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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 hind-brain 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.

Keywords

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

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similarities in genomic organization as well as 89.5% identity with the mouse GIR (De Moerloose 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.

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 $\times$ 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 $\times$ 5 min in 5 mM DEPC-treated potassium phosphate-buffered saline (KPBS), 2 $\times$ 5 min in PBS containing 0.2% glycine, followed by 2 $\times$ 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 $\times$ Denhardt's solution, 200 $\mu\text{g}/\text{ml}$ salmon sperm DNA, 150 $\mu\text{g}/\text{ml}$ 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 $\times$ SSC, and rinsed in fresh 0.2 $\times$ SSC for 10 min. Sections were then treated with RNase A (50 $\mu\text{g}/\text{ml}$) for 30 min at 37 °C, and transferred to fresh 2 $\times$ SSC and then rinsed three times in 0.2 $\times$ SSC (10 min/wash), followed by a 1 h wash in 0.2 $\times$ 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 $\mu\text{g}/\text{ml}$, 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 observed in the hippocampus, hypothalamus, prefrontal and cerebral cortices, amygdala, striatum, and cerebellum. Brain regions that did not contain measurable amounts of GIR mRNA by RT-PCR included the olfactory bulb, medulla, pons and spinal cord (Fig. 1). *In situ* hybridization was performed to obtain information on GIR expression at a sub-regional and cellular level.

Regional distribution of GIR in the rat brain by *in situ* hybridization

The vast majority of labeling was seen over cytoplasm-rich cells of neuronal morphology. GIR mRNA was not expressed in white matter-rich regions of brain, such as the corpus callosum, anterior commissure or internal capsule. In addition, there was no evidence for grain accumulation over glial cells. Control experiments indicated no specific signal following hybridization with sense-strand probes (data not shown). Table 1 shows a comparative distribution of GIR mRNA in various regions of the rat brain.

We hybridized brain tissue with probes complementary to sequences in the carboxy terminus of GIR mRNA, as well as using a full-length GIR probe. The distribution of GIR mRNA was identical with both probes.

Telencephalon

GIR mRNA was richly expressed throughout the cerebral cortex (Fig. 2). In neocortex, positively labeled cells were concentrated in layers 2, 3 and 5 (Fig. 2). Importantly, the presence of GIR was noted in all regions of limbic cortex, including the orbitofrontal regions; infralimbic, prelimbic and anterior cingulate regions of the medial prefrontal cortex; cingulate cortex (layers 2 and 3); entorhinal cortex (layers 2 and 3); and piriform cortex (layer 2). Expression of GIR mRNA was particularly dense in layer 2 of the piriform cortex, although this may be a product of the dense cellular packing in this region. Overall, it is apparent that GIR is preferentially expressed in pyramidal cell layers of the respective cortices. However, it should be noted that lower levels of expression are observed in layers 4 and 6, suggesting that some GIR-positive neurons are present in granule cell layers as well.

GIR mRNA was highly expressed in olfactory regions of the basal forebrain. Most notable was the nucleus of the lateral olfactory tract, which exhibited the highest levels of GIR expression in brain (Figs. 2C, 3A). Within this region, GIR mRNA was expressed at highest levels in layer 2, with slightly lower but still substantial expression evident in layer 3. Intense GIR mRNA expression was also evident in other olfactory-related structures, including the olfactory tubercle, islands of Calleja and deep layers of the tenia tecta. Positive GIR signal was also evident in the anterior olfactory nuclei and related olfactory cortical structures (see above).

Moderate hybridization was found in dorsolateral septal nuclei. Intermediate GIR mRNA expression was evident in the caudal division of the lateral septum (Fig. 2B), and, more posteriorly, in the septofimbrial nucleus, where signal was dispersed to intensely hybridized, scattered neurons. GIR mRNA could also be observed in the septohippocampal nucleus (Fig. 3B). No significant signal was observed in the ventral septal nucleus, medial septum or horizontal limb of the diagonal band of Broca.

Several subnuclei of the bed nucleus of the stria terminalis expressed intermediate levels of GIR mRNA (Fig. 2B). The highest level of expression was observed in the cell-rich principal subnucleus. GIR signal could also be appreciated in anterodorsal and anteroventral subdivisions, as well as in the transverse and intrafascicular nuclei of the caudal bed nucleus. No signal was observed in the CRH-rich oval or fusiform subnuclei.

Hybridization signal above background was observed in the caudate nuclei as well as the nucleus accumbens (Fig. 4). In the caudate, emulsion-dipped autoradiographs indicate that GIR mRNA is present at intermediate levels in scattered cells throughout the striatum and accumbens core. In these regions, grains were localized over relatively pale-staining nuclei (Fig. 4B, C); the morphological characteristics of the positive nuclei were not obviously different from other cell types in the striatum. In contrast, the nucleus accumbens shell showed more widespread GIR mRNA expression in darkly stained nuclei (Fig. 4D); the relative cellular abundance was in the low range (10–25 grains/neuron).

The pattern and levels of GIR expression in the rat striatum are distinct from the mouse, where very high levels of GIR were reported in the caudate nuclei (Adams et al., 2003; Pesini et al., 1998). The significant difference in GIR expression in striatum between the two species is interesting, given the close sequence homology between the mouse and rat GIR. Striatal levels of GIR expression as assessed by RT-PCR, however, were found to be comparable to other regions (Fig. 1). The aim of RT-PCR methods in the current study was to provide an initial qualitative assessment of GIR expression between different brain regions. *In situ* hybridization provides greater resolution and sensitivity, and was used to provide a quantitative assessment and to compare regional and cell specific expression of GIR.

Hybridization signal for GIR was observed in all areas of the hippocampus (Fig. 5). Hybridization was most intense in scattered neurons located within and immediately adjacent to the dentate gyrus and pyramidal cell layers, as well as in the stratum radiatum and dentate gyrus hilus. Grains were concentrated over large, pale nuclei, consistent with neuronal localization (Fig. 5D). No GIR mRNA was observed over glial nuclei. The pattern of localization is suggestive of GABAergic interneurons; however, double *in situ* hybridization is required to clarify this. Weaker expression was also observed throughout the pyramidal and granule cell layers, suggesting the potential for GIR to play a role in excitatory signaling within the trisynaptic circuitry.

Amygdaloid expression of GIR mRNA was present in the posterior dorsal medial amygdaloid nucleus, basolateral and lateral nuclei and the posterior cortical area (Fig. 3). In the latter three regions, GIR mRNA was expressed in scattered cells containing an intermediate grain density.

In contrast, GIR mRNA expression in the medial amygdala was expressed at low levels throughout the posterodorsal subdivision.

Diencephalon

The vast majority of intensely labeled cells in this region were localized in the various nuclei in the ventral thalamus (Figs. 2C, D; 6). In anterior sections, strong hybridization signal was observed in the anteromedial thalamic nuclei and the ventrolateral nucleus reuniens (Fig. 6A, B). Intense signal was observed throughout the rostrocaudal extent of the nucleus reuniens. Notably, signal in the anteromedial thalamus was differentially distributed, with the most intense hybridization observed in a crescent-shaped swath corresponding to the ventral region of this nucleus. Positive hybridization was also seen in the rhomboid and anterodorsal thalamic nuclei, with weak hybridization signal present in the ventral anterior nucleus. Low levels of GIR mRNA expression were observed throughout the rostrocaudal extent of the reticular thalamic nucleus, whereas the paraventricular, paratenial and centromedian nuclei were hybridization-negative. More posteriorly, positive GIR signal was also observed in the mediodorsal thalamus and in the ventral posterolateral nucleus. Of note, signal was particularly intense in the parvocellular division of the ventral posterolateral group (Fig. 6D), corresponding to the gustatory relay area of this nucleus. GIR mRNA expression was also seen in the ventromedial and submedial (gelatinosus) nuclei at this level. Finally, in the caudal thalamus limited GIR mRNA expression was observed in the posterior thalamic complex and in cells scattered throughout the medial geniculate nucleus. Scattered, intensely hybridized cells were observed in the lateral habenular nucleus of the epithalamus.

Expression of GIR mRNA was also noted in several hypothalamic regions of the diencephalon. At anterior levels, GIR positive neurons were widespread in the medial preoptic nucleus (Figs. 2B, 7A) with additional, more weakly hybridized cells scattered throughout the medial preoptic area. Intense hybridization signal was localized to neurons of the periventricular nucleus (Fig. 7C), particularly in its dorsal region. GIR-positive neurons were scattered through the lateral hypothalamic area (Fig. 7B) at all rostrocaudal levels of the hypothalamus, with a tendency for more intense hybridization in the posteromedial extent of this region, often referred to as the tuberomammillary area. The ventromedial and arcuate nuclei contained numerous neurons expressing intermediate GIR grain densities (Fig. 7D). GIR mRNA was not expressed in the paraventricular, supraoptic or suprachiasmatic nuclei of the hypothalamus.

By far the most intense hypothalamic GIR hybridization was observed in medial mammillary nuclei (Fig. 2F). Signal was particularly intense in the median and medial mammillary nucleus proper. Signal of slightly lesser intensity could be appreciated in the ventral premammillary nucleus and in scattered neurons of the posterior hypothalamus.

Mesencephalon, rhombencephalon and metencephalon

Mesencephalic expression of GIR mRNA was greatest in the region of the lateral division of superior central (median) raphe nucleus. Intermediate expression of GIR mRNA was noted in the substantia nigra pars compacta of the midbrain, corresponding to the region of dopaminergic neurons. Weaker GIR expression was also observed in the dorsolateral periaqueductal gray, the dorsal and central nucleus of raphe. In the tectum of the mesencephalon, signal was also observed in scattered neurons of the inferior colliculi.

At the level of the pons and medulla, intermediate to intense expression of GIR mRNA was observed in several motor cranial nerve nuclei, including the motor nucleus of the trigeminal nerve, facial nucleus and hypoglossal nucleus (Fig. 8). Abundant GIR mRNA was also expressed in neurons of the spinal trigeminal nucleus (Fig. 8). Low to intermediate expression

of GIR was observed in the locus coeruleus and in scattered neurons within medial and lateral divisions of the nucleus of the solitary tract (Fig. 8).

Expression in the cerebellum was limited to weak labeling of Purkinje cells.

DISCUSSION

The present study demonstrates widespread distribution of GIR mRNA in the rat brain. Brain areas showing high levels of GIR mRNA were primarily localized within fore-brain limbic and thalamic structures, including layers 2 and 3 of the nucleus of the lateral olfactory tract, hippocampal interneurons, nucleus reunions of the thalamus and the medial mammillary nuclei. Nonetheless, GIR was expressed at moderate to lower levels in numerous brain regions, including the neocortical mantle, striatum, multiple amygdaloid nuclei, ventromedial hypothalamic nucleus, arcuate nucleus, lateral hypothalamus and some cranial nerve nuclei of the brainstem. The dispersion of GIR mRNA positive neurons in hippocampus, cortex and striatum are consistent with localization to interneurons in these regions. Overall, the widespread localization of GIR mRNA suggests involvement of this receptor in numerous facets of CNS integration.

Species comparisons

In general, GIR expression in the rat showed a similar distribution profile as reported for the mouse and human brain (Brezillon et al., 2001; Pesini et al., 1998). Some regional differences and variability in mRNA expression levels were noted between species as discussed in the following sections.

Rat brain areas showing high levels of GIR mRNA expression included the nucleus of lateral olfactory tract, various cortical areas, dorsal and ventral hippocampus, thalamus, and hypothalamic nuclei. Comparison with mouse and human GIR studies reveals GIR expression and distribution in hippocampus, hypothalamus, cortex, and amygdalar regions. However, the microdistribution of GIR mRNA in this region varies among species. In rat and human hippocampus, intense labeling was observed in scattered neurons throughout the hippocampal formation, notably including large neurons in proximity to the basal dendrites of pyramidal cells and the hilar region of the granule cell layers of the dentate gyrus. The location and size of these neurons suggests that these may be GABAergic interneurons (Mugnaini and Oertel, 1985; Woodson et al., 1989), however, double *in situ* hybridization would be required to verify this. Notably, GIR mRNA was also expressed throughout the pyramidal cells layers of the hippocampus and in pyramidal layers of the subiculum, albeit at lower levels of expression. Interneuronal expression of GIR mRNA is also evident in the mouse; however, this species shows dense expression in pyramidal cell layers of CA3, suggesting that GIR is highly expressed in excitatory neurons in this species (Pesini et al., 1998).

Within the hypothalamus, all three species show dense expression of GIR mRNA in the mammillary bodies, with more moderate expression in ventromedial nuclei and the arcuate nucleus. These data suggest a possible role of GIR in neuroendocrine functions (such as feeding) and perhaps limbic integration and memory coding via the mammillary bodies (Sziklas and Petrides, 1998; Swanson, 2000).

A potential role for GIR in mediating the response to altered glucocorticoid levels has been reported previously, based on significant down-regulation of GIR message following dexamethasone treatment, particularly in various hypothalamic nuclei (Adams et al., 2003). Overlap in localization of the glucocorticoid receptor (GR) or mineralocorticoid receptor with GIR mRNAs occurs in several brain regions (Adams et al., 2003; Pesini et al., 1998), indicating a capacity for GIR regulation pursuant to glucocorticoid secretion. However, it should be noted

that GR-GIR overlap does not assume co-localization; for example, whereas pyramidal layers of the hippocampus express both GR and GIR, the former does not appear to be extensively localized in interneuron populations. In addition, there are numerous regions that are rich in GIR mRNA expression but do not contain appreciable levels of GR. Thus, in depth co-localization studies are required to definitively identify areas where GIR and GR overlap.

Expression of GIR was observed in amygdalar nuclei in rat, mouse and human brain, particularly in the medial amygdala. In conjunction with hippocampal and hypothalamic expression, this would suggest a role for GIR in emotional and stress regulatory behavior. We also observed high levels of GIR in immortalized rat amygdalar AR-5 cells, that have previously been shown to contain CRF and NPY signaling pathways (Mulchahey et al., 1999; Sheriff et al., 2001). Modulation of CRF expression following glucocorticoid administration has also been reported in these cells (Mulchahey et al., 1999). In recent studies, we observed a significant down-regulation of GIR expression in AR-5 cells following dexamethasone (Eaton et al., 2004), suggesting that amygdalar neurons expressing GIR may be involved in glucocorticoid regulatory pathways and stress integration.

The most striking species difference in the levels of GIR mRNA is the differential predominance of expression in the nucleus accumbens, olfactory tubercle, and ventromedial caudate putamen in mouse brain. Distribution in the mouse was also prevalent in the amygdalostratial transition area and the interstitial nucleus of the posterior limb of the anterior commissure which represent the anatomical continuation of the ventral striatum into the amygdala (Pesini et al., 1998). These are primary targets of the mesolimbic and nigrostriatal dopamine system, suggesting a possible association of GIR with dopaminergic neurotransmission. While GIR mRNA is present in nucleus accumbens and striatum of the rat, the distribution is limited to scattered cells located throughout the rostrocaudal extent of these structures. Interestingly, the expression of GIR in human brain striatum is also observed in scattered neurons (Brezillon et al., 2001), similar to that seen in the rat. The differences in GIR expression levels and distribution may represent species-specific diversity in GIR function; however, an investigation of GIR receptor protein levels is essential to support this finding. The existence of a predominant splice variant form of GIR in striatal regions of the mouse brain but not rat and human brain is also possible. In fact, splice variant forms of GIR have been reported in the mouse (De Moerlooze et al., 2000; Kawasawa et al., 2003), out of which splice variants RP82 and RP105 are unique to mouse (De Moerlooze et al., 2000). Whether these unique splice variants of GIR exhibit predominant expression in mouse striatum is speculative at present.

Variability in GIR expression between murine and rat brain is also observed in the thalamic nuclei. High level of GIR expression was observed in rat brain thalamic nuclei such as the nucleus reunions, ventral posteromedial and anterior thalamic nuclei. This is in agreement with human brain, where highest expression levels were reported in the nucleus reunions and the parafascicular thalamic nuclei (Brezillon et al., 2001). GIR may play a role in limbic integration and processing of sensory inputs to the cortex via thalamic subfields fields in the rat as well as in humans. However, thalamic regions of mouse brain did not express significant levels of GIR (Pesini et al., 1998).

Overall, the data suggest that expression of GIR mRNA in human more closely approaches that seen in rat than mouse. Notable parallels in expression patterns between human and rat are seen in the hypothalamus, medial thalamic nuclei and hippocampus, regions critical for limbic processing. Thus, the rat may be a more useful model with which to explore the role of GIR in control of CNS functions relevant to limbic-hypothalamic function, including memory and homeostatic regulation.

Functional implications of GIR localization

It is notable that GIR expression is robust in several regions relevant to control of ingestive behavior. A potential role of GIR in regulation of feeding behavior and sensory control of appetite is suggested by its presence in key nodes involved in ingestion (Norgren, 1995; Schwartz et al., 2000). For example, neurons of the nucleus of the solitary tract and principal sensory trigeminal nucleus are positioned to receive taste and tactile sensation from the oral cavity (Norgren, 1995; Waite and Tracey, 1995). Recent anatomical studies of the nucleus of the lateral olfactory tract suggest that this region receives extensive input from regions involved in higher-order processing of olfactory, gustatory and interoceptive information, including the basolateral amygdaloid complex, limbic cortices and olfactory cortices, and projects to regions involved in food reward (nucleus accumbens) and food-reward associations (agranular insular cortex, possibly basolateral amygdala; Santiago and Shammah-Lagnado, 2004). The medial division of the ventral posteromedial thalamus is also related to relay of sensory gustatory stimuli to cortical structures (Norgren, 1995). The ventromedial and arcuate nuclei are heavily implicated in modulation of food intake (Schwartz et al., 2000), suggesting GIR may be involved in motoric aspects of feeding as well. Indeed, expression of GIR in the motor trigeminal, facial and hypoglossal nuclei suggests involvement in jaw, facial and tongue movements associated with the process of ingestion.

Glucocorticoid induction of the GIR in the periphery suggests a role in central processing of glucocorticoid information. The link between GIR and hypothalamo–pituitary–adrenocortical function is supported somewhat by the expression of GIR mRNA in HPA regulatory sites, including the ventral subiculum, infralimbic/prelimbic cortices, medial/central amygdaloid nuclei and nucleus of the solitary tract (Herman et al., 2003). However, expression of GIR is not particularly pronounced in these regions. Notably, GIR mRNA is not expressed in the PVN or in basal forebrain or hypothalamic regions projecting to the PVN, with the possible exception of the arcuate nucleus and lateral hypothalamus.

Previous work from our group suggests involvement of GIR in drug reinforcement (Wang et al., 2001). This supposition is supported by current anatomical data showing GIR mRNA expression in key limbic reward pathways, such as the medial prefrontal cortex and to a lesser extent, the nucleus accumbens and basolateral amygdala. Indeed, GIR mRNA is up-regulated during behavioral sensitization to amphetamine in the prefrontal cortex, suggesting a correlation between GIR signaling and drug reward value.

Finally, GIR mRNA is expressed in several limbic structures implicated in learning and memory. Expression of GIR mRNA in the prefrontal cortex and hippocampus suggests involvement of this G-protein-coupled receptor in learning and memory. In rat, this involvement may be related to inhibition, as the scatter of cells in both regions is suggestive of localization with GABAergic interneurons (Mugnaini and Oertel, 1985; Woodson et al., 1989). In addition, GIR mRNA is also richly expressed in downstream targets of the hippocampus (lateral septum and medial mammillary nucleus) and prefrontal cortex (midline thalamic nuclei, including the nucleus reunions).

GIR signaling in brain: relationship to the NPY receptor family

GIR bears close resemblance to the NPY-Y2 receptor sub-type (38% homology). Comparison of GIR and NPY-Y2 receptor primary structure indicates the presence of amino acid residues in GIR that are unique to Y2 receptors, such as the DRH sequence after transmembrane domain 3 (Weinberg et al., 1996) as well as specific residues involved in Y2-ligand binding (Berglund et al., 2002). Notably, NPY-Y2 specific analogs bind GIR expressed *in vitro* (Sah et al., 2002, 2003), although the pharmacological profiles and rank order potencies were distinct from previously characterized NPY-Y2 receptors (Rose et al., 1995; Weinberg et al., 1996). Thus,

possible ligand cross-reactivity and functional interaction between GIR and the NPY family may exist. NPY-Y2 receptors play an important role in regulation of excitatory neurotransmission, feeding, and anxiety behavior (Batterham et al., 2002; Kask et al., 2002; Weiser et al., 2001). Y2 receptors are mostly localized in terminals and have been implicated in the modulation of synaptic transmission by inhibiting neurotransmitter release (Qian et al., 1997; King et al., 1999). Interestingly, GIR is expressed in brain regions previously shown to be enriched in NPY-Y2 receptors, particularly in the hippocampal CA3 region, medial pre-optic nucleus, ventromedial, and arcuate nuclei of the hypothalamus, lateral septum, bed nucleus of stria terminalis, amygdalar nuclei and the piriform cortex (Gustafson et al., 1997). However, co-localization studies of GIR and Y2 receptors are necessary to determine physiological interaction between these two receptors.

The endogenous ligand to GIR remains to be identified at present. Behavioral and transgenic animal models are therefore important in understanding the physiological functions of GIR. The current study outlines a complete mapping of GIR expression in the rat brain and reports certain areas where interesting differences in GIR mRNA distribution were noted between species, especially mouse. Whether these differences in mRNA levels will reflect changes in receptor protein expression and function will need to be investigated. Nevertheless, this information provides a guidance to investigators interested in studying GIR physiology and pharmacology. In particular, it is clear that knock-out models in mice and stress behavior models in rats will require proper development, drug administration and careful extrapolation of data between species. Our results will be useful for development and interpretation of appropriate models for understanding the physiology and pharmacological potentials of GIR.

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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.

Abbreviations used in the figures

ACC, nucleus accumbens
 ACg, anterior cingulate cortex
 AD, anterodorsal thalamic nucleus
 AId, agranular insular cortex, dorsal region
 AM, anteromedial thalamic nucleus
 AMv, anteromedial thalamic nucleus, ventral
 ARC, arcuate hypothalamic nucleus
 BLA, basolateral amygdaloid nucleus, anterior
 BSTad,dl, bed nucleus of stria terminalis, anterodorsal and dorsolateral divisions
 BSTif,tr, bed nucleus of stria terminalis, intrafascicular and transverse divisions
 BSTlv, bed nucleus of stria terminalis, lateral division, ventral
 BSTpr, bed nucleus of stria terminalis, principle nucleus
 CA1, field CA1 of hippocampus

CA3, field CA3 of hippocampus
 CoA, anterior cortical amygdaloid nucleus
 CP, caudate putamen
 CSI, superior central raphe nucleus, lateral part
 DG, dentate gyrus
 DMX, dorsal motor nucleus of the vagus nerve
 DRN, dorsal raphe nucleus
 ENT, entorhinal cortex
 IC, inferior colliculus
 IL, infralimbic cortex
 LA, lateral amygdaloid nucleus
 LC, locus coeruleus
 LHb, lateral habenula
 LHA, lateral hypothalamic area
 LM, lateral mammillary nucleus
 LSc, lateral septal nucleus, caudal division
 LSi, lateral septal nucleus, intermediate division
 MD, mediodorsal thalamic nucleus
 MeApd, medial amygdaloid nucleus, posterodorsal division
 MGN, medial geniculate nucleus
 MM, medial mammillary nucleus
 MMme, medial mammillary nucleus, median part
 MoV, motor trigeminal nucleus
 MPN, medial preoptic nucleus
 MPOA, medial preoptic area
 NLOT, nucleus of olfactory tract
 NTS, nucleus of the solitary tract
 NTSm, solitary tract nucleus, medial region
 OT, olfactory tubercle
 PA, posterior amygdaloid nucleus
 PAGdl, periaqueductal gray, dorsolateral area
 PAGvl, periaqueductal gray, ventrolateral area
 PeV, periventricular hypothalamic nucleus
 PIR, piriform cortex
 PL, prelimbic cortex
 PMv, ventral premammillary nucleus
 PO, posterior thalamic nucleus
 Re, nucleus reunions
 ReL, nucleus reunions, lateral subdivision
 Rh, rhomboid thalamic nucleus
 RT, reticular thalamic nucleus
 SF, septofimbrial nucleus
 SHi, septohippocampal nucleus
 SMT, submammillothalamic nucleus
 SNc, substantia nigra pars compacta
 SPV, spinal trigeminal nucleus
 SSp, primary somatosensory cortex
 SUBd, dorsal subiculum
 SUBv, ventral subiculum
 TTd, tenia tecta, dorsal
 VII, facial nucleus
 vlBST, ventrolateral division of the bed nucleus of the stria terminalis
 VM, ventromedial thalamic nucleus

VMH, ventromedial hypothalamic nucleus
 VPL, ventral posterolateral thalamic nucleus
 VPLp, ventral postero thalamic nucleus, parvicellular
 XII, hypoglossal nucleus

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Fig. 1.

Distribution profile of the GIR mRNA in rat brain by RT-PCR. Total RNA from various brain regions was reverse transcribed and PCR was performed using primers specific for GIR and GFAP as control. The expected sizes of bands were 293 and 126 bp, respectively. Sample lanes are labeled as follows: 2, hippocampus; 3, striatum; 4, amygdala; 5, hypothalamus; 6, prefrontal cortex; 7, cerebellum; 8, cerebral cortex; 9, GIR-transfected Chinese hamster ovary cells; 10, transformed amygdalar cell line (AR-5); 11, olfactory bulb; 12, pons-medulla; 13, spinal cord. Lane 1 shows 1 kb DNA ladder.

Fig. 2.

Film autoradiograms of coronal sections through rat brain progressing A–F from rostral to caudal regions, hybridized with ^{35}S -labeled antisense GIR probe (308–1575 bp). (A) GIR mRNA is expressed in all region of the prefrontal cortex (ACg, PL and IL) cortices and throughout the neocortex (SSp indicated here). Rich expression is also evident in OT and PIR. (B) At slightly more caudal levels, GIR mRNA is present in the caudal divisions of the LSc, the SHi, the vIBST and the MPN. Note punctate distribution of GIR mRNA throughout the CP at this level. (C) GIR mRNA was richly expressed in the anterior diencephalons, thalamic AD, AMv, RE and the periventricular zone of the hypothalamus. Notably, intense hybridization signal was present in the NLOT. (D) GIR mRNA is present in more caudal sections through the RE, as well as in the LHb. (E) GIR mRNA signal was present throughout the VPL, with the most intense labeling concentrated in its VPLp. GIR mRNA could also be observed throughout the CA1 and CA3 cell fields of the hippocampus as well as in the DG. (F) Intense GIR signal was observed in the MM. Lower levels of expression were observed in the LM. GIR mRNA expression was also observed in the dorsal and SUBv cortices.

Fig. 3. Distribution of GIR mRNA in the NLOT (A), LSc (B) and BLA (C). Note the intense hybridization signal throughout the NLOT. Expression of GIR mRNA was evident throughout the LSc as well as in the SHi. Expression in the BLA was evident as dense labeling in scattered neurons. Scale bar=200 μ m.

Fig. 4. Densely hybridized GIR mRNA-positive neurons were scattered throughout the CP (A) and ACC. In the CP (B) and ACC core (C), hybridization-positive cells possessed large, Nissl-poor nuclei that are consistent with striatal neurons. In the accumbens shell, GIR positive cells were more frequent, but less intensely labeled. In this region, grains were observed overlying smaller, more darkly stained nuclei. Scale bar=200 μ m (A), 20 μ m (B–D).

Fig. 5.

In hippocampus, GIR mRNA was highly expressed in neurons scattered throughout Ammon's horn and the DG (A). Higher power micrographs indicate that grain intensities over CA1 pyramidal cells (B) and DG granule cells (C) are above background, suggesting the GIR mRNA may be present in these cell populations. The scattered GIR mRNA positive neurons in CA1-4 and DG differed in character from pyramidal or granule cells, showing large, pale nuclei (shown in D). Their position within the hippocampus is consistent with that of large interneurons. Scale bars=500 μ m (A), 200 μ m (B, C), 20 μ m (D).

Fig. 6. Distribution of GIR mRNA in thalamus nuclei at various coronal levels. Substantial populations of intensely labeled GIR neurons were observed in anterior thalamic regions (A, B), including the Re, AMv and the Rh. At the mid-thalamic level, labeling was observed in the VM and SMT, as well as Re (C). More caudally, GIR mRNA was expressed in the VPL, particularly in its VPLp. Scale bars=500 μ m (A), 200 μ m (B–D).

Fig. 7. Expression of GIR mRNA in hypothalamic subdivisions, including the MPO, MPN (A), LHA (B), PeV (C), VMH and ARC. Note that cellular labeling in the VMH and ARC is less intense than that in other hypothalamic regions. Scale bar=200 μ m (A–D).

Fig. 8. GIR expression in hindbrain regions. GIR mRNA is richly expressed in cranial nerve nuclei, including the facial nucleus (A), the MoV (B), the SPV, the NTS and the DMX. Neurons in the LC were also GIR mRNA positive (B). Scale bar=200 μ m (A–D).

Table 1Distribution of GIR mRNA in the rat brain^a

Telencephalon		Diencephalon	
Cortex		Hypothalamus	
ILA	+ (2–3), ++ (5) ^b	MPN	+++
PL	+ (2–3), ++ (5)	MPOA	++
ACA	+ (2–3), ++ (5)	PeV	+++
Ald	+ (2–3), ++ (5)	LHA	++–+++
PIR	+++ (2)	VMH	++
ENT	+ (2–3), ++ (5)	ARC	++–++
Neocortical regions	+ (2–3, 5)	PMV	+++
Basal ganglia		MMme	+++
CP	++ (sc)	MM	+++
ACBcore	++ (sc)	LH	++ (sc)
ACBshell	+	Thalamus	
Basal forebrain nuclei		AM	+++
TTd	+++	AMv	+++
ISI	+++	AD	++
SHi	+++	RT	++–++
LSc	++	REI	++++
SF	+++	Rh	+++
NLOT	+++	SMT	++
BSTpr	++	VM	++
BSTif,tr	+	MD	++ (sc)
BSTad,dl	++–++	VPL	++
Amygdala		VPLp	+++
BLA	+++ (sc)	PO	+
MeApd	+	MGN	+++ (sc)
CoA	++	Midbrain	
LA	+	IC	++ (sc)
PA	++	PAGvl	++
Hippocampus		PAGdl	++
CA1–2	+ (pyr), +++ (sc)	DRN	+
CA3–4	+ (pyr), +++ (sc)	RN	+
DG	+ (gran), +++ (sc)	CSI	+++
SUBv	+++ (sc)	Hindbrain	
SUBd	+++ (sc)	LC	++–++
		MoV	++ (sc)
		SPV	+++ (sc)
		VII	+++
		XII	+++
		NTSm	++
		Cerebellum	
		Purkinje cells	+

pyr, pyramidal cells; gran, granule cells; sc, cells scattered in granule cell layers and surrounding white matter. For other abbreviations, please see the abbreviations used in the figures.

^a Relative densities have been rated: + = low level expression (10–20 grains/cell); ++ = intermediate expression (20–40 grains/cell); +++ = high level expression (>40 grains/cell).

^b Numbers in parentheses indicate localization in cortical layers.