

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.

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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 α and β (ER α and ER β), 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 1977, 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/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 the place of GPR30 antiserum.

In the case of double-labeling experiments, hypothalamic and medullary 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 fluorescein isothiocyanate (FITC). After rinsing with PBS, the hypothalamic sections were incubated with either oxytocin (OT) antibody (1:1000, a monoclonal antibody from Chemicon, Tamecula, CA, USA) or vasopressin (VP) antiserum (1:1500, a guinea pig polyclonal antibody from Chemicon); medullary sections were incubated with choline acetyltransferase (ChAT) antiserum (1:1000; a guinea pig polyclonal antibody from Bachem Bio Sciences Inc., King of Prussia, PA, USA) for 48 h in a cold room. In the case of double labeling with the neuronal marker neuron-specific enolase, cultured hypothalamic neurons were fixed in 4% paraformaldehyde/0.2% picric acid, blocked with 10% normal goat serum, and incubated with GPR30 antiserum (1:500 dilution). They were washed with PBS and incubated with biotinylated anti-rabbit IgG, followed by avidin FITC. After rinsing with PBS, the cells were blocked with normal donkey serum and incubated with neuron-specific enolase antiserum (1:150, a mouse monoclonal antibody from Chemicon). Tissues and cells were incubated in Avidin Texas Red conjugated to appropriate secondary antibodies, washed with PBS, and mounted in Citifluor and coverslipped. Sections and cells 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-arabino furanoside (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 $[Ca^{2+}]_i$

Administration of G-1 (100 nM), the ligand that selectively activates GPR30 but not the nuclear ERs ER α and ER β (Bologna *et al.* 2006), induced an increase in $[Ca^{2+}]_i$ by 964 ± 8.6 nM in 26 of 109 cells tested; an example is shown in Fig. 7A. The rise of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 non-genomic ER (Owman *et al.* 1996, Carmeci *et al.* 1997, Feng & Gregor 1997, Kvingedal & Smeland 1997, Takada *et al.* 1997, O'Dowd *et al.* 1998). GPR30 (CEPR or CMKRL2) is a 375 amino acid class-1 GPCR (SwissProt (<http://www.expasy.ch/sprot/>) primary accession number 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 (Filardo *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 α and ER β .

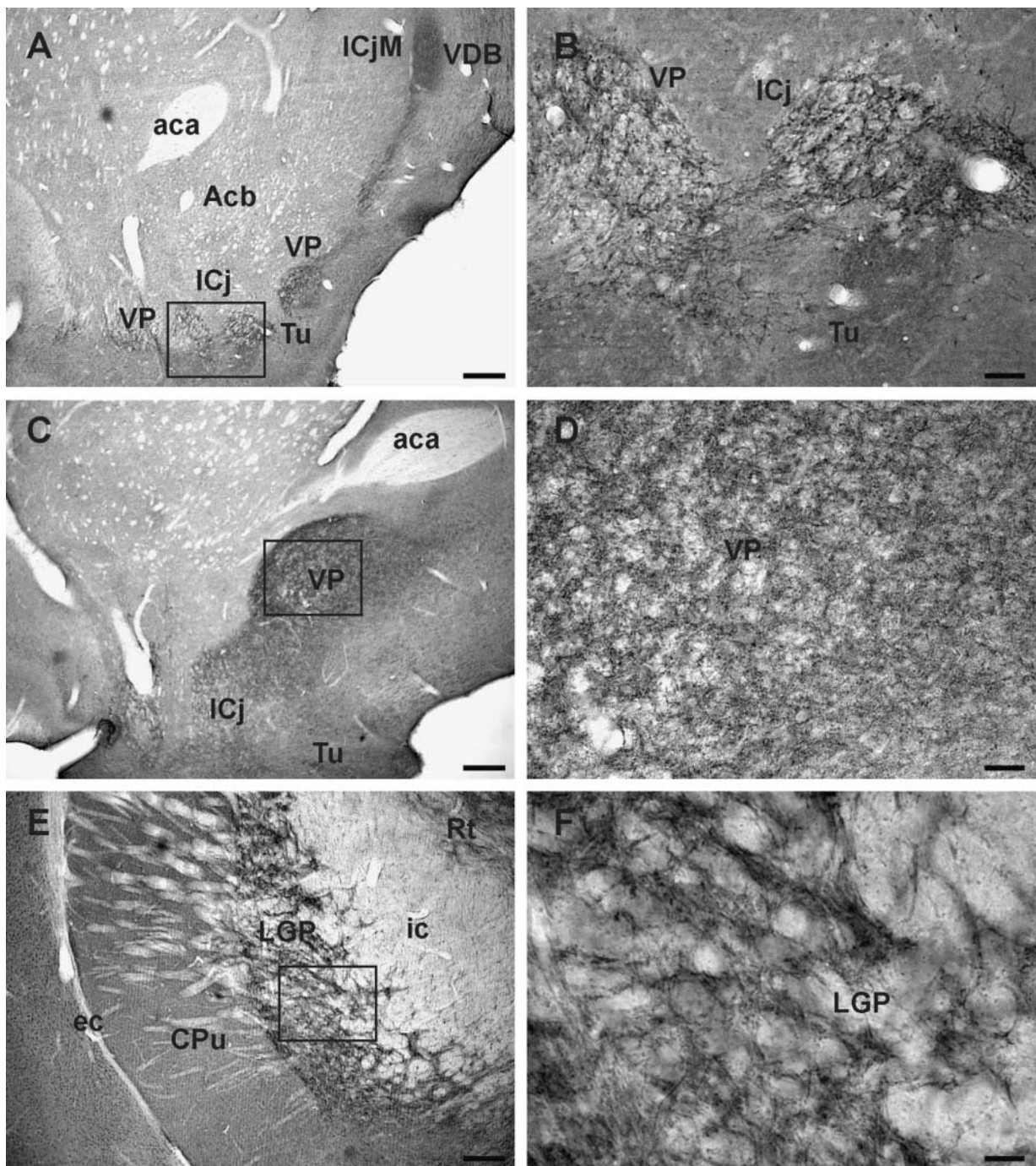


Figure 1 Distribution of irGPR30 in the rat forebrain. (A) A high density of irGPR30 is noted in the Islands of Calleja (ICj), major island (ICjM), ventral pallidum (VP), olfactory tubercle (Tu), nucleus of vertical limb diagonal band (VDB). (B) A higher magnification of the area outlined in A, depicting 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 μ m; B, D and F, 50 μ m.

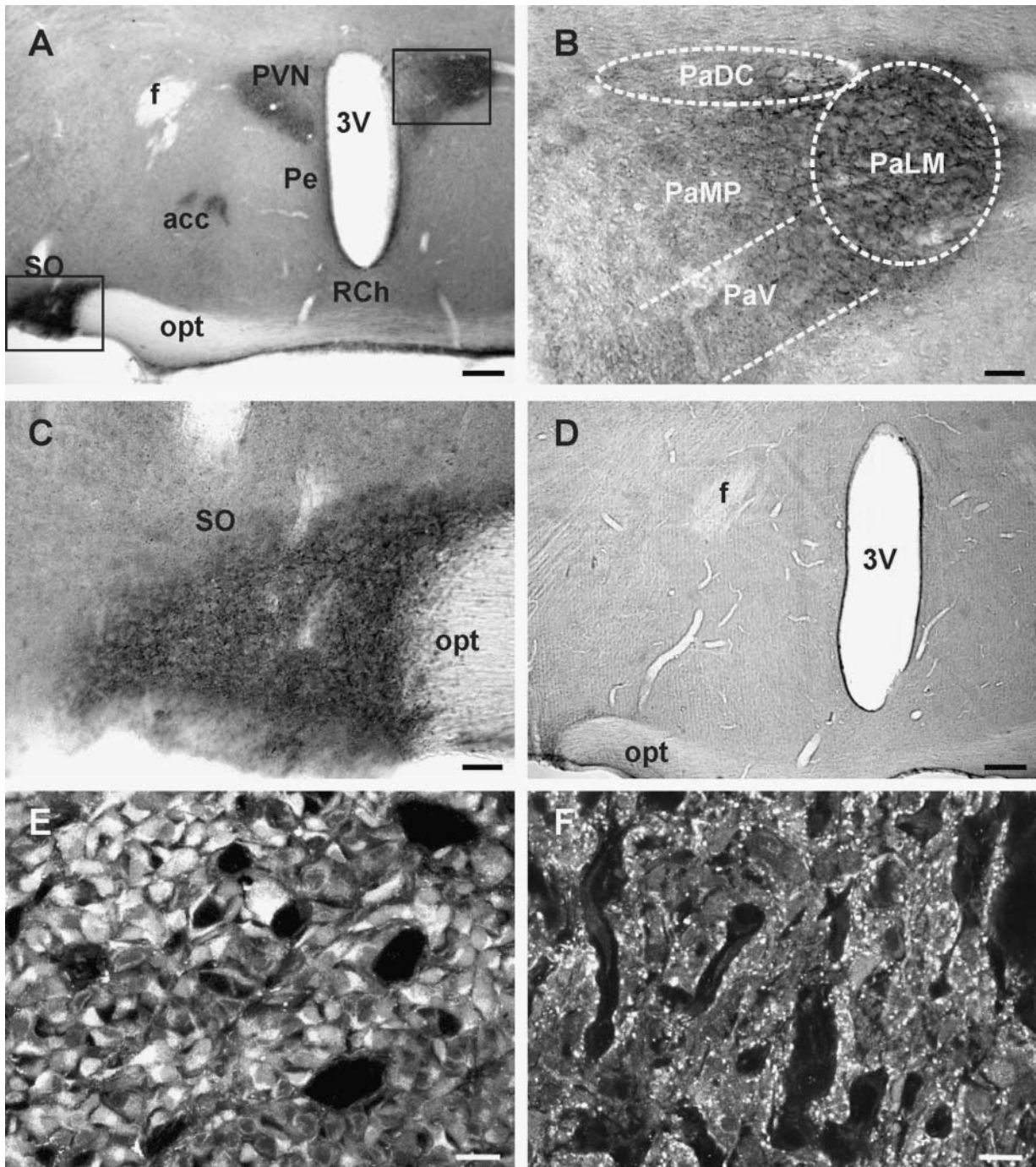


Figure 2 Distribution of irGPR30 in the rat hypothalamus and pituitary. (A) Dense irGPR30 is present in paraventricular hypothalamic nucleus (PVN), accessory neurosecretory nuclei (acc) and supraoptic nucleus (SO). Immunoreactivity is sparse in periventricular hypothalamic nucleus (Pe) and retrochiasmatic 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 μ m; B and C, 50 μ m; E and F, 20 μ m.

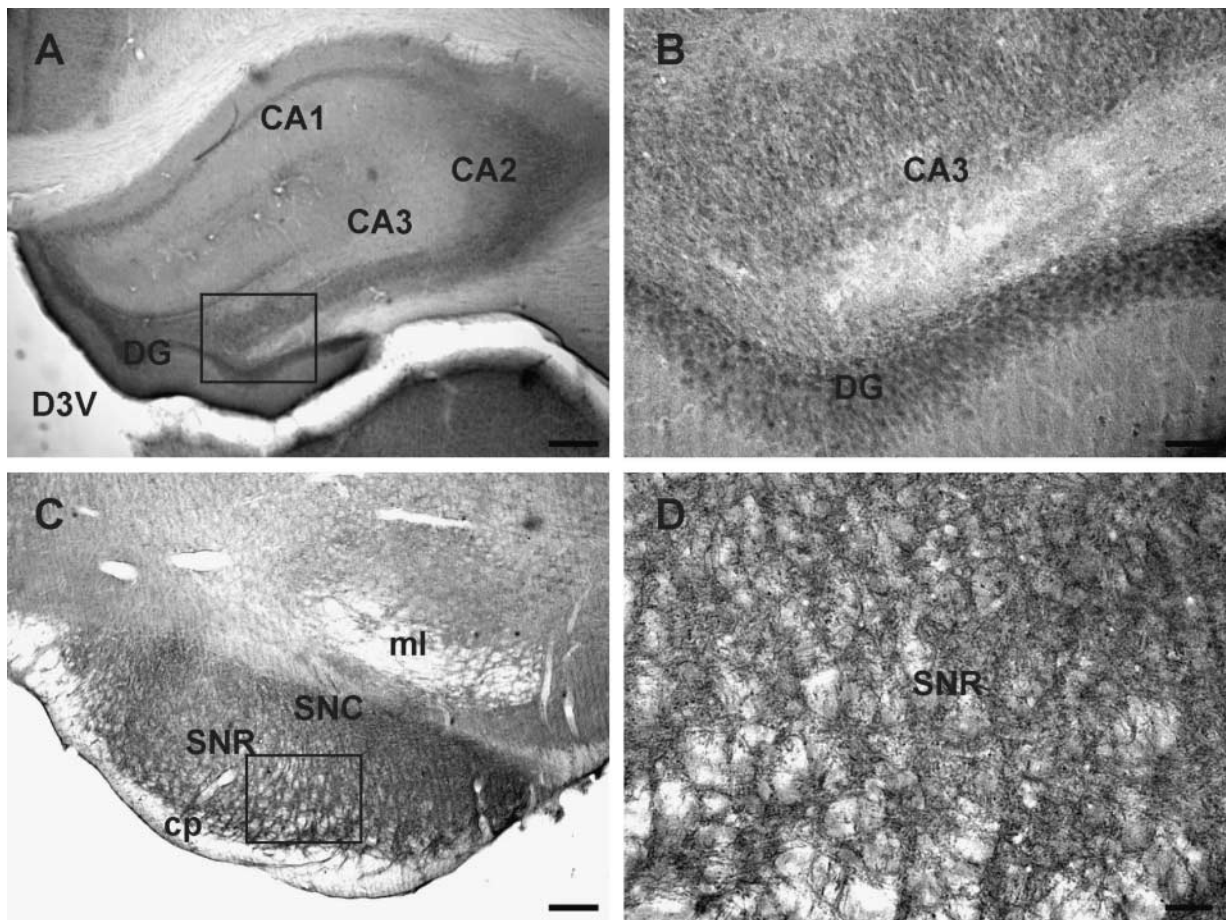


Figure 3 Distribution of irGPR30 in the hippocampus and substantia nigra. (A) Hippocampal formation expressing irGPR30 at the level of dentate gyrus (DG) and CA1, CA2, and CA3 fields. (B) A higher magnification of the area outlined in A, showing irGPR30 in DG and CA3. (C) irGPR30 is observed in substantia 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 μ m; B and D, 50 μ m.

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 ER α and ER β is not observed in the rat brain (Lafamme *et al.* 1998). Several areas, such as the ICj and Tu, express ER α and ER β (Lafamme *et al.* 1998) as well as irGPR30. Other areas, for example, the substantia nigra and AP express irGPR30 and ER α mRNA, whereas the paraventricular hypothalamic and supraoptic nuclei express irGPR30 and ER β mRNA. Several regions including the amygdala, ventromedial hypothalamic nucleus, and ventral premammillary nucleus, where a strong hybridization signal to ER α and ER β mRNA is detected (Lafamme *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 ER α and ER β 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 ER β are present in the paraventricular hypothalamic nucleus, they seem to be localized

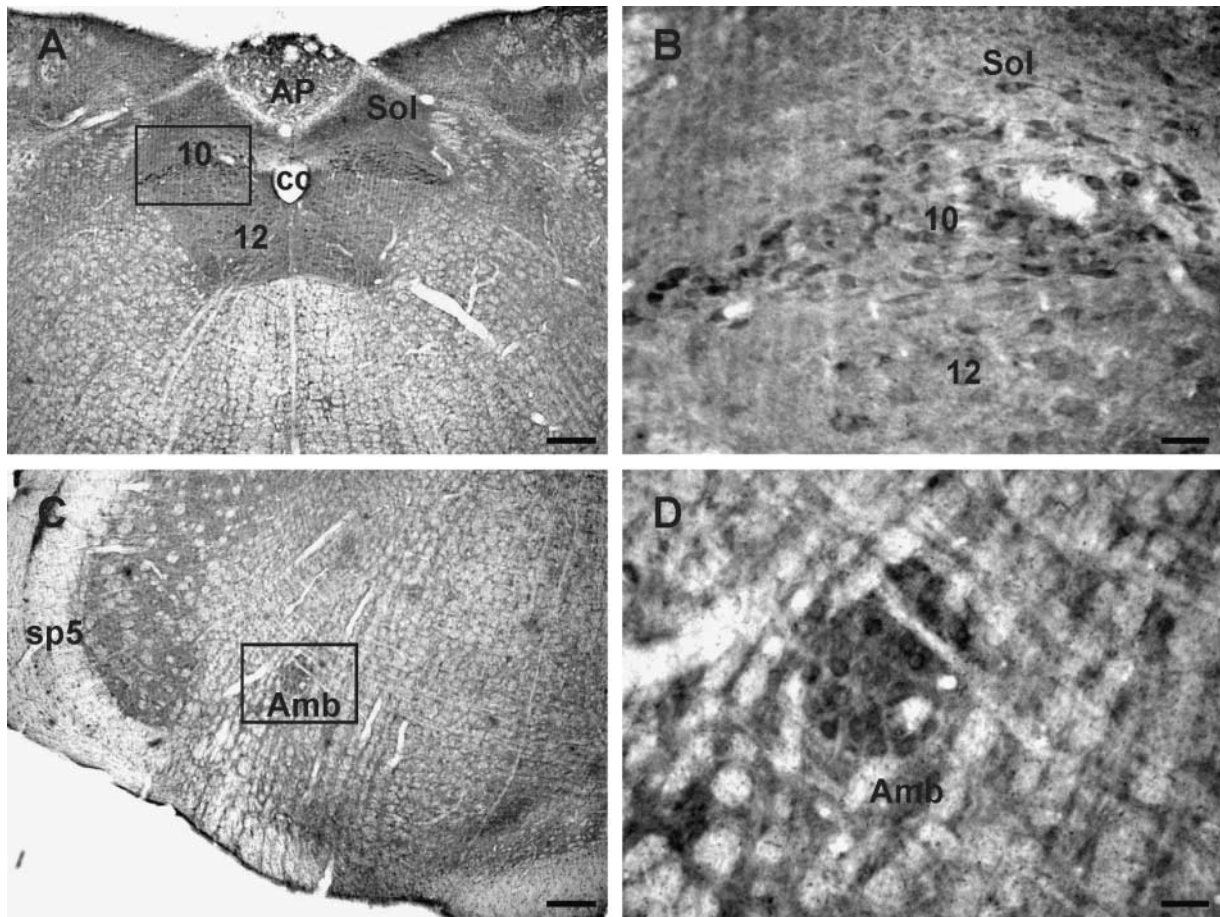


Figure 4 Distribution of irGPR30 in medulla oblongata. (A) irGPR30 cells are present in the dorsal motor nucleus of the vagus (10) and area postrema (AP); few labeled cells are noted in the nucleus of the solitary tract (Sol), and hypoglossal nucleus (12); and scattered cells are seen throughout the reticular formation. (B) A higher magnification of the area outlined in panel A. (C) A group of irGPR30 cells is present in the nucleus ambiguus (Amb). (D) A higher magnification of outlined area shown in C. Abbreviations: cc, central canal; sp5, spinal trigeminal tract. Calibration bar: A and C, 250 μ m; B and D, 50 μ m.

in different cell populations: ER β is reported to be highly (60–80%) expressed in corticotropin releasing factor-positive cells, whereas, the percentage of hypothalamic neurons expressing both ER β 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 ER β 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 endoplasmic reticulum of COS7 cells transfected with the receptor (Revankar *et al.* 2005). In another study, GPR30 is

shown to be 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.

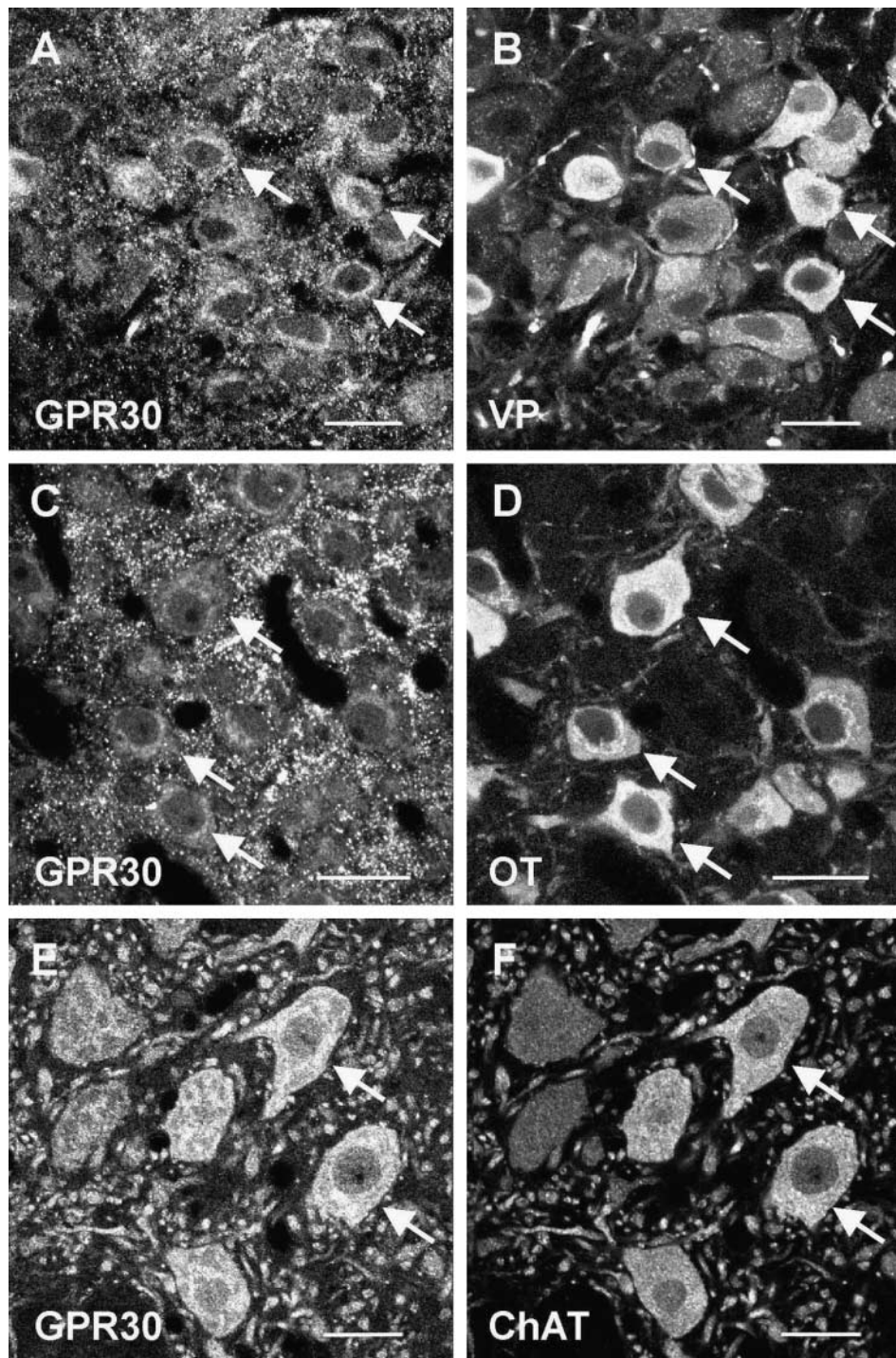


Figure 5 Confocal scanning images of hypothalamic and medullary sections double-labeled with GPR30 antiserum and vasopressin (VP), oxytocin (OT), or choline acetyltransferase (ChAT)-antiserum. (A and B) A hypothalamic section where the supraoptic hypothalamic nucleus is double-labeled with GPR30 antiserum 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 ambiguus is labeled with GPR30 antiserum and ChAT antiserum; cells expressing both irGPR30 and ChAT are indicated by arrows. Calibration bars, 20 μ m.

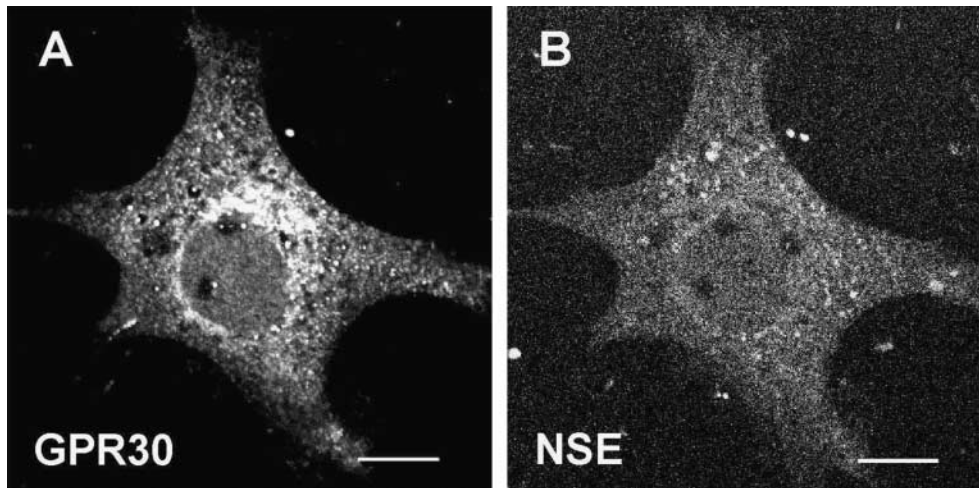


Figure 6 Confocal scanning images of cultured hypothalamic neurons double-labeled with GPR30 and neuron-specific enolase (NSE) antiserum. (A) irGPR30 is distributed throughout the cytoplasm, more intense in the perinuclear region. (B) NSE immunoreactivity is dispersed throughout the cytoplasm. Calibration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

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 ER α and ER β 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. ER α 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 ER α and ER β 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 $[\text{Ca}^{2+}]_i$ 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

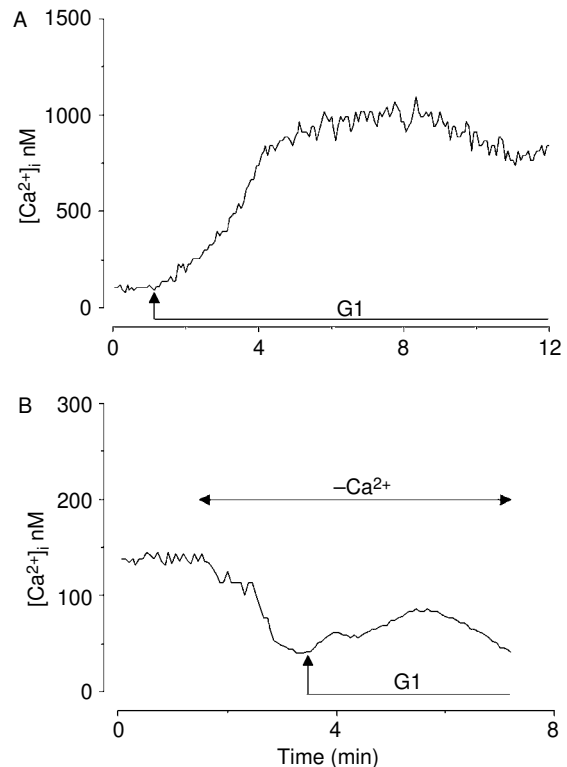


Figure 7 Calcium responses induced by G-1 in hypothalamic neurons. (A) G-1 (100 nM) induced a sustained increase in $[\text{Ca}^{2+}]_i$. (B) G-1 (100 nM) produced a much smaller increase in $[\text{Ca}^{2+}]_i$ in hypothalamic neurons perfused with a Ca^{2+} -free medium.

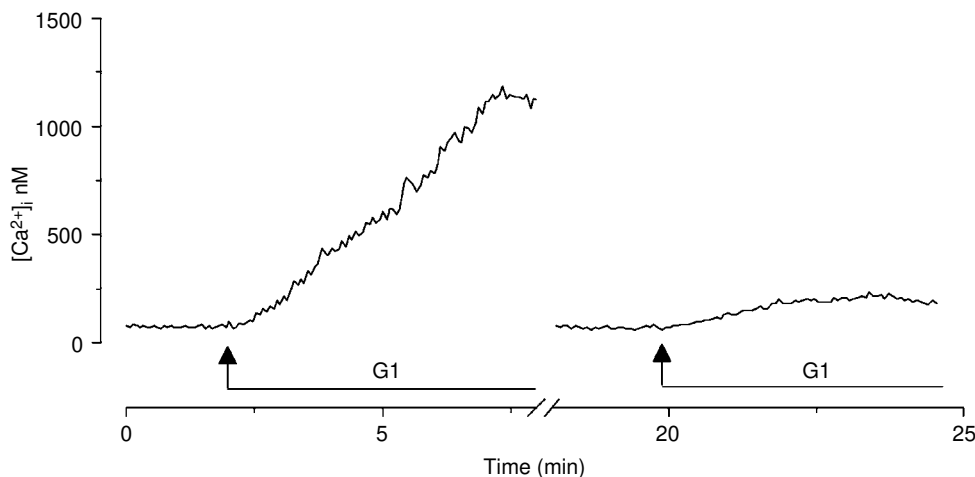


Figure 8 Calcium responses to two consecutive applications of G-1 to a hypothalamic neuron. (A) First application of G-1 (100 nM) produced a large increase in $[Ca^{2+}]_i$ in a hypothalamic neuron; a second superfusion of G-1 (100 nM) applied immediately after the Ca response had returned to the baseline, produced a much smaller increase in $[Ca^{2+}]_i$.

(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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