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EXPRESSION OF THE GLUCOCORTICOID-INDUCED RECEPTOR mRNA IN RAT BRAIN

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Abstract—The glucocorticoid-induced receptor (GIR) is an orphan G-protein-coupled receptor awaiting pharmacological characterization. GIR was originally identified in murine thymoma cells, and shows a widespread, yet not completely complementary distribution in mouse and human brain. Expression of the mouse GIR gene is modulated by dexamethasone in the brain and periphery, suggesting that GIR function is directly responsive to glucocorticoid signals. The rat GIR was cloned from rat prefrontal cortex by our group and was shown to be up-regulated following chronic amphetamine. The physiological role of GIR in the rat is not known at present. In order to gain a clearer understanding of the potential functions of GIR in the rat, we performed a detailed mapping of GIR mRNA expression in the rat brain. GIR mRNA showed widespread distribution in forebrain limbic and thalamic structures, and a more restricted distribution in hindbrain areas such as the spinal trigeminal nucleus and the median raphe nucleus. Areas with moderate to high levels of GIR include olfactory regions such as the nucleus of olfactory tract, hippocampus, various thalamic nuclei, cortical layers, and some hypothalamic nuclei. In comparison with previous studies, significant regional differences exist in GIR distribution in mouse and rat brain, particularly in the thalamus, striatum and in hippocampus at a cellular level. Overall, the expression of GIR in rat brain more closely approaches that seen previously in human than mouse, suggesting that rat models may be more informative for understanding the role of GIR in glucocorticoid physiology and glucocorticoid-related disease states. GIR mRNA distribution in the rat indicates a potential role of this receptor in the control of feeding and ingestive behavior, regulation of stress and emotional behavior, learning and memory, and, drug reinforcement and reward.

Key words: G protein-coupled receptor, *in situ* hybridization, forebrain limbic system, thalamus, feeding, stress.

The glucocorticoid-induced receptor (GIR) is an orphan G-protein-coupled receptor awaiting pharmacological characterization. GIR, also referred to as JP05, GPR72 or GPR83 was originally identified as a stress-responsive transcript from the murine T-cell line WEHI-7TG and normal thymocytes treated with glucocorticoids and forskolin (Harrigan et al., 1989; Baughman et al., 1991; Harrigan et al., 1991). Recent studies have reported CNS regulation of GIR mRNA following *in vivo* administration of dexamethasone, suggesting a potential role of this receptor in glucocorticoid-mediated effects such as, hypothalamic pituitary adrenal (HPA) function and stress regulation (Adams et al., 2003). GIR mRNA is detected at high levels in mouse brain regions such as the forebrain limbic structures, hypothalamic nuclei and the striatum (Pesini et al., 1998). Cloning and chromosomal mapping of human GIR elicited similarities in genomic organization as well as 89.5% identity with the mouse GIR (De Moerlooze et al., 2000; Parker et al., 2000).

The rat GIR was cloned from rat prefrontal cortex by our group, and was shown to be significantly upregulated following chronic amphetamine administration (Wang et al., 2001). Overall, the rat GIR elicits highest sequence identities with mouse GIR (99%), human GIR (88%), orphan G-protein-coupled receptor PGR15L (53%), neuropeptide Y (NPY)-Y2 receptor (38%), and the prolactin releasing peptide receptor (38%). We have observed that NPY C-terminus fragments can specifically bind and activate GIR expressed *in vitro* with moderate affinity (Sah et al., 2002, 2003). These observations suggest structural similarities between NPY compounds and GIR agonists. The physiological role of GIR in the rat is not known at present. Initial characterization of GIR expression in rat brain revealed a predominance in forebrain limbic regions (Wang et al., 2001), notably including numerous regions (hippocampus, prefrontal cortex, hypothalamus) that are critical to stress signaling in the CNS. The fact that GIR is regulated by glucocorticoids in both the periphery and brain suggests that this G-protein-coupled receptor may play an important role in transducing glucocorticoid information subsequent to stress. In order to gain a clearer understanding of the potential functions of GIR in the rodent brain, we performed a detailed, quantitative mapping of GIR mRNA in rat brain using *in situ* hybridization.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague–Dawley rats (Charles River Laboratories, Inc., Wilmington, MA, USA; 250–300 g) were used for *in situ* hybridization localization of GIR mRNA. Animals were maintained in constant temperature/humidity vivarium with standardized lighting and free access to rat chow and tap water. All procedures were approved by the institutional animal care and use committee (IACUC), University of Cincinnati, and conformed to the National Institutes of Health standards for the humane treatment of animals. All efforts were made to minimize the number of animals used and their suffering.

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Abbreviations: DEPC, diethylpyrocarbonate; EDTA, ethylene diamine tetraacetic acid; GIR, glucocorticoid-induced receptor; GR, glucocorticoid receptor; HPA, hypothalamic pituitary adrenal; KPBS, potassium phosphate-buffered saline; NPY, neuropeptide Y; RT-PCR, reverse transcription–polymerase chain reaction; SSC, standard saline citrate.

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GIR distribution analysis by reverse transcription–polymerase chain reaction (RT-PCR)

Distribution of GIR in various brain regions was investigated using RT-PCR experiments. Rat brains were obtained following cervical dislocation and specific brain regions were rapidly dissected out. Total RNA was isolated from each region by single step guanidine thiocyanate–phenol extraction using the TRI-REAGENT (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions. The concentrations of RNA samples were determined by spectrophotometric measurements at 260 and 280 nm. First strand cDNA was synthesized from total RNA using a random hexamer primer (Promega, Madison, WI, USA) following manufacturer's instruction. GIR specific primers 5′-TAC TTT GCC TTC CAC TGG TT 3′, 1283–1303 bp and 5′-CTA ACT CAC GGC CAC AGT GGG TT 3′, 1550–1568 bp, GenBank accession number AY029071, were synthesized (Integrated DNA Technologies, Coralville, IA, USA). Specific primers for GFAP, used as the internal control were 5′-GAA AAC CGC ATC ACC ATT CC-3′, 1139–1158 bp and 5′-GCA TCT CCA CCG TCT TTA CC-3′, 1245–1265 bp. PCR was run for 35 cycles at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, followed by one cycle at 72 °C at 10 min. PCR products were electrophoresed on 1.2% agarose gel and stained with ethidium bromide.

Preparation of cRNA probes

Two GIR probes were used for *in situ* experimentation. Oligonucleotides complementary to full length GIR (308–1575 bp), and a C-terminus fragment (1201–1568 bp) of GIR mRNA were synthesized (GenBank accession number for GIR: AY029071). Probes were transcribed *in vitro* from linearized plasmid constructs. Labeling reactions included 60 μCi of ^{35}S-UTP (specific activity 1800 Ci/mmol), 1× transcription buffer, 15 mM dithiothreitol, 200 μM GTP, CTP, and ATP, 10 μM UTP, 40 U of placental RNase inhibitor, 1 μg of linearized plasmid DNA, and 20 U of appropriate RNA polymerase (T3 or T7; Roche Molecular Biochemicals, Indianapolis, IN, USA). Reactions were incubated at 37 °C for 90 min. The DNA template was then removed by RNase-free DNase one digestion for 15 min at 37 °C, and the reaction mix was diluted to 100 μl with diethylpyrocarbonate (DEPC)-treated water and ethanol precipitated with 7.5 M ammonium acetate. The resulting pellet was re-suspended in DEPC-treated water. Successful labeling was confirmed by scintillation counting.

In situ hybridization

In situ hybridization studies were carried out as previously described (Wang et al., 2001; Ziegler et al., 2002). Rats were decapitated between the hours of 9:00 and 11:00 a.m., during the circadian nadir of corticosterone secretion and brains rapidly removed and frozen on dry ice. Coronal cryostat sections (14 μm thick) were cut through the brain, thaw-mounted onto Superfrost Plus slides, and stored at −20 °C until use. Prior to hybridization, sections were thawed to room temperature and fixed for 15 min in 4% paraformaldehyde. Sections were then rinsed 2×5 min in 5 mM DEPC-treated potassium phosphate-buffered saline (KPBS), 2×5 min in PBS containing 0.2% glycine, followed by 2×5 min in KPBS. Sections were acetylated for 10 min in triethanolamine (0.1 M, pH 8.0), containing 0.25% acetic anhydride, rinsed twice in standard saline citrate (SSC) buffer (0.25 M sodium chloride, 0.015 sodium citrate, pH 7.2) for 5 min, followed by dehydration in a graded ethanol series. Sections were re-hydrated to 70% ethanol and then air dried. Labeled probes were added to a hybridization buffer containing 50% formamide, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 335 mM NaCl, 1× Denhardt's solution, 200 μg/ml salmon sperm DNA, 150 μg/ml yeast transfer RNA, 20 mM dithiothreitol, and 10% dextran sulfate. Probes were denatured for 15 min at 65 °C and 50 μl (1×10^6 cpm) of diluted probe applied to each slide. Slides were coverslipped, placed in moistened chambers, and incubated overnight at 55 °C. After hybridization, coverslips were removed in 0.2× SSC, and rinsed in fresh 0.2× SSC for 10 min. Sections were then treated with RNase A (50 μg/ml) for 30 min at 37 °C, and transferred to fresh 2× SSC and then rinsed three times in 0.2× SSC (10 min/wash), followed by a 1 h wash in 0.2× SSC at 65 °C. Finally, sections were dehydrated in a graded ethanol series, dried at room temperature, and exposed for 14–21 days to Kodak BioMAX film (Eastman Kodak, Rochester, NY, USA). Following development sections were coated with Kodak photographic emulsion NTB2, diluted 1:1 with water, air dried, and stored at 4 °C in a light- and humid-free environment for 5 weeks. Following development in Kodak D-19 developer and Rapid Fix solutions, emulsion-dipped sections were counterstained with 0.25% Cresyl Violet, dehydrated, and coverslipped using DPX mountant (Fluka, Milwaukee, WI, USA). Controls run in parallel included sections hybridized with the sense-strand probe generated from the same vector construct, and sections preincubated in RNase A (50 μg/ml, 30 min, 37 °C) before hybridization with antisense probe. No hybridization signal was observed after either control procedure.

Image processing and analysis

Images from X-ray film autoradiographs were captured using a Hewlett-Packard 5300C flatbed scanner at maximum resolution (1200 dpi). Microscopic images were captured from emulsion-dipped, counterstained slides using a Zeiss Axiocam digital camera. All images were imported into Photoshop and brightness/contrast adjusted to provide optimal visualization.

Neuroanatomical regions were identified with reference primarily to Paxinos and Watson's (1997) rat brain atlas. Regions of interest were sampled on the basis of homogeneous expression profiles; in some cases, profiles represented clear nuclear divisions within the CNS (e.g. nucleus of the lateral olfactory tract); in others, expression was limited to subdivisions of particular nuclei (e.g. ventromedial region of the ventral thalamus). Subnuclear boundaries were confirmed by examination of emulsion-dipped autoradiographs of the same sections. In addition, GIR mRNA expression was also observed in many brain regions where due to scatter of cells or weaker signal, areal densitometry could not be performed. Expression in these regions was assessed in emulsion-dipped images using a graded scale: −=no labeling; +=weak diffuse labeling (<10 grains/cell) in a nucleus of interest; ++=10–25 grains/cell; +++=>25 grains per cell.

RESULTS

GIR mRNA distribution in rat brain: RT-PCR analysis

GIR mRNA was assayed by RT-PCR in several rat brain regions (Fig. 1). A strong band of expected size (293 bp) was detected in multiple regions indicating a widespread distribution of GIR in rat brain. This distribution profile is in agreement with previous reports on GIR expression in murine and human brain (Brezillon et al., 2001; Pesini et al., 1998). Chinese hamster ovary cells stably transfected with the full length GIR insert (GIRD7 cells) also revealed a PCR product of similar size (Fig. 1). Since these cells do not express other brain specific receptors, the product obtained in all tissues likely represents GIR. Expression was also observed in AR-5-transformed amygdalar cells (Kasckow et al., 1997) as well as dissected amygdalar tissue. These *in vitro* systems will be useful for investigating GIR pharmacology and regulation since they express similar forms of GIR as brain tissue. GIR expression was