

Localisation of GPR30, a novel G protein-coupled oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues

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Abstract

Recently, the G protein-coupled receptor GPR30 has been identified as a novel oestrogen receptor (ER). The distribution of the receptor has been thus far mapped only in the rat central nervous system. This study was undertaken to map the distribution of GPR30 in the mouse brain and rodent peripheral tissues. Immunohistochemistry using an antibody against GPR30 revealed high levels of GPR30 immunoreactivity (ir) in the forebrain (e.g. cortex, hypothalamus and hippocampus), specific nuclei of the midbrain (e.g. the pontine nuclei and locus coeruleus) and the trigeminal nuclei and cerebellum Purkinje layer of the hindbrain in the adult mouse brain. In the rat and mouse periphery, GPR30-ir was detected in the anterior, intermediate and neural lobe of the pituitary, adrenal medulla,

renal pelvis and ovary. *In situ* hybridisation histochemistry using GPR30 riboprobes, revealed intense hybridisation signal for GPR30 in the paraventricular nucleus and supraoptic nucleus (SON) of the hypothalamus, anterior and intermediate lobe of the pituitary, adrenal medulla, renal pelvis and ovary of both rat and mouse. Double immunofluorescence revealed GPR30 was present in both oxytocin and vasopressin neurones of the paraventricular nucleus and SON of the rat and mouse brain. The distribution of GPR30 is distinct from the other traditional ERs and offers an additional way in which oestrogen may mediate its effects in numerous brain regions and endocrine systems in the rodent.

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Introduction

Oestrogen (17- β -oestradiol or E₂) affects a variety of physiological processes including fertility, immune function, cardiovascular homeostasis, mood, locomotion, neuroprotection and cognition (Watson & Gametchu 2001, McEwan 2002, de Ronde *et al.* 2003). E₂ effects are typically mediated through two structurally related oestrogen receptors, the α and β (ER α and ER β) subtypes that function as ligand-activated transcription factors. On activation ERs can either bind directly to their target DNA sequences in the nucleus or interact with other nuclear proteins to alter gene activation, and this genomic action occurs slowly (hours–days). However, it is well documented that E₂ can also provoke many fast ‘non-genomic’ effects e.g. activation of large conductance calcium-activated potassium channels (K_{Ca}1.1; Valverde *et al.* 1999), and these could be mediated by extranuclear ERs or by non-classical membrane bound receptors (Levin 2005).

GPR30, a G protein-coupled receptor (GPCR) specifically binds E₂ with high (nanomolar) affinity and evokes rapid effects (Revankar *et al.* 2005, Thomas *et al.* 2005), including stimulating Ca²⁺ mobilisation from intracellular stores

directly or via epidermal growth factor receptor transactivation, *c-fos* expression, adenylyl cyclase and cAMP mediated signalling and ERK-1/2 in a variety of cell types (Prossnitz *et al.* 2008). Intriguingly, only a small fraction of total cellular GPR30 appears to be expressed on the cell surface with most of the receptor located intracellularly in the endoplasmic reticulum (Revankar *et al.* 2007). GPR30 acts as an atypical GPCR, in that, it is activated intracellularly by E₂ which readily diffuses across cell membranes (Revankar *et al.* 2007).

Recent studies in GPR30 knockout mice have indicated that GPR30 mediates E₂ stimulated insulin release in females, reduces blood pressure and is involved in bone development, but is not essential for reproduction (Mårtensson *et al.* 2009, Otto *et al.* 2008). In addition, GPR30 is expressed in oxytocin (OXT) neurones of the rat hypothalamic paraventricular nucleus (PVH) and supraoptic nucleus (SON), implicating a role for GPR30 in the fast, non-genomic actions of E₂ on OXT release (Wang *et al.* 1995, Revankar *et al.* 2005). Whether or not GPR30 is expressed in vasopressin (AVP) neurones remains unclear as one study found co-expression (Brailoiu *et al.* 2007) while another did not (Sakamoto *et al.* 2007), both these studies used the same antibody. A role for E₂ in regulating OXT and/or AVP release is supported by studies

showing that administration of E₂ to postmenopausal women increases circulating levels of OXT and AVP (Bossmar *et al.* 1995), and E₂ rapidly stimulates intrahypothalamic and peripheral OXT and possibly AVP release (Wang *et al.* 1995, Burbach *et al.* 2001).

As GPR30 provides an alternative model for E₂ signalling, and possibly E₂ stimulated release of OXT and AVP, the present study was undertaken to clarify the distribution of GPR30 in the rodent. As GPR30 provides an alternative model for E₂ signalling, and possibly E₂ stimulated release of OXT and AVP, the present study was undertaken to clarify the distribution of GPR30 in the rodent. We aimed to 1) map the distribution of GPR30 in the mouse brain to determine whether there are differences to that shown in the rat 2) identify and highlight regions that express high levels of GPR30 mRNA and protein in the rodent periphery, to provide additional insights into the role of GPR30.

Materials and Methods

Animals

Adult male and female Sprague–Dawley rats, weighing 200–250 g (Harlan, Bicester, UK), and adult (10–12 week) male and female wild-type mice (25–30 g) from our AVP V1b receptor knockout colony (Wersinger *et al.* 2002) were used in this study. For each separate experiment (immunohistochemistry, double immunofluorescence or *in situ* hybridisation histochemistry) three mice or rats of each gender were used. Animals were housed under a constant temperature (21 ± 2 °C), light (lights on from 0700 to 1900 h) and humidity (45–50%) regimens with access to food and water *ad libitum*. Animal care, maintenance and surgery were performed in accordance with the Animal Scientific Procedures Act (1986) United Kingdom and the appropriate University of Bristol ethical review process.

Immunohistochemistry

Animals were anaesthetised with sodium pentobarbital (100 mg/kg *i.p.*) and intracardially perfused with 0.1 M PBS followed by 4% paraformaldehyde in 0.1 M PBS. Tissues were post fixed overnight in 20% sucrose and 4% paraformaldehyde in 0.1 M PBS solution. Following overnight fixation, the tissues were rapidly frozen over liquid nitrogen and stored at –80 °C until processed. In single staining, rat and mouse peripheral cryostat sections (12 µm) were cut and thaw-mounted onto Superfrost Plus slides, or free-floating (40 µm) mouse brain sections were cut and processed for GPR30-immunoreactivity (GPR30-ir). Tissues were first washed in 0.1 M PBS, endogenous peroxidase activity was quenched with 3% H₂O₂ for 10 min, washed (3 × 10 min with 0.1 M PBS), and then blocked with 10% normal goat serum (NGS) and 0.3% Triton-X 100 (T-X) in 0.1 M PBS for 15 min. After washing (3 × 10 min

with 0.1 M PBS), sections were incubated overnight in an affinity-purified rabbit antiserum against GPR30 diluted (1: 1000–1:2000) in 1% NGS/0.3% T-X in 0.1 M PBS at 4 °C. The specificity of GPR30 antiserum has previously been confirmed (Revankar *et al.* 2005), and normal rabbit IgG serum (Vector Laboratories, Peterborough, UK) was used as a control at the same concentration as the antibody. After washing (3 × 10 min with 0.1 M PBS), sections were incubated at room temperature in secondary biotinylated affinity purified goat anti-rabbit (1:500, Vector Laboratories), in 1% NGS/0.3% T-X in 0.1 M PBS for 1 h, washed (3 × 10 min with 0.1 M PBS) and incubated in horseradish peroxidase streptavidin (1:500, Vector Laboratories) in 1% NGS/0.3% T-X in 0.1 M PBS for a further hour. The GPR30 signal was visualised with 3,3'-diaminobenzidine in peroxidase buffer (10 min at 1:10, Roche Diagnostics). Mouse brain sections were mounted onto Superfrost Plus slides with 0.5% gelatin solution, and along with the pre-mounted peripheral sections were cover-slipped and viewed under a light microscope (Leica DM IRB, Milton Keynes, UK).

For double immunofluorescence staining, hypothalamic free-floating sections (40 µm) were rinsed in 0.1 M PBS, and then blocked with 10% NGS/0.3% T-X in 0.1 M PBS for 15 min. After washing (3 × 10 min in 0.1 M PBS), sections were incubated overnight at 4 °C with rabbit antiserum against GPR30 (diluted 1:1000) and guinea pig antiserum against OXT or AVP (diluted 1:2000 (OXT), 1:500 (AVP); Peninsula Laboratories, San Carlos, CA, USA). Sections were washed and incubated for an hour at room temperature in biotinylated goat anti-rabbit antibody (as above), and Alexa fluor 488 goat anti-guinea pig antibody (diluted 1:500, Invitrogen) to detect OXT or AVP protein. Sections were further washed and incubated for an hour in streptavidin-conjugated Alexa Fluor 594 (diluted 1:500, Vector Laboratories). Sections were mounted onto Superfrost Plus slides, cover slipped with an antifade mounting medium (Vectashield Hard Set, Vector Laboratories) and viewed under a confocal laser scanning microscope (Leica TCS-NT microscope housing a Leica DM IRBE inverted epifluorescence with a two-line krypton/argon laser) and processed with Adobe Photoshop CS3 extended computer software.

In situ hybridisation histochemistry

Coronal cryostat sections (12 µm) were cut, thaw mounted onto polylysine-coated slides and stored at –80 °C until use. The mouse GPR30 riboprobe was generated by PCR using ~20 ng mouse GPR30 cDNA (cloned from a mouse AtT20 pituitary tumour cDNA library; SJL unpublished data) as template. Primers (upstream: 5'-GAGAGGATC-CGGTCAGGGGCGCAGGC-3'; downstream 5'-AAA-CAAGCTTTGTGAGAGGAGCATC-3') corresponding to bp 1739–2344 of a mouse GPR30 mRNA (Genbank Accession number NM_029771) were used to generate a 606 bp product. The primers contain the recognition sequences for the restriction endonucleases HindIII and

BamHI. The rat GPR30 riboprobe was generated using ~150 ng rat genomic DNA (Promega no. G313A) as a template. PCR primers (upstream: 5'-GAGAGGATC-CTCCTAGAGGAAAACGGA-3'; downstream 5'-AAA-CAAGCTTTGTGAGAGGAGCATC-3') corresponding to bp 15718722–15718072 of a rat GPR30 gene (Genbank Accession number NM_133573) were used to generate a 651 bp product. The primers contain the recognition sequences for the restriction endonucleases HindIII and BamHI. Primer restriction endonuclease sites allowed subcloning into the RNA generating vector pGEM4Z (Promega), and sense and antisense probes were generated using T7 and SP6 polymerases (mouse and rat antisense: linearised with BamHI and generated with T7 polymerase; sense: linearised with HindIII and generated with SP6 polymerase) with ³⁵S-UTP and the MAXIscript *in vitro* transcription kit (Ambion, Austin, TX, USA). The integrity of the probes was verified by DNA sequencing. All *in situ* hybridisation experiments were performed as described in detail (<http://intramural.nimh.nih.gov/lcmr/snge/Protocols/ISHH/ISHH.html>).

Hybridised sections were exposed to film (Amersham Hyperfilm MP) for 8 weeks, or emulsion dipped (Ilford K5) for 24 weeks and then counterstained with toluidine blue.

Results

Distribution of GPR30-ir in the adult mouse brain

The data presented here are semi-quantitative assessments based on subjective judgement of three independent observers. No apparent gender differences were observed in the distribution of GPR30 in the adult mouse brain. In most brain regions, GPR30-ir was expressed in cell bodies and fibres; in some regions mostly cell bodies (e.g. paraventricular nucleus of the thalamus, hypothalamic ventromedial nucleus (VMH)) or fibres (e.g. lateral mammillary nucleus) expressed GPR30-ir (see Table 1 and Figs 1–3).

In the forebrain GPR30-ir expression was high in the cingulate, motor (Fig. 1A) and somatosensory regions of the isocortex, piriform cortex (Fig. 1B), entorhinal cortex, the dentate gyrus hilus (Fig. 2A), and subiculum of the dorsal hippocampus formation and the zona incerta (Fig. 2B). Moderate to high expression was identified in the mitral layer of the olfactory bulb, dorsal endopiriform nucleus, CA1–3 of the ventral hippocampal formation, the medial habenular nucleus of the thalamus, anteroventral periventricular nucleus and the medial preoptic nucleus. The retrosplenial granular region of the isocortex, the lateral nucleus of the septal complex, the nucleus of the horizontal limb of the diagonal band of Broca, the olfactory tubercle, the anterior amygdaloid area of the amygdala and the paraventricular nucleus of the thalamus (Fig. 1D), all demonstrated moderate expression of GPR30. Low to moderate staining was noted in the granular insular, perirhinal and retrosplenial agranular regions of the

isocortex, medial nucleus of the septal complex and the CA2 of the dorsal hippocampal formation. Expression of GPR30 was low in many areas including the glomerular layer of the olfactory bulb, islands of Calleja, claustrum, caudate putamen of the striatum, shell of the accumbens nucleus, subfornical organ, CA1 and CA3 of the dorsal hippocampal formation, bed nucleus of the stria terminalis, and amygdalohippocampal nucleus and medial nucleus of the amygdala. The data are summarised in Table 1.

In the hypothalamus intense GPR30 expression was found in the arcuate nucleus, PVH (Fig. 1E), periventricular hypothalamic nucleus, SON (Fig. 1C), dorsomedial, central and ventrolateral divisions of the VMH with moderate to high expression in the suprachiasmatic nucleus and lateral mammillary nucleus. GPR30-ir was low in the dorsal–medial nucleus, medial mammillary nucleus and ventral tuberomammillary nucleus and GPR30 expression is sparse in the lateral hypothalamic area, posterior hypothalamic nucleus, subthalamic nucleus and supramammillary nucleus.

In the midbrain and pons, distinct GPR30-ir could be seen in the pontine nuclei, anterior tegmental nucleus, medioventral periolivary nucleus and superior paraolivary nucleus of the superior olive and the locus coeruleus (Fig. 3B). A moderate to high expression was noted in the interpeduncular nucleus, compact region of the substantia nigra (Fig. 2E) and the reticulotegmental nucleus of pons. The dorsal medial periaqueductal grey, inferior colliculus, caudal linear raphe nuclei, oral pontine reticular nucleus, the rostral, dorsal, caudal periolivary regions and the lateral superior olivary nucleus of the superior olive, nucleus of trapezoid body, lateral nucleus of the parabrachial region and the medial vestibular nucleus all express moderate levels of GPR30. On the other hand, low levels were found in areas such as the lateral and reticular regions of the substantia nigra, superior colliculus, dorsal raphe and the lateral vestibular nucleus.

In the hindbrain, high expression of GPR30 was found in the oral and dorsomedial region of the spinal trigeminal nucleus (Fig. 3C and D). GPR30-ir was also high in the Purkinje cells of the cerebellum. Moderate expression was seen in the facial motor nucleus, principal nucleus of the inferior olive and the dorsal motor nucleus of vagus with low to moderate expression in the middle cerebellar peduncle, nucleus ambiguus and nucleus of the solitary tract. Low to moderate staining was also found in the internal granule layer and molecular layers of the cerebellum. Low expression was detected in other regions including the posterodorsal tegmental nucleus, supratrigeminal, parapyramidal, intermediate reticular, gigantocellular, lateral reticular and the rosteroventrolateral reticular nuclei and the area postrema.

GPR30 co-localises with OXT and AVP neurones

The results for this part of the study were obtained from three male and three female rat and mouse brains, double labelled with antibodies to GPR30 and either OXT or AVP.

Table 1 Summary of the distribution of GPR30 immunoreactivity (ir) in the adult brain, determined using three males and three females. Data presented here are of semi-quantitative assessments and the intensity of staining is based on the subjective judgement of three observers. No apparent gender differences were detected

	GPR30
Brain region	
<i>Forebrain</i>	
Olfactory bulb	
Glomerular layer	+ /c
Mitral layer	++ /++ + /c
External plexiform layer	–
Granule layer	–
Islands of Calleja	+ /c
<i>Cortex</i>	
<i>Allocortex</i>	
Piriform	+++ /c
Dorsal endopiriform nucleus	++ /++ + /f
<i>Isocortex</i>	
Agranular insular	–
Cingulate	+++ /c
Dorsal peduncular	– /+ /c
Granular insular	++ /+ /c
Infra-limbic	– /+ /c
Prelimbic	– /+ /c
Motor	+++ /c
Somatosensory	+++ /c
Perirhinal	++ /+ /c
Retrosplenial agranular	++ /+ /c
Retrosplenial granular	++ /c
Visual	– /+ /c
Dorsal tenia tecta	–
Clastrum	+ /f
<i>Striatum</i>	
Caudate putamen	+ /f
Globus pallidus	–
<i>Septal complex</i>	
Lateral nucleus	++ /c
Medial nucleus	++ /+ +
Septohippocampal nucleus	–
Diagonal band of broca	
Horizontal limb diagonal band	++
Vertical limb diagonal band	+ /c
Accumbens nucleus	
Shell	+ /c
Core	– /+ /c
Ventral pallidum	– /+ /c
Subfornical organ	+ /c
<i>Hippocampal formation – dorsal</i>	
CA1	+
CA2	++ /+ +
CA3	+
Dentate gyrus hilus	+++
Subiculum	+++ /c
<i>Hippocampal formation – ventral</i>	
CA1–CA3	++ /+ + + +
Entorhinal cortex	+++ /c
Internal capsule	–
Bed nucleus of the stria terminalis	+
Interstitial nucleus post limb anterior commissure	+
Olfactory tubercle	++ /c
Substantia innominata	+

(continued)

Table 1 Continued

	GPR30
<i>Amygdala</i>	
Anterior amygdaloid area	++
Amygdalohippocampal nucleus	+ /c
Basolateral nucleus	–
Basomedial nucleus	–
Cortical amygdaloid nucleus	–
Central nucleus	–
Medial nucleus	+
<i>Thalamus</i>	
Anterodorsal nucleus	–
Mediodorsal nucleus	–
Central medial nucleus	– /+ /c
Paraventricular nucleus	++ /c
Rhomboid nucleus	–
Medial habenular nucleus	++ /+ + + /c
Precommissural nucleus	–
Pretectal nucleus	–
Lateral posterior thalamic nucleus	–
Ventral medial thalamic nucleus	–
Zona incerta	+++
Subincertal nucleus	+ /c
<i>Preoptic area</i>	
Anteroventral periventricular nucleus	++ /+ + + /c
Lateral preoptic nucleus	+ /c
Medial preoptic nucleus	++ /+ + + /c
Magnocellular preoptic area	–
<i>Hypothalamus</i>	
Anterior commissural nucleus	–
Anterior hypothalamic nucleus	–
Arcuate nucleus	+++
Dorsomedial nucleus	–
Lateral hypothalamic area	– /+ /c
Paraventricular hypothalamic nucleus	+++
Periventricular hypothalamic nucleus	++ + /c
Posterior hypothalamic nucleus	– /+
Subthalamic nucleus	– /+
Suprachiasmatic nucleus	++ /+ + +
Supraoptic nucleus	+++
Ventromedial hypothalamic nucleus	
Dorsomedial	++ + /c
Cental	++ + /c
Ventrolateral	++ + /c
Lateral mammillary nucleus	++ /+ + + +
Medial mammillary nucleus	+
Supramammillary nucleus	– /+
Ventral tuberomammillary nucleus	+
<i>Midbrain/pons</i>	
Deep mesencephalic nucleus	–
Edinger–Westphal nucleus	– /+
Interfascicular nucleus	– /+
Interpeduncular nucleus	++ /+ + + +
Supragenulate thalamic nucleus	– /+
Substantia nigra (caudal)	
Compact	++ /+ + + +
Lateral	+ /c
Reticular	+ /c
Ventral tegmental area	–
Nucleus brachium inferior colliculus	– /+
Parabrachial pigmented nucleus	– /+ /c
Periaqueductal gray	
Dorsal medial	++ /c
Superior colliculus	+

(continued)

Table 1 Continued

	GPR30
Inferior colliculus	++
Raphe nuclei	
Rostral linear	-/+
Caudal linear	++
Medial/paramedian	-/+
Dorsal raphe	+
Pontine nuclei	+++/c
Pontine reticular nucleus	
Oral	++/c
Caudal	-
Intercollicular nucleus	-
Lateral lemniscus	-/+
Parabigeminal nucleus	-
Dorsal tegmental nucleus	+/+++
Pedunclopontine tegmental nucleus	-
Reticulotegmental nucleus pons	+++++
Anterior tegmental nucleus	+++
Superior olive	
Medioventral periolivary nucleus	+++/c
Rostral periolivary region	++
Dorsal periolivary region	++/c
Superior paraolivary nucleus	+++/c
Caudal periolivary nucleus	++
Lateral superior olivary nucleus	++
Nucleus of trapezoid body	++
Locus coeruleus	+++/c
Subcoeruleus nucleus	-/+
Parabrachial	
Medial nucleus	-
Lateral nucleus	++
Medial vestibular nucleus	++
Lateral vestibular nucleus	+
<i>Hindbrain/cerebellum</i>	
Raphe nuclei	
Magnus	-/+
Pallidus	-/+
Obscurus	-/+
Middle cerebellar peduncle	+/+/f
Posterodorsal tegmental nucleus	+
Supratrigeminal nucleus	+
Facial motor nucleus	++
Parapyramidal nucleus	+
Intermediate reticular nucleus	+
Gigantocellular reticular nucleus	+
Lateral reticular nucleus	+
Paragigantocellular reticular nucleus	-/+
Rostroventrolateral reticular nucleus	+
Nucleus ambiguus	+/+/c
Prepositus nucleus	++
Nucleus solitary tract	+/++
Area postrema	+
Inferior olive, principal nucleus	++
Dorsal motor nucleus of vagus	++
Trigeminal nucleus, sensory	+++/c
Spinal trigeminal nucleus	+++/c
Spinal trigeminal nucleus, oral	+++/c
Cerebellum	
Molecular layer	-/+
Purkinje cell layer	+++/c
Internal granule layer	+/++

Intensity of label: +++, intense; ++, moderate; +, low; -, absent; (c), cells; (f), fibres; all other regions contain both immunoreactive cells and fibres.

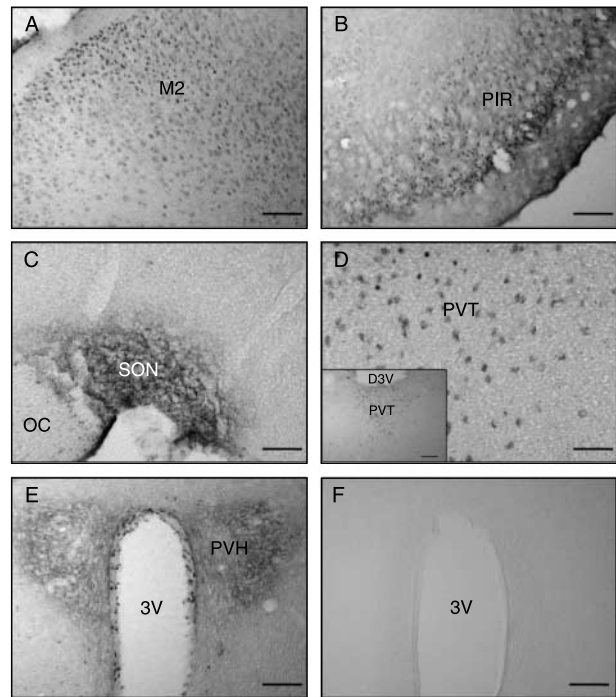


Figure 1 Immunoreactivity for GPR30 in the adult male or female mouse brain: labelled cells in the supplementary motor cortex (A), piriform cortex (B), labelled cells and fibres in the SON (C), with moderate labelling of cells in the paraventricular nucleus of the thalamus (D), labelled cells and fibres in the PVH (E) with an absence of labelling in the PVH with a section incubated with IgG in place of GPR30 antiserum (F). M2, supplementary motor cortex; PIR, piriform cortex; SON, supraoptic nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; PVH, paraventricular nucleus of the hypothalamus; OC, optic chiasm; 3V, third ventricle. Scale bars, 100 μ m in (A–B and E–F); 50 μ m in (C–D). A lower magnification of the PVT is inserted in (D) (Scale bar = 100 μ m). D3V, dorsal third ventricle.

Pictures of GPR30 immunofluorescence were acquired and merged with the corresponding immunofluorescence pictures of OXT or AVP. Neurones containing both GPR30-ir (red) and OXT-ir or AVP-ir (green) appeared yellow (merged) and were counted, and a co-expression percentage was calculated against the total number of OXT-ir or AVP-ir neurones.

In the rat PVH, GPR30-ir is prominent in the magnocellular PVH with low-level labelling in the parvocellular PVH (Fig. 4). Dual labelling of hypothalamic sections revealed that GPR30-ir was present in both OXT and AVP magnocellular neurones and 40–60% of OXT neurones and 50–70% co-expressed GPR30-ir in the PVH and SON (see Table 2 and Figs 4 and 5). In the PVH and SON of the mouse both OXT and AVP neurones were positive for GPR30-ir, with 60–80% of OXT and AVP neurones expressing the receptor (Figs 4 and 5). Co-localisation of GPR30-ir with OXT and AVP was also observed in fibres in the internal zone of the median eminence of both species.

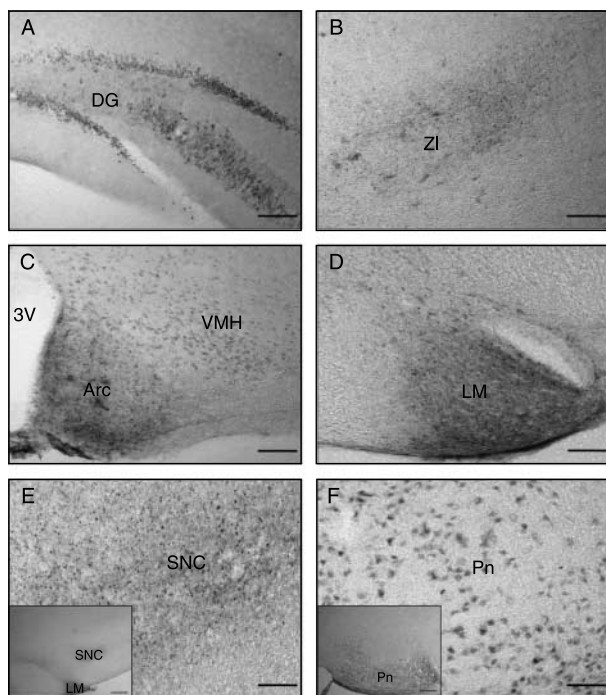


Figure 2 Immunoreactivity for GPR30 in the adult male or female mouse brain: labelled cells and fibres in the dentate gyrus of the hippocampus (A), zona incerta (B), arcuate nucleus and cell bodies of the ventromedial hypothalamic nucleus (C), GPR30-ir in cells and fibres of the lateral mamillary nucleus of the hypothalamus (D), labelled cells and fibres of the pars compacta of the substantia nigra (E) and labelled cells of the pontine nuclei (F). DG, dentate gyrus of the hippocampus; ZI, zona incerta; Arc, arcuate nucleus of the hypothalamus; VMH, ventromedial hypothalamic nucleus; LM, lateral mamillary nucleus of the hypothalamus; SNC, pars compacta of the substantia nigra; Pn, pontine nuclei; 3V, third ventricle. Scale bars, 100 μ m in (A–D); 50 μ m in (E–F). In E and F, lower magnifications of the SNC and Pn respectively, are shown (scale bars=200 μ m).

Peripheral distribution of GPR30

In the anterior lobe of the pituitary gland, ~50% of cells were intensely stained for GPR30-ir. The majority of cells in the pituitary intermediate lobe stained positively and there was prominent staining of fibres in the neural lobe (Fig. 6A). The adrenal medulla displayed strong GPR30-ir; occasional GPR30-ir cells were also observed in the zona glomerulosa of the cortex. In the kidney, GPR30-ir was present in the smooth-muscle cells of the pelvic region and to a lesser extent the medulla, with only a few positive cells in the cortex (Fig. 6B). In the ovary, dense staining was found within the granulosa cells with moderate to low staining in the theca cells (Fig. 6C). Staining was absent in corpora lutea. There were no species or gender differences observed in the peripheral distribution of GPR30 protein expression in the three male and three female rats and mice analysed.

Distribution of GPR30 mRNA

We determined the expression of GPR30 mRNA in tissues that displayed abundant GPR30-ir in tissues from three male and three female rats and mice. In the rat central nervous system, a hybridisation signal for GPR30 was detected in the PVH and SON particularly in the magnocellular region of the PVH (Fig. 7A and C). In the pituitary gland, the intermediate lobe was intensely labelled (Fig. 7D–F) and scattered cells exhibited a moderate signal in the anterior lobe. The neural lobe was unlabelled. In the adrenal glands, high levels of GPR30 mRNA were present in the adrenal medulla and cells within the zona glomerulosa of the adrenal cortex (Fig. 8A and B). In the kidney, intense labelling was present in the smooth muscle of the renal pelvis, and to a lesser extent in the medulla, with some sparse labelling in the cortex (Fig. 8C). A few developing follicles of the rat ovary exhibited a hybridisation signal (data not shown). A similar distribution of GPR30 transcripts was observed in mouse tissues– the SON, the adrenal medulla and pituitary intermediate lobe expressed the highest amount of GPR30 mRNA compared with kidney, ovary and PVH based on signal intensities observed for the same film exposures. Figure 8E shows GPR30 mRNA expression in developing follicles of the mouse ovary, with silver grains accumulated mainly in the granulosa and theca cells. No signal was detected in the corpora lutea. Rat and mouse sections hybridised with sense GPR30 riboprobes as controls showed only background level of labelling (Figs 7B and 8D and E).

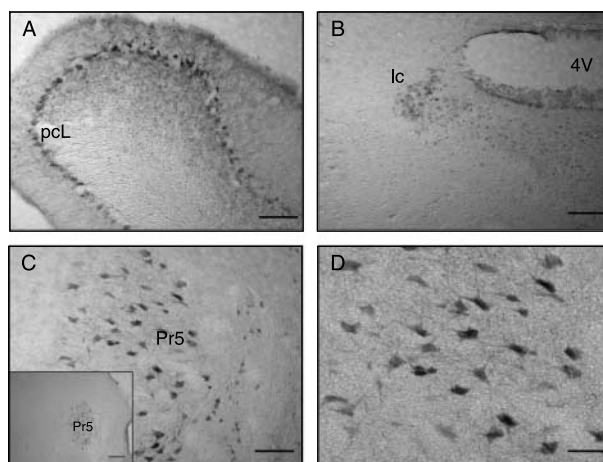


Figure 3 Immunoreactivity for GPR30 in the adult male or female mouse brain: labelled cells of the Purkinje cell layer of the cerebellum (A), locus coeruleus (B) and sensory trigeminal nucleus (C) enlarged in (D). pcL, Purkinje cell layer of the cerebellum; lc, locus coeruleus; Pr5, sensory trigeminal nucleus; 4V, fourth ventricle. Scale bars, 100 μ m in (A–C); 50 μ m in (D). In (C), a lower magnification of Pr5 is shown (scale bar=200 μ m).

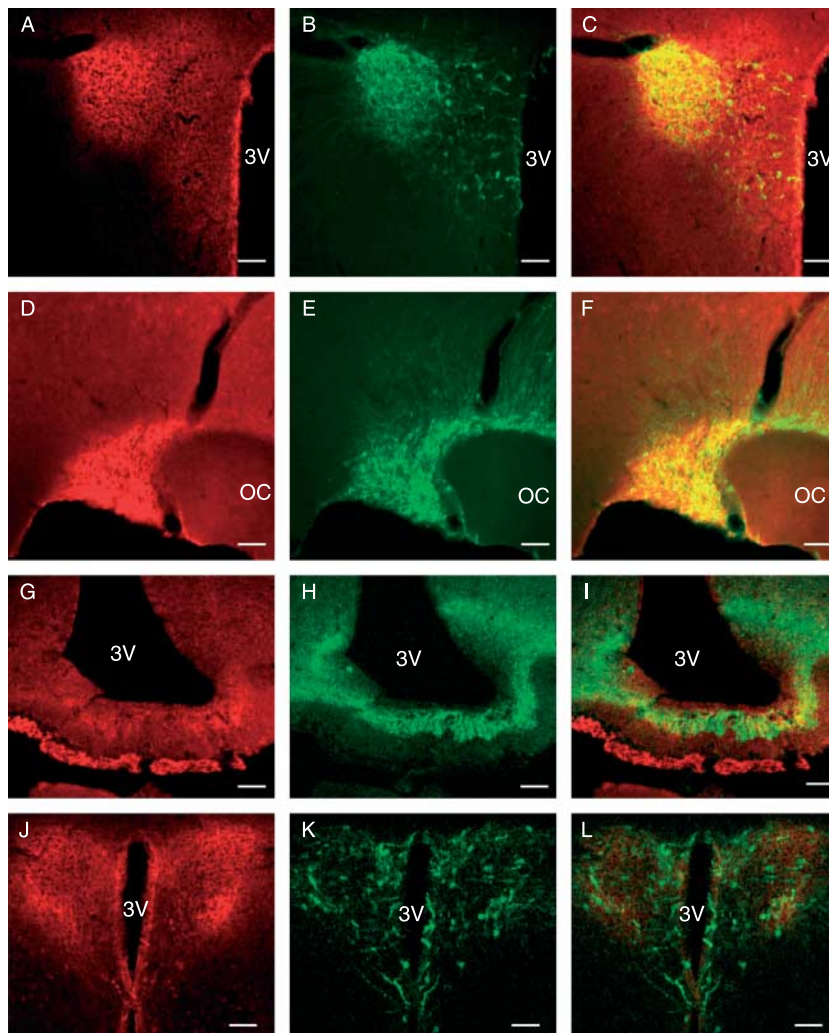


Figure 4 Double label immunofluorescence for GPR30 and AVP in the adult rat PVH (A–C), SON (D–F) and median eminence (G–I), and GPR30 and OXT in the adult mouse PVH (J, K and L). Immunoreactivities against GPR30 (A, D, G and J; red) and AVP (B, E and H; green) or OXT (K; green) were merged in each right panel (C, F, I and L; overlap yellow) respectively. G–L were captured using a laser confocal microscope. 3V, third ventricle; OC, optic chiasm. Scale bars, 100 μ m in (A–F); 80 μ m in (G–L).

Discussion

GPR30 was originally cloned by us as an orphan GPCR over 10 years ago (Owman *et al.* 1996) and subsequently identified as an ER in a number of laboratories (Filardo *et al.* 2000, 2002, Revankar *et al.* 2005, Thomas *et al.* 2005). Unlike many GPCRs, GPR30 appears to be predominantly expressed and functions intracellularly at least *in vitro* (Revankar *et al.* 2007). It has been reported that GPR30 is also found on the plasma membrane where it appears to be functionally relevant (Filardo *et al.* 2007). Further studies on GPR30 internalisation and trafficking are required to address this issue. The suggestion that a GPCR is primarily localised intracellularly is not without precedent—e.g. the proportion of human GnRH

receptors at the cell surface is extremely low (<1%; Finch *et al.* 2008) and depends upon the cellular context (the proportion of human GnRH receptors at the cell surface is five times greater in a gonadotroph cell line (L β T2) than MCF7 breast cancer cells (Finch *et al.* 2008)). Homologues of the human, rat and mouse GPR30 are present in many species including rhesus monkey, chimpanzee, dog, chicken and zebrafish (see NCBI or Ensembl databases at www.ncbi.nlm.nih.gov or www.ensembl.org respectively). The GPR30 gene is widely distributed in human and rat tissues as shown by northern blot and immunohistochemical analyses (Owman *et al.* 1996, Bonini *et al.* 1997, Carmeci *et al.* 1997, Feng & Gregor 1997, Kvingedal & Smeland 1997, Brailoiu *et al.* 2007) and see EST profile Hs.20961 in NCBI Entrez

Table 2 Summary of GPR30 co-localisation with oxytocin and vasopressin neurones in the rodent paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus

	OXT	Co-localised GPR30 and OXT (%)	AVP	Co-localised GPR30 and AVP (%)
Rat PVN	223	50.6 ± 10.4	160	59.1 ± 6.3
Rat SON	148	54.6 ± 8.3	181	62.1 ± 7.4
Mouse PVN	191	70 ± 6.5	152	70.6 ± 8.2
Mouse SON	219	74.6 ± 8.9	131	64.2 ± 7.8

Values represent the total number of OXT and AVP neurones counted in sections of PVN and SON obtained from three male and three female animals, and the average percentage (\pm s.d.) that also express GPR30-ir. There was no apparent difference in the number of co-expressing neurones between the anterior, middle and posterior PVN in either rat or mouse.

Gene database). We have extended these observations in the present study using immunohistochemistry and *in situ* hybridisation histochemistry to obtain the cellular context of GPR30 expression in mouse brain and peripheral tissues.

Distribution of GPR30-ir in the adult mouse brain

The present study provides the first full comprehensive characterisation of GPR30 distribution in the mouse central nervous system. Previously, the distribution of GPR30 has been illustrated in the rat brain using the same GPR30 antiserum as described here (Brailoiu *et al.* 2007). In agreement with our findings in mice, there are no apparent gender differences in GPR30 expression in the rat and this appears common to all E₂ receptors (Laflamme *et al.* 1998). Brailoiu *et al.* (2007) found predominant GPR30 expression in the hypothalamic PVH and SON, hippocampal formation, substantia nigra in the rat brain and this correlates well with our observations in the mouse brain. However, GPR30 is also highly expressed in the nucleus ambiguus where only low to moderate expression is found in the equivalent area in the mouse brain, suggesting some species differences. A comparison of GPR30 mRNA versus protein localisation in both rat and mouse brains may reveal whether receptor transcription and translation coincide or whether there are mismatches.

Comparison of ER distribution in the adult mouse brain

There appears to be only a few brain regions that express high levels of GPR30 and high amounts of either ER α or ER β and even fewer regions that express all three receptors (see Table 3) to a high degree. For example, GPR30 is most abundant in the hypothalamus of the mouse, particularly the arcuate nucleus, PVH, periventricular nucleus, SON and the dorsomedial, central and ventrolateral regions of the VMH. Of these nuclei, ER α is highly expressed in the arcuate nucleus and in the ventrolateral portion of the VMH, and in rats priming the VMH with E₂ over days promotes lordosis, seemingly through ER α (Pfaff & Sakuma 1978). In the rat and

mouse, hypothalamus ER β is the predominant ER isoform found in the PVH (Mitra *et al.* 2003, Merchenthaler *et al.* 2004). Interestingly, E₂ has been shown to activate the (MAPK) ERK 1/2 pathway in the PVH and SON of the mouse (Ábrahám *et al.* 2004). In the PVH, this is potentially mediated via either ER β and/or GPR30, and presumably GPR30 in the SON.

In the forebrain, GPR30 is also highly expressed in the cingulate, motor and somatosensory regions of the isocortex, piriform cortex, the hippocampal dentate gyrus hilus, subiculum and entorhinal cortex, and the zona incerta where ER α and ER β expression is either low or absent. Interestingly, E₂ has been shown to induce *c-fos* protein expression in some brain regions (e.g. cingulate cortex) of double ER $\alpha\beta$ knockout mice. As GPR30 is abundant in this region, GPR30 or another E₂-binding protein may mediate the effects of E₂ (Dominguez-Salazar *et al.* 2006). On the other hand, ER α and β are both highly expressed in the bed nucleus of the stria terminalis and the medial nucleus of the amygdala, where only minimal amounts of GPR30 can be detected.

In the midbrain and pons, high expression of GPR30 is found in the pontine nuclei, anterior tegmental nucleus, the medioventral periolivary nucleus and superior paraolivary nucleus of the superior olive, and the locus coeruleus. Of these, only the locus coeruleus contains high levels of the ERs. Interestingly in the mouse, noradrenaline neurones of the locus coeruleus have been shown to be responsive to E₂ stimulation with an upregulation in tyrosine hydroxylase transcription in females, and attenuation of transcription in males (Thanky *et al.* 2002). This gender difference could be indicative of the varying roles of all the three ERs that are highly expressed in this brain region.

GPR30 co-localises with OXT and AVP neurones

The present study also aimed to clarify the inconsistencies in the literature regarding the distribution of GPR30 in hypothalamic magnocellular neurones. Our findings confirm that in the rat and mouse hypothalamus both OXT and AVP neurones express GPR30. This is in agreement with observations of Brailoiu *et al.* who found co-localisation of GPR30 with OXT and AVP in magnocellular neurones of the rat PVH and SON, but contrast with those of another study that detected GPR30 only in OXT neurones of the PVH and SON of the same species (Brailoiu *et al.* 2007, Sakamoto *et al.* 2007). The reasons for these discrepancies are not readily apparent, especially since all three studies used the same antiserum directed against the GPR30 protein (Revankar *et al.* 2005). To confirm the specificity of GPR30-ir in the present study we substituted normal rabbit IgG serum for primary antibody at the same concentration, and did not observe any staining (Fig. 1F).

E₂ mediates the release of OXT and AVP *in vivo* and *in vitro*. It rapidly stimulates intrahypothalamic OXT and AVP release and rapid peripheral OXT and possibly AVP release

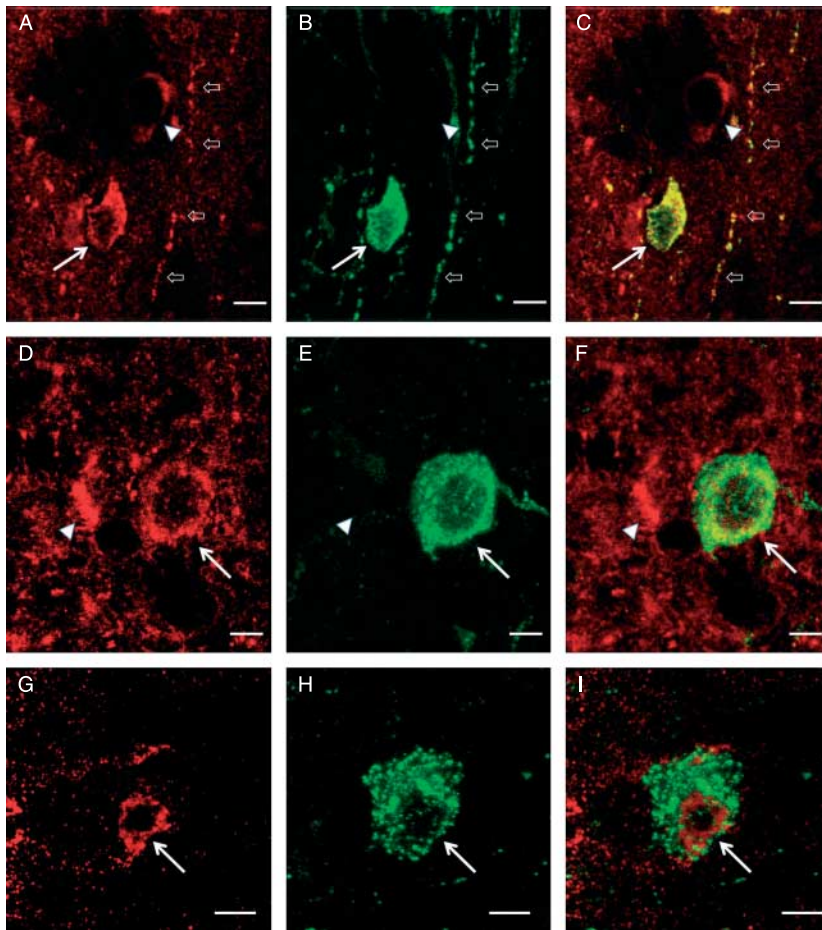


Figure 5 Double label immunofluorescence for GPR30, OXT and AVP in the adult rat SON and mouse PVH. Immunoreactivities against GPR30 (A, D and G; red) and OXT (B; green) or AVP (E and H; green) were merged in each right panel (C, F and I; overlap yellow) respectively. Arrows indicate OXT or AVP immunoreactive cell bodies that also contain GPR30-immunoreactivity. Note that some GPR30-ir cells (arrowhead) do not express OXT-ir or AVP-ir. In addition to staining of cell bodies, there also appears to be co-expression of GPR30-ir and OXT-ir in processes (that may be axons or dendrites), suggesting that the receptor may be axo-dendritically transported, as has been well-established for the neuropeptide (see open arrows). The presence of possible intracellular staining of GPR30 within OXT and AVP neurones which is consistent with the predominantly cytoplasmic distribution of GPR30 that has been observed in various cell types *in vitro* (see discussion for details). Pictures captured at the same low magnification using a laser confocal microscope. Scale bars, 10 μ m in (A–I).

(Wang *et al.* 1995, Burbach *et al.* 2001). In addition, E_2 inhibits the release of both peptides from hypothalamo-neurohypophysial explants stimulated hyperosmotically via a non-genomic action (Swenson & Sladek 1997) and rapidly alters the electrophysiological properties of OXT cells in the SON of lactating or morphine-dependent rats (Israel & Poulain 2000, Brown *et al.* 2008). OXT and AVP neurones of the rat PVH and SON, express $ER\beta$ (Hrabovszky *et al.* 2004). It is well established that ERs can stimulate second messenger pathways, perhaps via receptors tethered to the plasma membrane, as well as alter gene activation in the nucleus, suggesting that the rapid E_2 effects on OXT/AVP synthesis

and release could be modulated through extranuclear $ER\beta$ at the level of the hypothalamus (Razandi *et al.* 2003, 2004, Levin 2005). Alternatively, E_2 could regulate AVP and OXT neuronal activity by indirectly activating $ER\alpha/\beta$ -expressing neurones that project to the PVH and SON (Shughrue *et al.* 1997, Hrabovszky *et al.* 2004, Sladek & Somponpun 2004). The relative contribution of GPR30 in E_2 effects on hypothalamo-neurohypophysial activity is not known. However, $ER\beta$ immunoreactivity is absent from the neural lobe suggesting that E_2 does not directly mediate peripheral release of OXT and AVP at the level of the pituitary via this receptor (Pelletier *et al.* 2000). Interestingly in the present

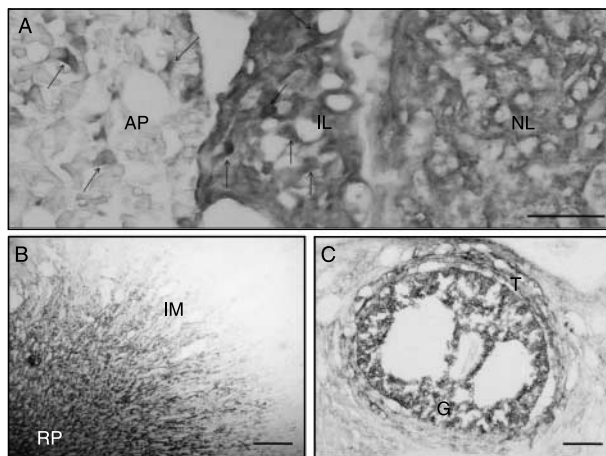


Figure 6 Immunohistochemical analysis of GPR30 in rodent peripheral tissues. (A) GPR30-ir in rat pituitary is prominent in the nerve terminals of the neural (posterior) lobe (NL) and is present in most melanotrophs of the intermediate lobe (IL) (e.g. indicated by arrows). GPR30-ir is also found in scattered (~50%) cells in the anterior pituitary (AP) lobe. Whether these represent endocrine cells (e.g. prolactotrophs and corticotrophs) was not determined. (B) High levels of GPR30-ir are present in the rat renal pelvis (RP), an extension of the ureter, with projections into the renal inner medulla (IM). (C) In the rat ovary, GPR30-ir is found mainly in the granulosa cells (G), with some staining of theca cells (T) of the developing follicle. Scale bars, 400 μ m in (B); 50 μ m in (A and C).

study, we found high levels of GPR30-ir in the neural lobe, indicating that E_2 could modulate the peripheral release of OXT and AVP through GPR30 signalling. As we were unable to detect GPR30 mRNA in the neural lobe, it can be assumed that the receptor had been transported, presumably from the magnocellular neurones in the PVH and SON, as we also observed co-localisation of GPR30 with OXT and AVP fibres in the internal zone of the median eminence. It is possible that GPR30 regulates OXT and AVP synthesis in PVH and SON magnocellular cell bodies, and/or modulates OXT and AVP release from PVH and SON magnocellular neurone dendrites or from fibres that terminate in the posterior pituitary. Our studies also suggest that GPR30 could mediate E_2 effects on the hypothalamic–pituitary adrenal axis by modulating the release of OXT, AVP or perhaps CRF into portal blood from neurones originating from the parvocellular region of the PVH.

Peripheral distribution of GPR30

The present study demonstrates high expression of GPR30 mRNA and protein in the pituitary gland, adrenal medulla, renal pelvis and ovary of the rat and mouse. There is a strong correlation between the tissue distribution of both GPR30 mRNA and protein, apart from that in the neural lobe of the pituitary.

While GPR30 protein is expressed in all three lobes of the pituitary with the highest mRNA expression was observed in

the intermediate lobe. $ER\alpha$ and $ER\beta$ are also expressed in the anterior and intermediate lobes, but are absent from the posterior lobe of the pituitary (Mitchner *et al.* 1998, Pelletier *et al.* 2000, González *et al.* 2008). It is well established that the anterior pituitary is a target tissue for E_2 , regulating gonadotrophin and prolactin (PRL) secretion from gonadotrophs and lactotrophs, and this response can be rapid, with E_2 increasing excitability in PRL-secreting pituitary cell lines within minutes (Duffy *et al.* 1979). High expression of GPR30 mRNA and protein in the intermediate lobe and the presence of the traditional E_2 receptors suggests a possible role for E_2 in pro-opiomelanocortin synthesis and α -melanocyte stimulating hormone (α -MSH) and ACTH-like peptide release. A previous study has shown that E_2 stimulates the release of α -MSH from the intermediate lobe which in turn appears to exert a rapid stimulatory effect on PRL secretion (Ellerkmann *et al.* 1992).

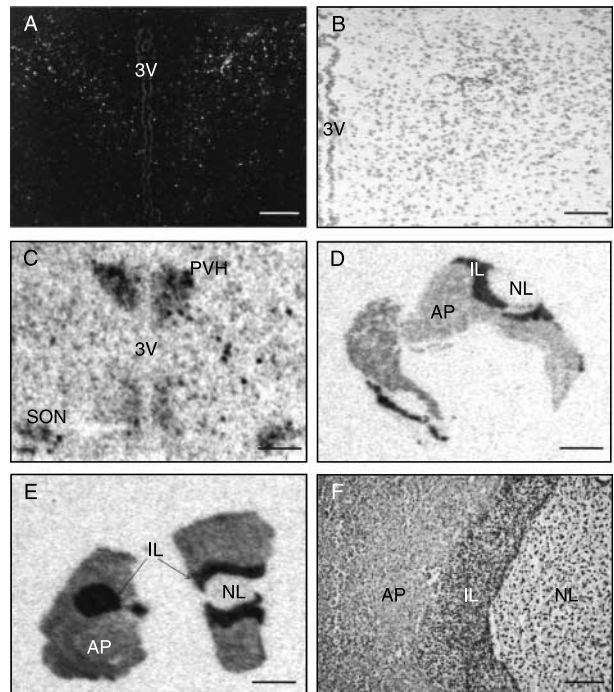


Figure 7 *In situ* hybridisation of GPR30 mRNA in the rodent PVH, SON and pituitary gland: reversed image of emulsion dipped section of the rat PVH the GPR30 hybridisation grains appear white in the picture (A) (but black on the actual slide); the corresponding sense slide is absent of black GPR30 hybridisation grains (B); low-magnification photographs of film autoradiographic images of a slide mounted hypothalamic section hybridised with a GPR30 probe, with signal in the PVH and SON of the rat brain (C), and pituitary sections hybridised with a GPR30 probe in rat (D) and mouse (E) with intense signal in both intermediate lobes; emulsion dipped section of the mouse pituitary, hybridisation signal for GPR30 mRNA appears as black grains (E). 3V, Third ventricle; PVH, paraventricular nucleus; SON, supraoptic nucleus; AP, anterior pituitary; IL, intermediate lobe of the pituitary; NL, neural lobe of the pituitary. Scale bars, 1 mm in (C–E); 100 μ m in (A and F); 50 μ m in (B).

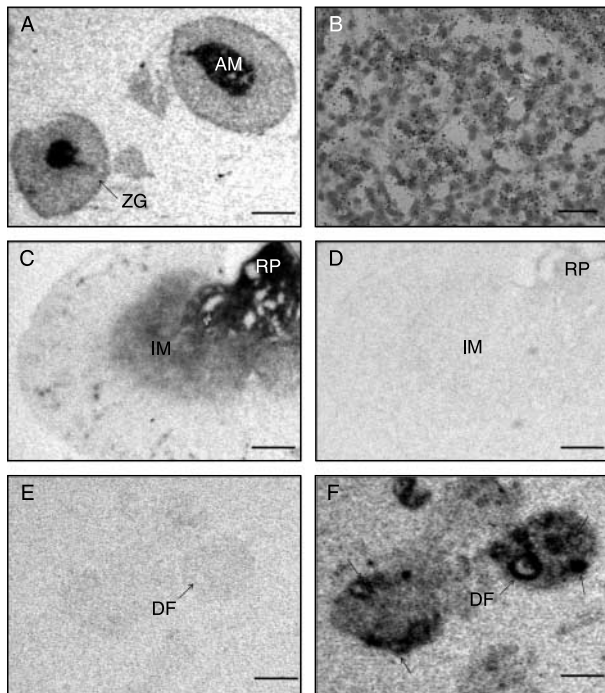


Figure 8 *In situ* hybridisation of GPR30 mRNA in rodent peripheral tissues: low-magnification photograph of a film autoradiographic image of a slide mounted, rat adrenal section hybridised with a GPR30 probe (A); emulsion dipped section of the mouse adrenal medulla-hybridisation signal for GPR30 mRNA appears as black grains on toluidine blue counterstained cells (B); low-magnification photograph of a film autoradiographic image of a slide mounted, rat kidney section hybridised with a GPR30 probe (C), with an absence of signal in the sense control (D); low-magnification photograph of a film autoradiographic image of a slide mounted, mouse ovary section hybridised with a GPR30 probe, with signal localised predominantly in the developing follicles (E), with an absence of signal in the sense control (F). AM, adrenal medulla; ZG, zona glomerulosa; RP, renal pelvis; IM, renal medulla; DF, developing follicle. Scale bars, 1 mm in (A and C–F); 25 µm in (B).

In agreement with the present study, GPR30 has been reported to be strongly expressed in the zona glomerulosa and the medulla of the human adrenal glands (Baquedano *et al.* 2007). The fast effects of E₂ have previously been described in primary cultures of bovine adrenal medullary cells with an up regulation in catecholamine synthesis within 20 min of E₂ stimulation (Yanagihara *et al.* 2006). This suggests a role for GPR30 in E₂ signalling either independently or synergistically with extranuclear ERβ which is also expressed in cells of the rat adrenal cortex and medulla (Saunders *et al.* 1997).

In the kidney, ERα/β are found mainly in the glomeruli and arterioles of the renal cortex where E₂ treatment has been shown to upregulate angiotensin type II receptors that regulate sodium and water reabsorption, renal blood flow and glomerular filtration rate (Baiardi *et al.* 2005, Oestreicher *et al.* 2006). We found high expression of GPR30 mRNA and

protein in the contracting muscle of the pelvis and to a moderate extent in the medulla. In the renal pelvis, the role of E₂ is presently unclear but there are suggestions that prolonged E₂ administration can encourage renal pelvic tumour growth (Oberley *et al.* 1991, Adsay *et al.* 2000). In other carcinomas (e.g. breast, endometrial), GPR30

Table 3 An overall comparison of the distribution of GPR30, oestrogen receptors (ER) α and β, in the mouse brain (Results for ERα and β are a summary of the mouse brain atlases from Mitra *et al.* (2003) and Merchenthaler *et al.* (2004))

	GPR30	ERα	ERβ
Brain region			
Allocortex			
Piriform	+++	+	+
Isocortex			
Cingulate	+++	–	–/+
Motor	+++	–	–/+
Somatosensory	+++	–/+	–/+
Hippocampus			
Dentate gyrus hilus	+++	+	–
Subiculum	+++	–/+	+/++
Entorhinal cortex	+++	–	+
Bed nucleus of the stria terminalis	+	+++	+++
Preoptic area			
Medial preoptic nucleus	++/+++	+++	++
Amygdala			
Medial nucleus	+	+++	+++
Hypothalamus			
Arcuate nucleus	+++	+++	+
Paraventricular nucleus	+++	+	+++
Periventricular nucleus	+++	++/+++	–/+
Supraoptic nucleus	+++	–	+
Ventromedial hypothalamic nucleus			
Dorsomedial	+++	–/+	–
Central	+++	++	–
Ventrolateral	+++	+++	+/+++
Substantia nigra			
Reticular	+	–	+++
Ventral tegmental area	–	–	++
Periaqueductal gray	++/+++	++/+++	+
Raphe nuclei			
Dorsal raphe	+	+	+++
Pontine nuclei	+++	–	+
Anterior tegmental nucleus	+++	–	+
Superior olive			
Medioventral periolivary nucleus	+++	–	+
Superior paraolivary nucleus	+++	–	+
Locus coeruleus	+++	++	++
Spinal trigeminal nucleus	+++	++	–/+
Cerebellum			
Purkinje layer	+++	–	–

Intensity of label: + + +, intense; ++, moderate; +, low; –, abs.

expression has been demonstrated (Filardo *et al.* 2006, Smith *et al.* 2007). Taken together, it is tempting to speculate that GPR30 is an alternative oestrogen responsive receptor in the production and maintenance of renal pelvic tumours.

E₂ has been shown to promote cell division and survival of granulosa cells in developing follicles of the ovary (Adashi 1994). E₂ also induces rapid (s) increases in cytosolic calcium concentration of human granulosa cells *in vitro* (Younglai *et al.* 2005). While this could be mediated through the ER β which has been shown to be highly expressed within the granulosa cells of developing follicles and corpora lutea of the rat (Saunders *et al.* 1997), our results also indicate a role for GPR30. GPR30 mRNA and protein have been found in both granulosa and theca cells of the hamster and expression is sensitive to cyclic changes in FSH and LH (Wang *et al.* 2007). It has also been suggested that GPR30 is required for primordial follicle formation (Wang *et al.* 2008). These observations have been appeared to contradict a recent report which concluded that GPR30 did not mediate E₂ responses in reproductive organs owing to the fertile phenotype of the GPR30 knockout mice (Otto *et al.* 2008). In GPR30 knockout animals, the normal phenotypes in E₂ responsive tissues such as the ovary, uterus and mammary gland does not necessarily preclude the possibility that GPR30 binds E₂ *in vivo*. For example, in the knockout model, ER α and/or ER β may compensate for the loss of GPR30 especially in tissues of co-expression where there is potential for receptor cross-talk (Siriani *et al.* 2008). In addition, it is well known that ER β knockouts remain fertile; it is only the deletion of ER α which appears to create the infertile phenotype (Walker & Korach 2004).

While this manuscript was in preparation, four papers relevant to the possible function of GPR30 in the brain and adrenal have been published online. Isensee *et al.* (2009) analysed the distribution of GPR30 using GPR30-LacZ reporter mice and found predominant expression in sub populations of cells in the cortex and the dentate gyrus of the brain, in the intermediate and anterior lobe of the pituitary gland and in the adrenal medulla. As shown in Table 1, GPR30-ir is also highly expressed in regions of the cortex and in the dentate gyrus of the adult mouse brain. Our results also confirm the presence of GPR30 mRNA in the anterior and intermediate lobes of the pituitary and the adrenal medulla. However, in contrast to our data that clearly demonstrate both GPR30 mRNA and protein in the PVH and SON, GPR30 was only identified in the hypothalamic vasculature and not neurones in the GPR30-LacZ reporter mice (Isensee *et al.* 2009). Xu *et al.* implicate a role for GPR30 in the rat hypothalamus based on the selective GPR30 agonist G-1 attenuating 5-HT_{1A} signalling within the PVH. They also confirmed GPR30 expression in OXT neurones (Xu *et al.* 2009). Another recent study has shown that GPR30 may have been mediated E₂-stimulated release of LHRH in primate neurones (Noel *et al.* 2009). In the rodent brain, co-localisation of GPR30 with LHRH neurones or a possible role of GPR30 in E₂-stimulated LHRH release has yet to be

described. Finally, the expression of GPR30 in the adrenal medulla of the rat has been confirmed by Dun *et al.* (2009) with GPR30 located in tyrosine hydroxylase-positive chromaffin cells, further suggesting a likely role for GPR30 in E₂-mediated noradrenaline release from the adrenal glands.

Conclusion

The distribution of GPR30 in the adult mouse brain appears distinct from ER α and ER β receptors and offers an additional site for E₂ action. The expression of GPR30 in OXT and AVP neurones in the PVH, SON and in the median eminence, and its presence in the pituitary gland provides a likely means by which E₂ can modulate fast 'non-genomic' effects on these specific neuropeptide systems. In the periphery, GPR30 may be involved in multiple functions including hormone release and development. Whether E₂ initiates responses through GPR30 alone or in concert with the other ER₂ receptors (e.g. in regions of co-expression) has yet to be investigated, but the cellular distribution of GPR30 in the brain and peripheral tissues provides a basis for further studies on E₂ mediated signalling.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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