0.64 mV ($n=23$); a representative example is shown in Fig. 6A. The amplitude of depolarizations was dose-dependent, as G-1 (1 nM, 10 nM and 100 nM) depolarized spinal motoneurons by 0.84 ± 0.76 mV ($n=14$), 3.7 ± 0.48 mV ($n=19$) and 8.6 ± 0.64 mV ($n=23$), respectively (Fig. 6B).

Discussion

Results from RT-PCR and immunohistochemical studies show that the recently identified non nuclear estrogen receptor GPR30 is expressed in the rat spinal cord, adrenal gland, autonomic and sensory ganglia. In the spinal cord, irGPR30 is detected in cells as well as cell processes of the dorsal horn, and in a population of ventral horn neurons. With respect to the peripheral nervous tissues, irGPR30 is expressed in neurons of the trigeminal, nodose and dorsal root ganglia, major pelvic ganglia and myenteric ganglia; and it is undetectable in sympathetic ganglia. In the adrenal gland, irGPR30 is associated with cells of the zona glomerulosa and TH-containing chromaffin cells. The GPR30 antiserum used in this study was the same used in our previous study where the expression of irGPR30 in the rat central nervous system was investigated (Brailoiu et al., 2007). As in the previous study, tissues processed with pre-immune serum were devoid of irGPR30, supporting the specificity of the antiserum. RT-PCR results corroborate the immunohistochemical observations in that GPR30 transcripts can be detected in the spinal cord, and sensory ganglia; whereas the level appears to be very low in sympathetic ganglia. Expression of GPR30 mRNA in the rat dorsal root ganglia has recently been reported (Kuhn et al., 2008).

There is evidence that estrogen can induce rapid signaling via activation of non-nuclear ER α and ER β receptors. For example, estrogen mediates rapid actions on gonadotropin-releasing hormone neurons (Abraham et al., 2003), reduces calcium currents in rat neostriatal neurons (Mermelstein et al., 2003), modulates inositol phospholipid hydrolysis by norepinephrine (Favit et al., 1991) and inhibits L-glutamate uptake activity of astrocytes (Sato et al., 2003). Moreover, in mouse midbrain neurons and astrocytes, estrogen elicits fast and transitory Ca²⁺ responses (Beyer and Raab, 1998; Chaban et al., 2004) from thapsigargin-sensitive calcium stores. In addition to the rapid effects mediated by non-nuclear ER α and ER β and GPR30, another estrogen receptor, which mediates fast events, has been described. ER-X is present in developing brain but not in adult and is located intracellularly in caveolar-like microdomains. This location allowed ER-X to rapidly interact with the kinases of the MAPK cascade (Toran-Allerand et al., 2002).

In our previous study, the pattern of irGPR30 expression in the female and male brain is found to be similar (Brailoiu et al., 2007). A similar observation is noted relative to the distribution pattern of irGPR30 in the spinal cord, adrenal gland, autonomic and sensory ganglion of male and female rats. Similarly, the pattern of distribution of genomic estrogen receptors in the central nervous system of male rats is comparable to that of female rats (Laflamme et al., 1998) as well as the brainstem and spinal cord of male and female mice (VanderHorst et al., 2005). While a gender difference is not apparent in the pattern of distribution of genomic and non genomic estrogen receptors in the nervous system, the density or pharmacological property of estrogen receptors may be substantially different in females as compared to males. The observation that the pattern of distribution of estrogen receptors is similar in male and female rat central and peripheral nervous systems lends further support to the notion that estrogens modulate a range of biological activities in males and females, and may not be restricted to reproductive or gender-related behaviors (Zoubina and Smith, 2002).

While there is a general agreement that GPR30 is a non nuclear estrogen receptor (Revankar et al., 2005; Thomas et al., 2005), the site where GPR30 is concentrated; i.e., plasma membrane versus cytoplasm, in neuronal or non neuronal cells, has yet to be clearly resolved (Revankar et al., 2005; Funakoshi et al., 2006; Filardo et al., 2007; Revankar et al., 2007). In the case of rat spinal neurons, sensory and autonomic ganglion cells examined here, irGPR30 seems to be distributed throughout the cytoplasm, excluding the nucleus. Similarly, irGRP30 is found to be present in the cytoplasm of central neurons of the rats (Brailoiu et al., 2007). Unlike the ER α - and ER β -immunoreactivity that is present in the nucleus (Papka et al., 1997; VanderHorst et al., 2005), irGPR30 is non nuclear in the central and peripheral neurons.

When comparing the expression of GPR30 and ER α or ER β in the rat brain, several areas, such as the olfactory tubercle and Islands of Calleja, express ER α and ER β . (Laflamme et al., 1998) as well as GPR30 (Brailoiu et al., 2007). Overall, the pattern of GPR30 expression appears to be distinct from that of ER α and ER β . in the rat brain (Brailoiu et al., 2007). With respect to the rat spinal cord, ER α - and ER β -immunoreactive neurons are highly expressed in the superficial layers of the dorsal horn, with fewer cells in the ventral horn and, this pattern of distribution is similar in male and female rats (Papka et al., 1997; VanderHorst et al., 2005). In the case of irGPR30, the superficial layers of the dorsal horn and ventral horn contain positively labeled cells. The function of GPR30 and genomic estrogen receptors in the spinal cord is largely unknown. The presence of irGPR30 in the superficial layers of the dorsal horn suggests that activation of GPR30 may be involved in the processing of sensory and/or nociceptive information. There is evidence supporting a modulatory role for estrogens in pain processing at the spinal level (McCarthy et al., 1990; Hucho et al., 2006; Fan et al., 2007; Spooner et al., 2007). More recently, GPR30 agonists have been shown to induce mechanical hyperalgesia in the rat (Kuhn et al., 2008). Further, aromatase, the enzyme responsible for the synthesis of estradiol from testosterone, is also present in the superficial layers of the dorsal horn of different species, suggesting a local synthesis of estrogen (Evrard, 2006). Similarly, rat cultured motoneurons express genomic estrogen receptors as well as aromatase (Rakotoarivelo et al., 2004). Similar to testosterone, estradiol is shown to be neuroprotective on motoneurons *in vivo* and *in vitro* and promotes regeneration following injury (Tanzer and Jones, 1997; Nakamizo et al., 2000; Huppenbauer et al., 2005; Ahmed et al., 2006). The question whether estradiol exerts its neuroprotective effect via genomic (ER α and ER β) estrogen receptors or GPR30 or both remains to be investigated.

Several studies showed that the genomic estrogen receptors ER α and ER β proteins and mRNAs are expressed in the rat autonomic (major pelvic) and sensory (nodose and dorsal root) ganglia (Papka et al., 1997, 2001; Bennett et al., 2003; Purves-Tyson et al., 2007). The present result shows that GPR30 is expressed in a population of neurons of the autonomic (major pelvic and myenteric) and sensory ganglia (dorsal root, nodose and trigeminal) examined here. Rapid actions of estrogen via cyclic AMP response element binding protein (CREB) were reported in dorsal root ganglion neurons (Purves-Tyson and Keast, 2004). Sympathetic (superior cervical) ganglia seem to contain very low levels of GPR30 transcripts or irGPR30 as shown here. In contrast, sympathetic postganglionic neurons express ER α and ER β (Zoubina and Smith, 2002). In the sensory ganglia, irGPR30 appears to be present in ganglion cells of different sizes, suggesting that GPR30 expressing ganglion cells participate in nociceptive as well as non-nociceptive transmission. Whether or not nuclear estrogen receptors and GPR30 are expressed in the same subset of autonomic and sensory ganglion cells is not known. Similarly, the functional role of activation of nuclear estrogen receptors and GPR30 in autonomic and sensory pathways remains to be studied. Estrogen has been shown to increase sensory nociceptor innervation of arterioles (Blacklock et al., 2004), promote neuritogenesis in dorsal root ganglion neurons via either an NGF-

independent mechanism (Blacklock et al., 2005) or the renin-angiotensin system (Chakrabarty et al., 2008)

In the adrenal gland, irGPR30 is noted in a population of cells of the zona glomerulosa as well as adrenal chromaffin cells. GPR30 and aromatase, but not ER α or ER β , are expressed in the zona glomerulosa in human adrenal tissue from subjects older than 6–20 years (Baquedano et al., 2007). Zona glomerulosa cells have a high proliferation index from early infancy to late puberty (Baquedano et al., 2005). It has been suggested that estrogens, in an autocrine and/or paracrine manner, may stimulate adrenal progenitor cell proliferation and migration via a GPR30-dependent mechanism (Baquedano et al., 2007).

Chromaffin cells have two known phenotypes: epinephrine- and norepinephrine-containing. Double-labeling studies show that irGPR30 is expressed in a population of TH positive, presumably norepinephrine-containing, chromaffin cells. The functional significance of differential expression of GPR30 in norepinephrine-containing chromaffin cells is not clear. Environmental estrogenic pollutants, such as p-nonylphenol and bisphenol A, are compounds that are shown to have estrogenic effects on fetal development and the reproductive system (Bechi et al., 2006). A recent study shows that in bovine adrenomedullary cells environmental estrogenic pollutants stimulated catecholamine synthesis by activating tyrosine hydroxylase; the latter was not antagonized by the nuclear estrogen receptor antagonist ICI182,780 (Yanagihara et al., 2005). This observation raises the possibility that the stimulatory effect of environmental estrogenic pollutants on catecholamine synthesis may be related to the activation of GPR30.

The observations that the selective GPR30 agonist G-1 (Bologa et al., 2006) depolarizes cultured spinal neurons support the contention that GPR30 expressed in spinal neurons is functional. Our finding that activation of GPR30 by G-1 is excitatory in spinal neurons may explain the mechanical hyperalgesia induced by G-1 injected into the hind paw (Kuhn et al., 2008).

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Fig. 1.

RT-PCR results of GPR30 mRNA in various tissues. The total RNA was extracted by Trizol reagent. 3 μ g of total RNA was used for RT-PCR as described under "Experimental Procedures." Housekeeping gene β -actin was used as an internal control. The product size of rat GPR30 was 987 bp and that of β -actin was 876 bp. The figure is the representative of three experiments with similar results.

Fig. 2.

Fluorescence images of rat spinal cord sections labeled with GPR30 antiserum or pre immune serum. A, lumbar spinal section where irGPR30 is detected in terminals and neurons of small diameter (arrows) in the dorsal horn. B, lumbar spinal cord section processed with pre immune serum, where immunoreactivity is not detected. C, few intensely and many moderately labeled ventral horn cells are noted. D, higher magnification of the boxed ventral horn area shown in C; irGPR30 is detected in the cytoplasm. Scale bar: A– C, 50 μ m; D, 10 μ m.

Fig. 3.

Images of rat trigeminal, nodose, dorsal root and superior cervical ganglion sections labeled with GPR30 antiserum. A, section of trigeminal ganglion where numerous ganglion cells are irGPR30. B, section of nodose ganglion where ganglion cells of varying intensities are detected. C, numerous irGPR30 cells of large and small diameter are noted in this lumbar dorsal root ganglion section. D, section of superior cervical ganglion where little or no irGPR30 is detected. Scale bar: A and D, 25 μ m; B and C, 50 μ m.

Fig. 4. Sections of rat major pelvic ganglion, myenteric plexus and adrenal gland labeled with GPR30 antiserum. A, numerous irGPR30 cells are noted in the major pelvic ganglion. B, several irGPR30 cells in a myenteric ganglion; moderately labeled cell processes extend from some of these cells. C, section of adrenal cortex where cells of the zona glomerulosa (arrows) located underneath the adrenal capsule, are labeled. D, section of adrenal medulla where groups of irGPR30 chromaffin cells intermix with non labeled or lightly labeled chromaffin cells. Scale bar: A, B and D, 50 μ m; C, 100 μ m.

Fig. 5. Confocal images of rat adrenal medulla sections double-labeled with GPR30 antiserum and tyrosine hydroxylase (TH) or phenylethanolamine-N-methyltransferase (PNMT) antiserum and major pelvic ganglion and spinal cord sections labeled with GPR30 and choline acetyltransferase (ChAT) antisera. A and B, section double-labeled with GPR30 and TH antiserum; some irGPR30 chromaffin cells (green fluorescence) are also TH-positive (red fluorescence). C, a merged image of A and B, where double-labeled cells appear orange/yellow. D and E, section double-labeled with GPR30 (green fluorescence) and PNMT antiserum (red fluorescence). F, a merged image of D and E where there is a clear distinction between irGPR30 and irPNMT cells. G and H, pelvic ganglion section double-

labeled with ChAT (green fluorescence) and GPR30 (red fluorescence) antiserum. irGPR30 was present in some cholinergic cells (solid arrows); while other irGPR30 cells (open arrows) do not express ChAT, but are surrounded by ChAT immunoreactive fibers. I, a merged image of G and H; several double-labeled cells (solid arrows) are irGPR30 and ChAT positive, and several irGPR30 cells are ChAT negative (open arrows). J and K, spinal cord sections double-labeled with ChAT (green fluorescence) and GPR30 (red fluorescence). L, merged image of J and K; few double-labeled cells are irGPR30 and ChAT positive. Scale bar: A–F, 20 μ m, G–L, 50 μ m

Fig. 6. Depolarization of cultured spinal neurons by G-1. A, administration of G-1 (100 nM) produced a depolarization with the amplitude of 8 mV in this neuron. B, G-1-induced depolarizations were concentration-dependent; G-1 (1 nM, 10 nM, 100 nM) depolarized cultured spinal neurons by 0.84 ± 0.76 mV ($n=14$), 3.7 ± 0.48 mV ($n=19$), and 8.6 ± 0.64 mV ($n=23$). One asterisk indicates statistically significant difference as compared to control ($P<0.05$).