The neuropeptide galanin has been implicated in the regulation of processes such as nociception, cognition, feeding behavior, and hormone secretion. Multiple galanin receptors are predicted to mediate its effects, but only two functionally coupled receptors have been reported. We now report the cloning of a third galanin receptor distinct from GALR1 and GALR2. The receptor, termed GALR3, was isolated from a rat hypothalamus cDNA library by both expression and homology cloning approaches. The rat GALR3 receptor cDNA can encode a protein of 370 amino acids with 35% and 52% identity to GALR1 and GALR2, respectively. Localization of mRNA by solution hybridization/RNase protection demonstrates that the GALR3 transcript is widely distributed, but expressed at low abundance, with the highest levels in the hypothalamus and pituitary. We also isolated the gene encoding the human homologue of GALR3. The human GALR3 receptor is 90% identical to rat GALR3 and contains 368 amino acids. Binding of porcine ∼125I-galanin to stably expressed rat and human GALR3 receptors is saturable (rat $K_D = 0.08 \text{ nm}$ and human $K_D = 2.23 \text{ nm}$) and displaceable by galanin peptides and analogues in the following rank order: rat galanin, porcine galanin = M32, M35 = porcine galanin-(7 to +29), galantide, human galanin > M40, galanin-(1–16) > [d-Trp2]galanin-(1–29), galanin-(3–29). This profile resembles that of the rat GALR1 and GALR2 receptors with the notable exception that human galanin, galanin-(1–16), and M40 show lower affinity at GALR3. In Xenopus oocytes, activation of rat and human GALR3 receptors co-expressed with potassium channel subunits GIRK1 and GIRK4 resulted in inward $K^+$ currents characteristic of $G_i/G_o$-coupled receptors. These data confirm the functional efficacy of GALR3 receptors and further suggest that GALR3 signaling pathways resemble those of GALR1 in that both can activate potassium channels linked to the regulation of neurotransmitter release.

The neuropeptide galanin modulates a variety of physiological processes including cognition/memory (1), sensory/pain processing (2, 3), hormone secretion (4, 5), and feeding behavior (6, 7) (for review, see Ref. 8). The endogenous and exogenous effects of galanin are mediated by multiple receptor subtypes.

To date, two cloned galanin receptors, GALR1 and GALR2, have been shown to functionally couple to their intracellular effectors through distinct signaling pathways: GALR1 inhibits adenylate cyclase via a pertussis toxin-sensitive G-protein of the $G_i/G_o$ family (9–11), whereas GALR2 stimulates inositol phospholipid turnover and intracellular calcium mobilization through a pertussis toxin-insensitive $G_i/G_{11}$-type mechanism (11, 12). The pharmacological profiles of GALR1 and GALR2 are similar to one another in that both show a preference for the N-terminal portion of the galanin peptide, and chimeric “antagonist” peptides such as M35 and M40 act as full agonists at both cloned receptors (11, 13). These properties of GALR1 and GALR2 do not fully explain aspects of galanin pharmacology such as the reported agonist activity of galanin-(3–29) in the pituitary (14), guinea pig stomach (15, 16), and hypothalamus (17) or the antagonist properties of the chimeric galanin analogues M15, M35, and M40 in physiological models of galanin-mediated luteinizing hormone-releasing hormone release (18), facilitation of the spinal flexor reflex (19), and feeding (7, 20), respectively (for review, see Ref. 21). Together, these data suggested that additional galanin receptor subtypes beyond GALR1 and GALR2 existed. Recently, the cloning of a third galanin receptor cDNA was described by Wang et al. (22), but the pharmacological characterization of this receptor was limited by an extremely low apparent $B_{max}$ in porcine ∼125I-galanin binding assays, and no functional coupling was reported.

We now report the cloning and functional characterization of a third galanin receptor subtype, termed GALR3, from the rat hypothalamus based on a combination of expression and homology cloning methods. We have also isolated and characterized the functional human homologue of GALR3. Both human and rat GALR3 receptors exhibit pharmacological profiles distinct from GALR2 and GALR1. Moreover, we have shown that galanin acting at GALR3 receptors can activate GIRK currents in Xenopus oocytes, demonstrating that GALR3 can couple to a G-protein of the $G_i/G_o$ class. The cloning and characterization of rat and human GALR3 receptors will facilitate the association of functional roles with specific galanin receptor subtypes and will help to circumscribe the properties of any remaining uncloned galanin receptors.

**EXPERIMENTAL PROCEDURES**

Isolation of a Novel Rat Galanin Receptor: Convergence of Homology and Expression Cloning—A rat hypothalamus cDNA library in AZAP II (Stratagene, La Jolla, CA) was screened using overlapping oligonucleotide probes representing the transmembrane domains of the rat GALR2 receptor cDNA (11). The oligomers were labeled, and hybridizations were performed at reduced stringency as described (23). The cDNA
insert from one hybridizing clone, rHy35a, was sequenced on both strands by cycle sequencing with AmpliTaq DNA polymerase FS (Perkin-Elmer), and products were run on an automated fluorescent sequencer (Applied Biosystems, Inc.). Sequence analysis (GCC Version 9.1, Genetics Computer Group, Inc., Madison, WI) indicated that rHy35a encoded a fragment of a galanin receptor.

A rat hypothalamic plasmid cDNA library consisting of 1.2 × 10⁶ primary clones (average insert size of ~3.2 kilobase pairs) was constructed, processed, and screened for porcine 125I-galanin binding/photomulsion detection as described (11). Groups of primary hypothalamic cDNA library pools (10 pools of ~3200 colony-forming units/pool) were screened by polymerase chain reaction using primers specific for rHy35a; primary pools (20) from two positive superpools were inspected for galanin binding using the photomulsion binding assay (11) and screened by polymerase chain reaction for rHy35a. The slide corresponding to pool K163 exhibited positive galanin binding and also contained the rHy35a sequence. The cDNA encoding the new galanin receptor was isolated by sib selection (24) and sequenced on both strands using AmpliTaq DNA polymerase FS.

Cloning and Sequencing a Novel Human Galanin Receptor Fragment—A human placenta genomic library in ADASH II (~1.5 × 10⁹ total recombinants; Stratagene) was screened at reduced stringency using GALR2 transmembrane domain probes as described above. One positive phage clone, plc21a, contained a 2.7-kilobase pair KpnI/EcoRI fragment that hybridized with the rat GALR2 transmembrane domain II probe. Subcloning of the sequence analogously indicated that the fragment encoded the first three transmembrane domains of the human GALR3 receptor as well as a large intron. To obtain additional exons encoding the receptor, the original phage clone plc21a (~18-kilobase pair insert) was hybridized with probes directed to the third extracellular loop or transmembrane IV regions of the rat GALR3 cDNA. The full-length human GALR3 construct was prepared by ligating a polymerase chain reaction-derived product of the 5′-exon, representing the starting Met through the III/IV loop, with a genomic fragment containing the 3′-exon.

Cell Culture and Expression—The calcium phosphate technique (25) was used to co-transfect receptor cDNA plus a G418-resistant plasmid into mouse fibroblast LMTK cells for human GALR3 studies or into human embryonic kidney 293 cells for rat GALR3 studies. Stable clones were selected with G418 and screened as membrane preparations for specific porcine 125I-galanin binding. By the same protocol, rat GALR1 receptors were stably expressed in Chinese hamster ovary cells, and rat GALR2 receptors in LMTK cells. Supplements (10% bovine calf serum, 4 mM l-glutamine, and 100 units/ml penicillin and 100 μg/ml streptomycin) were added to Dulbecco’s modified Eagle’s medium for the culture of mouse fibroblast LMTK and human embryonic kidney 293 cells or to Ham’s F-12 medium for Chinese hamster ovary cells. Cells were grown on 150-mm plates at 37 °C and 5% CO₂. Stock plates were split with trypsin every 3–4 days at a ratio of 1:6 for 293 cells, 1:8 for Chinese hamster ovary cells, or 1:10 for LMTK cells.

Radioligand Binding Assay—Membranes were prepared from transfected cells as described previously (11). Membranes, peptides, and porcine 125I-galanin (2200 Ci/mmol; NEN Life Science Products) were diluted in galanin binding buffer supplemented with 0.1% bovine serum albumin and 0.1% bacitracin and then distributed into 96-well Millipore GF/C filter plates (pretreated for 30 min with 1% polyethyleneimine in 50 mM Tris, pH 7.4) so that the distributed into 96-well Millipore GF/C filter plates (pretreated for 30 min). Solution hybridization assays were performed as described previously (30). Autoradiographs of assays were generated by exposing dried gels to PhosphorImager screens (Molecular Dynamics, Inc.) or Eastman Kodak BioMax film at ~70 °C.

Materials—Cell culture media and supplements were from Specialty Media (Lavallette, NJ). Cell culture plates (150-mm and 96-well microtiter plates; Corning Inc., NY). Ex-Cell 400 medium containing 1 g/l d-glucose and 1 g/l aminoadenine was purchased from JRH Biosciences. Polypropylene 96-well microtiter plates were from Costar Corp. (Cambridge, MA). All radioligands were from NEN Life Science Products. Galanin and related peptide analogues were from either Bachem California (Torrance, CA) or Peninsula Laboratories, Inc. (Belmont, CA) or were synthesized by custom order from Chiron Mimotopes Peptide Systems (Clayton, Victoria, Australia). Bio-Rad reagent was from Bio-Rad. Bovine serum albumin (ultra fat-free, A-7511) was from Sigma. All other materials were reagent-grade.

RESULTS AND DISCUSSION

Cloning of Rat and Human GALR3 Receptors—Recent evidence indicates that GALR1 and GALR2 receptor mRNAs were present in the rat hypothalamus (10, 11, 31), but not all aspects of the cloned GALR1 and GALR2 pharmacological profiles matched those observed for galanin-mediated feeding (7, 20) as well as other physiological effects of galanin analogues (14, 21, 32). Based on the potential presence of multiple galanin receptors in the hypothalamus, we attempted to isolate additional galanin receptors using both homology and expression cloning strategies. The convergence of these strategies resulted in the isolation of a partial galanin receptor-like cDNA fragment (rHy35a) by reduced stringency homology cloning and the discovery of the same fragment in a galanin-binding library pool (K163, ~3200 primary clones) identified by expression cloning. Isolation of the full-length cDNA from pool K163 of the rat hypothalamus expression library and subsequent transfection/radioligand binding confirmed that the cDNA encoded a novel rat galanin receptor, termed GALR3. The human homologue of GALR3 was subsequently isolated from a human placenta genomic library and subcloned for sequencing and expression studies.

The rat GALR3 cDNA can encode a protein of 370 amino acids with 35% amino acid identity to rat GALR1 (10) and 52% identity to rat GALR2 (11), the receptor to which it is most closely related. The sequence similarity to GALR2 is higher within transmembrane domains II–IV, where amino acid identities range from 70 to >90%. The human GALR3 receptor comprises 368 amino acids with 90% identity to rat GALR3 (Fig. 1); compared with human galanin receptor subtypes, GALR3 is 37 and 57% identical to human GALR1 (9) and human GALR2 (33), respectively. In accord with its high level of sequence identity to GALR2, the human GALR3 gene contains an intron within the coding region at the same location as reported for rat GALR2 (11, 34). Notably, the amino acid sequences for the rat and human GALR3 receptors shown here (Fig. 1) differ from previous preliminary reports: the sequence of human GALR3 differs by 6 amino acids from that of an unannotated receptor-like sequence in GenBank™ (EM_HTG: HS8112), and the rat GALR3 receptor differs by 4 amino acids from another galanin receptor also described as GALR3 by Wang et al. (22). Both this GALR3 receptor cDNA sequence contains a single consensus site for N-linked glycosylation in the N terminus (see Fig. 1) and several predicted intracellular domains for phosphorylation by protein kinases; one putative phosphorylation site common to rat and human GALR3 in the third intracellular loop is absent in GALR1 and GALR2.

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Galanin GALR3 Receptors Activate K⁺ Channels

To assess the distribution of GALR3 mRNA in the rat, we carried out solution hybridization/RNase protection assays on poly(A)⁺ RNA isolated from a variety of tissues and brain regions (Fig. 2). GALR3 transcripts were broadly distributed, but present at low abundance within the rat central nervous system and many peripheral tissues. Among all tissues examined, the highest levels of GALR3 mRNA were found in the rat hypothalamus and pituitary gland. Within the central nervous system, lower levels were observed in the olfactory bulb, cerebral cortex, medulla oblongata, hippocampal formation, and spinal cord; no GALR3 mRNA was detected in the hippocampus or substantia nigra. In peripheral tissues, areas containing low levels of GALR3 included the liver, kidney, stomach, testicle, and adrenal cortex. Additionally, GALR3 mRNA was found in the lung, adrenal medulla, spleen, and pancreas (data not shown). GALR3 transcripts were not detected in RNA extracted from the heart, uterus, vas deferens, choroid plexus, or dorsal root ganglion. This localization pattern suggests that GALR3 may contribute more to galanin-mediated physiology in the rat hypothalamus and pituitary than in other areas. However, the up-regulation of galanin peptide expression in a variety of pathophysiological states (2, 35–37) leaves open the possibility that GALR3 receptor expression could be similarly plastic.

Pharmacological Characterization of Rat and Human GALR3 Receptors—Radioligand binding assays were conducted to characterize the cloned GALR3 receptor homologues. Porcine ¹²⁵I-galanin bound in specific and saturable fashion to membranes from cells stably transfected with cloned galanin receptors (CHO, Chinese hamster ovary) and the rat GALR1 receptor (CHO). Data generated from experiments in which porcine ¹²⁵I-galanin concentrations ranged from 0.5 pm to 3.0 nM were best fit to a one-site model. Apparent Kd and Bmax values are listed in Table I. For comparison, rat GALR1 and GALR2 receptors were studied under similar conditions. Data in Table I show that both the observed binding affinity of porcine ¹²⁵I-galanin and the measurable Bmax values are lowest for the GALR3 receptors.

The rat GALR3 receptor bond galanin and related peptide analogues in porcine ¹²⁵I-galanin binding assays with a distinctive rank order of binding affinity (Table II). Rat galanin, porcine galanin, porcine galanin-(1–16) > porcine galanin-(3–29). The chimeric peptides bound to rat GALR3 with a distinctive rank order: M32, C7, M35, galantide > M40. Comparison with the other cloned rat galanin receptor subtypes indicates that apart from quantitative differences, rat GALR3 shares certain features in common with rat GALR1 despite the high degree of sequence divergence. For example, both receptor subtypes display the

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**TABLE I**

**Saturation binding studies with porcine ¹²⁵I-galanin.** Membranes from cells stably transfected with cloned galanin receptors were analyzed in filtration binding assays as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell</th>
<th>Apparent pKd ± S.E.(Kd)</th>
<th>Apparent Bmax ± S.E. (fmol/mg)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat GALR1</td>
<td>CHO</td>
<td>9.84 ± 0.08 (0.145 nM)</td>
<td>21,400 ± 4500</td>
<td>5</td>
</tr>
<tr>
<td>Rat GALR2</td>
<td>LMTK-</td>
<td>9.53 ± 0.07 (0.293 nM)</td>
<td>5800 ± 490</td>
<td>3</td>
</tr>
<tr>
<td>Human GALR3</td>
<td>293</td>
<td>9.01 ± 0.08 (0.981 nM)</td>
<td>460 ± 30</td>
<td>6</td>
</tr>
<tr>
<td>Human GALR3</td>
<td>293</td>
<td>8.65 ± 0.05 (2.23 nM)</td>
<td>1530 ± 170</td>
<td>3</td>
</tr>
</tbody>
</table>

a CHO, Chinese hamster ovary.

---

**FIG. 1.** Amino acid sequence comparison of human and rat galanin GALR3 receptors. Shown is the amino acid sequence alignment of the human GALR3 receptor (top row) and the rat GALR3 receptor (bottom row). Putative transmembrane domains are indicated by brackets. Identical residues are shaded; consensus sites for N-linked glycosylation are indicated (¶).

**FIG. 2.** Localization of rat GALR3 mRNA by solution hybridization/RNase protection assay. Shown is an autoradiograph demonstrating protection of a radiolabeled rat GALR3 RNA probe by a single band (arrow) from various rat tissues in a solution hybridization/nuclease protection assay. The single band (arrow) represents levels of rat GALR3 receptor mRNA in the tissues indicated: sc, spinal cord; ad ctx, adrenal cortex; cblm, cerebral cortex; choroid, choroid plexus; ctx, cerebral cortex; drg, dorsal root ganglia; hif, hippocampal formation; medulla, medulla oblongata; olf bulb, olfactory bulb; sn, substantia nigra; pit, pituitary, duod, duodenum; vas def, vas deferens; trna, transfer RNA.
lowest affinity for M40 out of all the chimeric peptides studied, and both receptors are sensitive to galanin C-terminal truncation, with lower affinity for porcine galanin-(1–16) versus porcine galanin. Also, GALR3 and GALR1 receptors are sensitive to N-terminal modification in having decreased affinity for [n-Trp]galanin versus galanin and no detectable binding affinity for porcine galanin-(3–29), the latter being a common feature of all three cloned galanin receptor subtypes. In accord with its high level of amino acid identity to GALR2, GALR3 maintains moderate affinity for galanin-(2–29) (22). In sharing a general preference for the conserved N-terminal portion of the galanin peptide, all three cloned receptors are thus distinct from the galanin receptor subtypes activated by C-terminal fragments of galanin (14, 16, 17, 38) as well as galanin-(1–15)-preferring subtypes in the dorsal hippocampus and brain stem (39, 40).

The human GALR3 receptor bound galanin and related peptide analogues with a rank order resembling that derived in rat GALR3 binding studies (Table II). A noteworthy feature of both human and rat GALR3 receptor profiles is the lower affinity for human galanin versus rat and porcine galanin, in contrast to the profiles of GALR1 and GALR2. This is interesting in the case of human GALR3 and suggests the possibility of an additional galanin-like peptide in humans. Although the evidence for such a peptide is lacking, recent data suggest the existence of a novel galanin-like peptide in rat pancreatic islets (41). One obvious candidate would be the galanin message-associated peptide (GMAP) or a GMAP fragment; however, human GALR3 did not bind fragments of GMAP, including GMAP-(1–41), GMAP-(16–41), GMAP-(25–41), and GMAP-(44–59) (data not shown).

### Functional Coupling of the GALR3 Receptor—Clues about GALR3 Function

Galanin GALR3 receptors activate K+ channels. Xenopus oocytes expressing GIRK channels have been widely used to demonstrate functional activation of receptors coupled to the Gα16 class of heterotrimeric G-proteins (27–29). Under voltage clamp conditions, oocytes injected with mRNAs for human GALR3, GIRK1, and GIRK4 responded to 1 μM porcine galanin with inward currents of 154 ± 20 pA (n = 25) (Fig. 3). Oocytes injected with GIRK mRNAs produced only little or no inward current (5.5 ± 2.5 pA, n = 12) in response to 1 μM galanin; therefore, the responses in oocytes resulted from the heterologous expression of galanin receptors. The rat GALR3 receptor also supports a galanin-dependent activation of GIRK currents in oocytes; average currents were 34 ± 6 pA (n = 6) in the presence of 1 μM porcine galanin (data not shown). Evidence that galanin-induced currents were mediated by GIRK channels (Fig. 3) included the following: 1) dependence on elevated external K+, 2) strong inward rectification of the current-voltage relationship, 3) reversal potential (~24 mV) close to the predicted equilibrium potential for K+ (~23 mV in high K+ medium), 4) sensitivity to blockade by 300 μM Ba2+, and 5) lack of galanin sensitivity in oocytes injected with only GALR3 mRNA (data not shown). Injection of pertussis toxin (2 ng/oocyte) caused a 98% reduction (n = 10) of galanin currents in oocytes expressing GALR3, supporting the conclusion that, in oocytes, GALR3 receptors couple to a pertussis toxin-sensitive Gαi5/Gβγ pathway. This functionality is similar to GALR1 receptors, which also activate GIRK channels (Fig. 4C) in a pertussis toxin-sensitive manner (data not shown), but is different from GALR2 receptors, which, in oocytes, activate inward Cl− currents characteristic of Gαq-coupled receptors (11). Thus, the cloned GALR3 receptor appears to use a G-protein of the Gαi5/Gβγ class to modulate a channel (GIRK) associated with hyperpolarization and inhibition of neurotransmitter release.

A series of galanin and galanin-related peptides was tested for agonist activity at GALR3 receptors expressed in Xenopus oocytes. Of these peptides, porcine galanin, human galanin, M32, C7, M35, M40 (galantide), galanin-(7 to +29), galanin-(1–16), and M40 evoked agonist activity at a fixed dose of 1 μM; [n-Trp]galanin and galanin-(3–29) were inactive. These last two peptides were also tested for their ability to block galanin-evoked currents, but no antagonism was detected. EC50 values obtained for selected peptides from cumulative concentration-response measurements (Fig. 4B) were 56 nM for M32, 206 nM for porcine galanin, 343 nM for C7, 1907 nM for human galanin, 1678 nM for galanin-(7 to +29), 3270 nM for galanin-(1–16), and 3999 nM for M40. The rank order of potency was similar to that observed for displacement of 125I-galanin in binding assays using LMTK− cells stably expressing human GALR3 (Table II). The apparent overall low potency of galanin and related peptides in oocytes did not seem to be related to a low efficiency.
of receptor coupling inherent to oocytes since galanin exhibited an EC_{50} of 2 nM in oocytes expressing GALR1 (Fig. 4C). Instead, the low potency may reflect the somewhat lower affinity of galanin peptides for GALR3 (Tables I and II). Overall, the electrophysiological data reveal a functional similarity between GALR1 and GALR3 in oocytes despite a low level of primary sequence identity; exactly which G-proteins are involved in the oocyte or would be involved in mammalian cells remain to be determined for each receptor subtype. We are now in the process of extrapolating these findings from the oocyte to the study of GALR3 receptors transplanted into mammalian cells.

The localization, functional coupling, and pharmacology of GALR3 suggest a number of physiological roles for this receptor in the regulation of feeding, inhibition of neurotransmitter release (i.e. acetylcholine, serotonin, and norepinephrine), regulation of pituitary endocrine release, inhibition of glucose-stimulated insulin release, and regulation of spinal cord excitability (47). The involvement of GALR3 mRNA in spinal cord function is particularly intriguing. GALR3 shows high binding affinity (relative to galanin) for the alternately processed galanin-(7 to +29). This peptide and galanin-(9 to +29) are found in the adrenal gland (48) and modulate spinal excitability, albeit with weaker potency than full-length galanin (49). Furthermore, a galanin-dependent inward current appears in dorsal root ganglia only after axotomy, when galanin mRNA is up-regulated, but GALR1 and GALR2 mRNAs are decreased (50, 51). It would be interesting to determine whether the novel current is mediated by GALR3 or supports the existence of additional galanin receptor subtypes.

The ability of the chimeric peptides to bind GALR3 (M32, C7, M35, galantide > M40) and to activate GALR3 in oocytes prompts a re-evaluation of the actions of these peptides. The chimeras were classified as antagonists in tissues such as hypothalamus, hippocampus, pituitary, spinal cord, and pancreas (21, 52), but are agonists for the cloned GALR1, GALR2, and GALR3 receptors (Refs. 11 and 13 and this paper). The discrepancy may be explained by a system-dependent spectrum of partial agonist/antagonist activity, or the chimeric peptides may be degraded in vivo to fragments having different properties than the parent peptides. Alternatively, it may also indicate the existence of additional receptor subtypes. The pharmacology of cloned GALR1, GALR2, and GALR3 receptors cannot explain the binding of galanin-(3–29) to a receptor in the pituitary (14) or gastric smooth muscle (16), nor can it explain a galanin-(1–15)-preferring receptor in the hippocampus (39) or locus ceruleus (40). Continued cloning efforts, combined with the identification of stable subtype-selective ligands, should help define the contributions of GALR1, GALR2, and GALR3 to the broad picture of galanin-dependent physiology.

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Gerald and Kenneth A. Jones

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