Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system

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Abstract

The G protein-coupled receptor 30 (GPR 30) has been identified as the non-genomic estrogen receptor, and G-1, the specific ligand for GPR30. With the use of a polyclonal antiserum directed against the human C-terminus of GPR30, immunohistochemical studies revealed GPR30-immunoreactivity (irGPR30) in the brain of adult male and non-pregnant female rats. A high density of irGPR30 was noted in the Islands of Calleja and striatum. In the hypothalamus, irGPR30 was detected in the paraventricular nucleus and supraoptic nucleus. The anterior and posterior pituitary contained numerous irGPR30 cells and terminal-like endings. Cells in the hippocampal formation as well as the substantia nigra were irGPR30. In the brainstem, irGPR30 cells were noted in the area postrema, nucleus of the solitary tract, and dorsal motor nucleus of the vagus; a cluster of cells were prominently labeled in the nucleus ambiguus. Tissue sections processed with pre-immune serum showed no irGPR30, affirming the specificity of the antiserum. G-1 (100 nM) caused a large increase of intracellular calcium concentrations \( [Ca^{2+}]_i \) in dissociated and cultured rat hypothalamic neurons, as assessed by microfluorometric Fura-2 imaging. The calcium response to a second application of G-1 showed a marked homologous desensitization. Our result shows a high expression of irGPR30 in the hypothalamic–pituitary axis, hippocampal formation, and brainstem autonomic nuclei; and the activation of GPR30 by G-1 is associated with a mobilization of calcium in dissociated and cultured rat hypothalamic neurons.

Introduction

The steroid hormone estrogen produces a variety of cell responses, many of which can be attributed to the activation of two known estrogen receptors \( \alpha \) and \( \beta \) (ER\( \alpha \) and ER\( \beta \)), which function as transcription factors (Jensen & DeSombre 1973, Mosselman et al. 1996). However, a number of cell responses are induced in seconds to minutes following estrogen stimulation, for example, nitric oxide generation, calcium mobilization, and activation of tyrosine kinases (Morley et al. 1992, Brubaker & Gay 1999, Karpuzoglu et al. 2006). For this reason, the existence of non-genomic ERs mediating rapid cell signaling has been proposed in a number of studies (Lagrange et al. 1997, Pietras & Szego 1997, Singh et al. 2000, Toran-Allerand et al. 2002, Morales et al. 2003, Qiu et al. 2003).

Two groups independently proposed that the G protein-coupled receptor (GPR 30), is the non-genomic ER (Revankar et al. 2005, Thomas et al. 2005). A high-affinity, displaceable, single binding site specific for estrogen was detected respectively in plasma membranes of SKBR3 breast cancer cells that express GPR30 but lack nuclear ERs, and in COS7 cells transfected with GPR30 (Revankar et al. 2005, Thomas et al. 2005). As GPR30 is expressed in cell lines in these studies (Revankar et al. 2005, Thomas et al. 2005), the distribution of GPR30 in the brain has not been investigated. An earlier study demonstrates that COS7 cells transfected with GPR30 responded to estrogen with a large influx of \( Ca^{2+} \) (Revankar et al. 2005). The present study was undertaken to determine the distribution of GPR30 in the central nervous system of adult rats of either sex, and to characterize the calcium response of native GPR30 in dissociated and cultured rat hypothalamic neurons in response to the GPR30-specific ligand G-1 (Bologa et al. 2006).

Materials and Methods

Immunohistochemistry

Adult male and female Sprague–Dawley rats, weighing 225–250 g (Ace Animals Inc., Boyertown, PA, USA), were used in this study. Animal protocols were reviewed and approved by the Institution Animal Care and Use Committee.
Animals were anesthetized with urethane (1·2 g/kg, i.p.) and intracardially perfused with 0·1 M PBS followed by 4% paraformaldehyde/0·2% picric acid in PBS. Brains and pituitary glands were removed, post-fixed for 2 h, and stored in 30% sucrose/PBS solution overnight.

In single-staining, tissues were processed for GPR30-immunoreactivity (irGPR30) by the avidin–biotin complex procedure (Brailoiu et al. 2005). 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). 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 several washes in Tris-buffered saline, sections were incubated in 0·05% diaminobenzidine/3% H2O2 solution and washed for at least 2 h with Tris-buffered saline. Sections were mounted on slides with 0·25% E BRAILIOIU and others.

In double-staining, brain sections were examined under a confocal scanning laser microscope (Leica Microsystems Inc., Exton, PA, USA) with excitation/emission wavelengths set to 488/520 nm for FITC and 543/620 nm for Texas Red in the sequential mode.

Neuronal cell culture

Cells were isolated from the hypothalamus of postnatal 1- to 3-day-old rats by enzymatic digestion with 0·5 mg papain/100 mg tissue (Dun et al. 2006). Cells were plated at a density of 103/mm2 in a Neurobasal-A medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen), and maintained at 37 °C in a humidified atmosphere with 5% CO2. Glial cell growth was inhibited by the mitotic inhibitor cytosine β-arabinofuranoside (1 µM; Sigma). Neurons were cultured for 5 days. Cells were transferred to a medium without fetal serum 12 h prior to Ca2+ measurements.

Cytosolic Ca2+ concentrations

Cytosolic Ca2+ measurements were performed as described previously (Brailoiu et al. 2006). Cells were incubated with 5 µM Fura-2 AM (Molecular Probes, Eugene, OR, USA) in Hanks' balanced salt solution (HBSS) at room temperature for 45 min in the dark, washed thrice with dye-free buffer, and then incubated for another 45 min to allow for complete de-esterification of the dye. Coverslips were subsequently mounted in a custom-designed bath on the stage of an Eclipse TE 2000-U Nikon inverted microscope equipped with a Roper Scientific CCD camera (Optical Apparatus Co., Ardmore, PA, USA). Cells were routinely superfused with HBSS at a flow rate of 1 ml/min. Fura-2 fluorescence (emission = 510 nm), following alternate excitation at 340 and 380 nm, was acquired at a frequency of 0·33 Hz. Images were acquired and analyzed using Metafluor software. For Ca2+-free experiments, HBSS without Ca2+ supplemented with 2·5 mM EGTA was used.

Chemicals

G-1 was a gift from Chemical Diversity Inc. (San Diego, CA, USA).

Statistical analysis

In calcium measurement experiments, statistical significance between groups was tested using one-way ANOVA followed by Bonferroni test, P<0·05 being considered significantly different.

Results

Immunohistochemistry

Examination of sections prepared from four female and five male rats revealed a similar pattern of distribution of irGPR30.
in the brain and pituitary. Results described below are derived from male rats unless stated otherwise. The nomenclature follows that used in Paxinos & Watson (1998).

**Forebrain**

A high density of irGPR30 was detected in the Islands of Calleja (ICj), major island (ICjM), ventral pallidum (VP), and olfactory tubercle (Tu); a lesser density of irGPR30 was present in the nucleus of vertical limb diagonal band (VDB; Fig. 1A–D). A high density of irGPR30 was detected in the rostro-caudal extent of lateral globus pallidus (Fig. 1E and F). In the thalamus, irGPR30 was detected in the reticular thalamic nucleus (Fig. 1E) and medial habenular nucleus (not shown).

With respect to the hypothalamus, cells in the paraventricular nucleus (PVN), supraoptic nucleus (SO), and accessory neurosecretory nuclei (acc) were strongly labeled (Fig. 2A–C). Within the PVN, irGPR30 was identified mostly in the lateral magnocellular part (PaLM), medial parvicellular part (PaMP), ventral part (PaV), dorsal cap (PaDC), and less in the anterior and posterior parvicellular parts (Fig. 2B). Few cells in the suprachiasmatic nucleus, retrochiasmatic nucleus, and periventricular hypothalamic nucleus were immunoreactive (Fig. 2A). The arcuate nucleus showed a moderate number of irGPR30 cells (not shown).

Incubation with pre-immune serum resulted in little or no irGPR30 in hypothalamic sections; an example is shown in Fig. 2D.

**Pituitary gland**

With respect to the pituitary gland, the anterior pituitary contained a large number of immunoreactive cells (Fig. 2E); numerous terminal-like endings were irGPR30 in the posterior pituitary (Fig. 2F).

**Hippocampus**

Cells in the hippocampal formation including CA1, CA2, CA3 regions and dentate gyrus (DG) were irGPR30 (Fig. 3A and B).

**Midbrain and medulla oblongata**

At the level of pons, a high concentration of irGPR30 was seen in the substantia nigra reticular and compact parts (SNR, SNC; Fig. 3C and D). In the medulla, positively labeled cells were noted in the area postrema (AP), dorsal motor nucleus of the vagus (10), nucleus of the solitary tract (Sol), and hypoglossal nucleus (12, Fig. 4A and B). A cluster of irGPR30 cells was conspicuously present in the nucleus ambiguus (Amb; Fig. 4C–D). In addition, irGPR30 cells were distributed sparsely throughout the reticular formation (Fig. 4A).

**Co-expression**

Double-labeling of the hypothalamic sections with GPR30-antiserum and OT or VP antiserum revealed that ~40 and 32% irGPR30 cells were OT-positive, and about 60 and 70% irGPR30 cells were VP-positive, in the hypothalamic paraventricular and SOs respectively (Fig. 5A–D). In the medulla oblongata, nearly all irGPR30 cells in the Amb, dorsal motor nucleus of the vagus, and hypoglossal nucleus were ChAT-positive (Fig. 5E and F). With respect to cultured hypothalamic neurons, irGPR30 was co-localized with neuron-specific enolase (Fig. 6A and B), affirming that irGPR30 was expressed in cultured neurons.

**Intracellular \([Ca^{2+}]\)**

Administration of G-1 (100 nM), the ligand that selectively activates GPR30 but not the nuclear ERs ER\(\alpha\) and ER\(\beta\) (Bologa et al. 2006), induced an increase in \([Ca^{2+}]\), by 964 ± 86 nM in 26 of 109 cells tested; an example is shown in Fig. 7A. The rise of \([Ca^{2+}]\), was rapid and sustained, reaching a plateau in about 3–6 min. In hypothalamic neurons perfused with a Ca\(^{2+}\)-free medium, G-1 (100 nM) elevated \([Ca^{2+}]\), by 58 ± 2-2 nM (n=6); a representative experiment is shown in Fig. 7B.

The next series of experiments was designed to evaluate whether or not GPR30 undergoes desensitization, a feature common to GPRs, by monitoring the Ca\(^{2+}\) responses to two consecutive perfusions of G-1. In those hypothalamic cells responding to the first application of G-1 (100 nM), a second application of G-1 induced an average increase of \([Ca^{2+}]\), of 174 ± 3-6 nM, which was significantly smaller than that elicited by the first G-1 application (n=10, P<0.05); a representative experiment is shown in Fig. 8.

**Discussion**

The gene that encodes GPR30, also known as CEPR, FEG-1, CMKRL2 or LyGPR, was identified many years prior to the recent proposal that the receptor may serve as the chemokine receptor family, the C–C chemokine 8 (CMKRL2) is a 375 amino acid class-1 GPCR (SwissProt Q99527 (http://www.expasy.org/uniprot/Q99527)) with highest sequence identity (<35%) with members of the chemoattractant receptor family, the C–C chemokine 8 receptor, the chemokine receptor CXCR1 (~29% overall identity) and the somatostatin receptor 4. For this reason, a chemokine was first thought to be the endogenous ligand (O’Dowd et al. 1998). Evidence from an early study (Fildaro et al. 2000) and two recent studies (Revankar et al. 2005, Thomas et al. 2005) indicates that GPR30 is an ER, but distinct from the classic ERs, ER\(\alpha\) and ER\(\beta\).
Figure 1  Distribution of irGPR30 in the rat forebrain. (A) A high density of irGPR30 is noted in the Islands of Calleja (ICj), major island (ICjM), ventral pallidum (VP), olfactory tubercle (Tu), nucleus of vertical limb diagonal band (VDB). (B) A higher magnification of the area outlined in A, depicting irGPR30 in the ICj, VP, and Tu. (C) Intense irGPR30 is present in VP. (D) A higher magnification of the area outlined in C, where a high density of irGPR30 is detected in the VP. (E) irGPR30 is detected in the lateral globus pallidus (LGP) and reticular thalamic nucleus (Rt). (F) a higher magnification of the area outlined in E. Abbreviations: aca, anterior commissure anterior part; Acb, accumbens nucleus; CPu, caudate putamen; ec, external capsule; ic, internal capsule. Calibration bar: A, C and E, 250 µm; B, D and F, 50 µm.
Figure 2 Distribution of irGPR30 in the rat hypothalamus and pituitary. (A) Dense irGPR30 is present in paraventricular hypothalamic nucleus (PVN), accessory neurosecretory nuclei (acc) and supraoptic nucleus (SO). Immunoreactivity is sparse in periventricular hypothalamic nucleus (Pe) and retrochiasmatic nucleus (RCh). (B) A higher magnification of PVN, where irGPR30 is seen in the lateral magnocellular part (PaLM), medial parvicellular part (PaMP), ventral part (PaV), and dorsal cap (PaDC). (C) A higher magnification of SO where dense irGPR30 is detected. (D) A hypothalamic section processed with pre-immune serum; immunoreactivity is not detected in this section. (E) irGPR30 cells are detected in the anterior pituitary. (F) Punctate-like elements, presumably nerve terminals, are observed in the posterior pituitary. E and F are immunofluorescent images. Abbreviations: f, fornix; 3V, 3rd ventricle; opt, optic tract. Calibration bar: A and D, 250 μm; B and C, 50 μm; E and F, 20 μm.
Using an antiserum directed against the human C-terminus GPR30, irGPR30 was detected in the Tu, hypothalamus, hippocampal formation, substantia nigra, medulla oblongata, and pituitary. As tissues processed with pre-immune serum, instead of GPR30 antiserum, resulted in no positive labeling, the irGPR30 appears to be specific. Further, the expression pattern of GPR30 (or CMKRL2 or CEPR) mRNA in human brain tissues (Owman et al. 1996, Feng & Gregor 1997, O’Dowd et al. 1998) is similar to the receptor distribution profile observed here in the rat brain. For example, GPR30 mRNA is highly expressed in the Tu, hippocampus, supraoptic, paraventricular, arcuate, and suprachiasmatic nuclei; the signal is less abundant in the cortex and caudate nucleus (O’Dowd et al. 1998). CEPR mRNA is highly expressed in the caudate nucleus, hippocampus, substantia nigra, subthalamic nucleus, thalamus, hypothalamus, medulla oblongata, and spinal cord (Feng & Gregor 1997).

Our results show that the pattern of irGPR30 expression is similar in the adult male and female rat brain. This is in agreement with the report in which a gender difference relative to the distribution of ERα and ERβ is not observed in the rat brain (Laflamme et al. 1998). Several areas, such as the ICj and Tu, express ERα and ERβ (Laflamme et al. 1998) as well as irGPR30. Other areas, for example, the substantia nigra and AP express irGPR30 and ERα mRNA, whereas the paraventricular hypothalamic and supraoptic nuclei express irGPR30 and ERβ mRNA. Several regions including the amygdala, ventromedial hypothalamic nucleus, and ventral premammillary nucleus, where a strong hybridization signal to ERα and ERβ mRNA is detected (Laflamme et al. 1998), contain little or no irGPR30. In spite of partial overlapping with one or the other ER mRNA in different brain regions, our results indicate that the pattern of irGPR30 expression is distinct from that of ERα and ERβ in the rat brain.

The co-localization study provides information relative to the phenotype of cells expressing irGPR30 in the hypothalamus and brainstem. Even if GPR30 and ERβ are present in the paraventricular hypothalamic nucleus, they seem to be localized

**Figure 3** Distribution of irGPR30 in the hippocampus and substantia nigra. (A) Hippocampal formation expressing irGPR30 at the level of dentate gyrus (DG) and CA1, CA2, and CA3 fields. (B) A higher magnification of the area outlined in A, showing irGPR30 in DG and CA3. (C) irGPR30 is observed in substantia nigra reticular part (SNR) and compact part (SNC). (D) A higher magnification of the area outlined in C. Abbreviations: cp, cerebral peduncle; D3V, dorsal 3rd ventricle; ml, medial lemniscus. Calibration bar: A and C, 250 μm; B and D, 50 μm.
in different cell populations: ERβ is reported to be highly (60–80%) expressed in corticotropin releasing factor-positive cells, whereas, the percentage of hypothalamic neurons expressing both ERβ and VP varies with different studies, ranging from 5% (Laflamme et al. 1998) to 88–99% (Hrabovszky et al. 2004). In our study, approximately 60% of irGPR30 neurons expressed VP in the paraventricular hypothalamic nucleus. Co-expression of OT and ERβ is reported to be about 40% (Laflamme et al. 1998), which is similar to the percentage of co-localization of irGPR30 and OT in the paraventricular hypothalamic nucleus. Estrogens regulate the activity of OT (Akaishi & Sakuma 1985) and VP neurons (Hartley et al. 2004), and activation of GPR30 could be one of the signaling pathways. With respect to the nucleus Amb, irGPR30 neurons are ChAT-positive, thus are cholinergic.

There is evidence that GPR30 is localized to the plasma membrane of human breast cancer cells (Thomas et al. 2005). A more recent study shows that GPR30 is localized to the plasma membrane of rat hippocampal CA2 neurons, and in GPR30-transfected HeLa cells the receptor is translocated to the cytoplasm upon agonist stimulation (Funakoshi et al. 2006). In the case of our immunohistochemical study, irGPR30 seems to be distributed throughout the cytoplasm in some of the neurons; e.g. dorsal motor nucleus of the vagus (DMNV; Fig. 4B) and Amb (Figs 4D and 5E). On the other hand, irGPR30 may be affiliated with plasma membranes of hypothalamic and hippocampal neurons (Figs 2B, C and 3B). Immunofluorescent staining clearly shows GPR30 is localized intracellularly; i.e. perinuclear in cells shown in Figs 5A and 6A, and dispersed throughout the cytoplasm in cells illustrated in Fig. 5E. Electron microscopic examination will be needed to resolve the issue of receptor localization. The possibility of a cell type-specific or state-dependent localization of GPR30 is intriguing.
Figure 5 Confocal scanning images of hypothalamic and medullary sections double-labeled with GPR30 antiserum and vasopressin (VP), oxytocin (OT), or choline acetyltransferase (ChAT)-antiserum. (A and B) A hypothalamic section where the supraoptic hypothalamic nucleus is double-labeled with GPR30 antiserum and VP antiserum; some of the cells that express both irGPR30 and VP are indicated by arrows. (C and D) A hypothalamic section where the paraventricular nucleus is double-labeled with GPR30 antiserum and OT antiserum; several cells expressing both irGPR30 and OT are indicated by arrows. (E and F) A brainstem section where nucleus ambiguous is labeled with GPR30 antiserum and ChAT antiserum; cells expressing both irGPR30 and ChAT are indicated by arrows. Calibration bars, 20 μm.
The physiological consequence of activation of GPR30 in a specific neural axis is largely unknown. In addition to its well-documented hormonal action, estradiol is being considered as a neurotransmitter in the brain (Balthazart & Ball 2006). Estradiol by intrahippocampal injection enhanced spatial memory in male rats (Packard et al. 1996). Since the expression of ERα and ERβ mRNA in the hippocampus is limited (Laflamme et al. 1998), GPR30 may prove to be the major receptor subtype through which estrogen produces its enhancing effect.

In the midbrain, distribution of irGPR30 appears to be restricted to the substantia nigra, compact and reticular parts. ERα is detected in low levels in the substantia nigra compact part of the rat (Laflamme et al. 1998). Estrogen is reported to have a protective role on dopaminergic neurons in a mouse model (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced) of Parkinson’s disease. However, both the ERα and ERβ appear to be absent in neurons of the substantia nigra of the mouse (Shughrue 2004). Viewed in this context, the neuroprotective effect of estrogen on dopaminergic neurons may be related to activation of GPR30 and independent of nuclear receptors.

With respect to the brainstem, irGPR30 is detected in the AP, nucleus of the solitary tract, nucleus Amb, and dorsal motor nucleus of the vagus. These nuclei are directly or indirectly associated with autonomic regulation of cardiovascular/gastrointestinal activity. Moreover, estradiol administered directly to the nucleus Amb increased the phenylephrine-induced baroreflex within 5 min of injection (Saleh et al. 2000). A participation of GPR30 in this response may be anticipated.

Similar to the COS7 cells transfected with GPR30 (Revankar et al. 2005), G-1 elicited a large increase of $[Ca^{2+}]_i$ in dissociated and cultured hypothalamic neurons. Under physiological conditions, circulating estrogens are bound to the carrier proteins, sex hormone-binding globulin, and albumin (Anderson 1974); however, only free estrogen is thought to enter the cell (through diffusion). Unlike the nuclear ERs that activate transcription, protein-bound estrogens mediate non-nuclear receptor signaling (Ramirez et al. 1996, Zheng et al. 1996, Kahn et al. 2002). Estradiol-BSA

![GPR30 and NSE images](image-url)

**Figure 6** Confocal scanning images of cultured hypothalamic neurons double-labeled with GPR30 and neuron-specific enolase (NSE) antiserum. (A) irGPR30 is distributed throughout the cytoplasm, more intense in the perinuclear region. (B) NSE immunoreactivity is dispersed throughout the cytoplasm. Calibration bar, 10 μm.

![Calcium responses](image-url)

**Figure 7** Calcium responses induced by G-1 in hypothalamic neurons. (A) G-1 (100 nM) induced a sustained increase in $[Ca^{2+}]_i$. (B) G-1 (100 nM) produced a much smaller increase in $[Ca^{2+}]_i$ in hypothalamic neurons perfused with a Ca²⁺-free medium.
(E2-BSA) has been shown to elevate [Ca^{2+}]_i through non-genomic ERs in several cell models; for example, E2-BSA elevates [Ca^{2+}]_i in gonadotropin-releasing hormone-1 neurons (Temple & Wray 2005), human granulocytes (Stefano et al. 2000a), and cultured human pre-osteoclastic cells (Fiorelli et al. 1996). Fast calcium mobilization by E2-BSA has also been reported in rat aorta endothelial cells (Rubio-Gayosso et al. 2000), as well as in thoracic and human arterial endothelia (Stefano et al. 2000b). The observation that G-1, the GPR30 selective agonist (Bologa et al. 2006), rapidly elevates [Ca^{2+}]_i in hypothalamic cells is consistent with a non-nuclear effect of estrogen. Another novel observation is that the Ca^{2+} response elicited by a second application of G-1 was significantly smaller as compared with that evoked by the first application, indicating that the non-genomic ER undergoes rapid desensitization. Calcium mobilization and receptor desensitization are two common features of GPRs. Viewed in this context, our observations support the thesis that GPR30 is a GPR.

In a Ca^{2+}-free medium, G-1 causes an elevation of [Ca^{2+}]_i, with a magnitude much smaller than that elicited in a Ca^{2+}-containing medium, suggesting that activation of GPR30 mobilizes extracellular as well as intracellular Ca^{2+} stores. Increase of [Ca^{2+}]_i in neurons may lead to neurotransmitter release (Brailoiu et al. 2003), modulation of neurite outgrowth (Jacques-Fricke et al. 2006), and hormone secretion (Stojilkovic et al. 2005). Activation of GPR30 by estrogen may influence cell physiology according to the type of cells in question.

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