Cloning and Characterization of a cDNA Encoding a Novel Subtype of Rat Thyrotropin-releasing Hormone Receptor*

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A cDNA encoding a thyrotropin-releasing hormone (TRH) receptor expressed in the pituitary was previously cloned (De La Pena, P., Delgado, L. M., Del Camino, D., and Barros, F. (1992) Biochem. J. 284, 891–899; De La Pena, P., Delgado, L. M., Del Camino, D., and Barros, F. (1992) J. Biol. Chem. 267, 25703–25708; Duthie, S. M., Taylor, P. L., Anderson, J., Cook, J., and Eidne, K. A. (1993) Mol. Cell Endocrinol. 95, R11–R15). We now describe the isolation of a rat cDNA encoding a novel subtype of TRH receptor (termed TRHR2) displaying an overall homology of 50% to the pituitary TRH receptor. Introduction of TRHR2 cDNA in HEK-293 cells resulted in expression of high affinity TRH binding with a different pharmacological profile than the pituitary TRH receptor. De novo expressed receptors were functional and resulted in stimulation of calcium transient as assessed by fluorometric imaging plate reader analysis. The message for TRHR2 was exclusive to central nervous system tissues as judged by Northern blot analysis. Studies of the expression of TRHR-2 message by in situ hybridization revealed a pattern of expression remarkably distinct (present in spinothalamic tract, spinal cord dorsal horn) from that of the pituitary TRH receptor (present in hypothalamus, and ventral horn of the spinal cord, anterior pituitary). Therefore, we have identified a novel, pharmacologically distinct receptor for thyrotropin-releasing hormone that appears to be more restricted to the central nervous system particularly to the sensory neurons of spinothalamic tract and spinal cord dorsal horn, which may account for the sensory antinociceptive actions of TRH.

The thyrotropin-releasing hormone (TRH) is a tripeptide (pyroglutamatic acid-histidine-proline-amide) synthesized from a precursor polypeptide whose sequence contains 5 copies of the TRH sequence (5–7).

Originally isolated from the hypothalamus, TRH is present in the central nervous system (thalamus, cerebral cortex, and spinal cord,) as well as in the periphery (pancreas, gastrointestinal tracts, and placenta). In the hypothalamus, TRH is synthesized by peptidergic neurons of supraoptic and paraventricular nuclei. It is then axonally transported to be stored in the median eminence. When secreted in the bloodstream, it reaches the pituitary where it stimulates the production of thyroid stimulating hormone which in turn stimulates the production of thyroxin (T₄) in the thyroid gland (8).

In addition to this pivotal role in controlling the synthesis and secretion of thyroid stimulating hormone and other hormones from the anterior pituitary, TRH has been implicated as a neurotransmitter (9). TRH abundantly exists in the central nervous system and exogenous administration of TRH elicits a variety of behavioral changes (see Ref. 10 for a review).

The distribution of TRH containing cells, fibers, or receptors suggests a potential role of TRH in the perception of nociceptive stimuli. TRH is present in the periaqueductal gray, the nuclei raphe magnus and pallidus, and the dorsal horn of the spinal cord. TRH-binding sites have been described in the brain, the pituitary, in both the dorsal and ventral horns of the spinal cord as well as in peripheral tissues. When injected centrally, TRH induces a short lasting supraspinal antinociception. The analgesia induced by intracerebroventricular TRH injection is powerful since it is twice as great, on a molar basis, as that of morphine (11). This TRH-induced antinociception is detected in models of chemically and mechanically, but not thermally induced pain. On the other hand, intrathecal TRH injections do not affect basal antinociceptive thresholds (11). However, it is known that TRH enhances spinal reflexes (both in vivo and in vitro) and modulates pain transmission (4, 12, 13). Although the mode of action of TRH at the level of the spinal cord is unclear, there is evidence suggesting that the TRH-induced facilitation of spinal transmission involves the activation of the N-methyl-D-aspartate receptor (14).

TRH actions are mediated by the stimulation of specific cell surface receptors. Studies of pituitary TRH receptors have suggested that the TRH₁ receptor triggers the phospholipase C-protein kinase C transduction pathway (3, 15). A cDNA sequence encoding a G-protein-coupled TRHR was originally isolated from mouse pituitary using an expression cloning strategy (15). Subsequently, several groups have described the cloning of rat TRH receptor cDNAs expressed in a pituitary tumor cell line (GH₃) or in the pituitary gland (1, 3). In addition, two isoforms of the rat TRHR have been shown to be generated from a single gene by alternative splicing (2). These isoforms are 387 and 412 amino acids in length and they differ in their C-terminal cytoplasmic tail. Functional expression in Xenopus laevis oocytes indicated that both cDNAs encode functional TRHRs with indistinguishable electrophysiological responses to TRH (16). Using an homology screening approach, a human TRHR cDNA was also cloned (17). The predicted amino acid sequence of the human TRHR showed high homology with the rat and mouse TRHRs with the exception of the C-terminal region (17). The level of sequence homologies between the putative protein sequences encoded by these cDNAs, excluding...
the C-terminal sequence, is more than 93%. It seems therefore that the currently available cloned TRHR receptor cDNA encode species variants of the same functional subtype of TRH receptor.

Pharmacological observations, however, suggest the existence of TRH receptor subtypes. A dissociation of endocrine from CNS effects was observed using synthetic TRH analogues. Indeed some TRH analogues containing the C-terminal thioamide group and norvaline or norleucine in position 2 neither bind to pituitary nor to brain receptors and generate no thyroid stimulating hormone release activity. Yet, both analogues affected sleeping time and breathing frequency (18). The differential effects evoked by TRH analogues with a modified COOH terminus, compared with TRH analogues that have an intact COOH terminus, further support the existence of TRH receptor subtypes (19). Comparatively, the TRH analogues in which the integrity of the COOH terminus is preserved (CG-3509, CG-3703, and YM-14673) have shown some efficacy in the treatment of CNS trauma. Whereas compounds with a modified COOH terminus have proven to be ineffective in treating traumatic spinal cord injury despite the fact that such analogues possess similar profile of activity with regard to their endocrine effects (19). Biochemical experiments also suggested the existence of TRH receptor subtypes. In particular, isoelectric focusing studies of receptors solubilized from the brain or the pituitary revealed respective pI values of 5.5 and 4.9. Although these differences may be caused by tissue differences in post-translational processing, one possible interpretation is that receptors in the pituitary and the brain differ in amino acid sequence (10). Interestingly, electrophysiological experiments and measurements of intracellular calcium concentration suggested that TRH and TRH metabolites present in the brain may act at different subtypes of TRH-binding sites (16). We describe here the isolation and characterization of a novel rat TRH receptor clone which we have designated TRHR2.

**EXPERIMENTAL PROCEDURES**

Cloning and Sequencing of a Rat Thyrotropin-releasing Hormone Receptor (rTRHR2)

The templates for PCR amplification were synthesized using GeneAmp RNA PCR kits (Perkin-Elmer) using 200 ng of spinal cord poly(A) RNA and were amplified using the following degenerate primers designed against several G protein-coupled receptors: TM3-4, 5'-GC- CAT/C or T/GA or G/C/G or T/A or G/TA/G/A or C/GA or G/TA-3'; TM4-5, 5'-AG or C/A or T/GCC/T or T/A or G/TA/G/A or C/GA or C/GGA or G/TT-3'. The reaction mixture contained 200 μmol of each of TM3-4 and TM7-4 primers and 2.5 units of Taq DNA polymerase in 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl, 200 μM dNTPs, pH 9.0. The reaction tubes were heated at 95 °C for 1 min and subjected to 39 cycles of denaturation (95 °C/1 min), annealing (42 °C/1 min), and extension (72 °C/1 min). The PCR amplification products of the reactions between 500 and 800 bp were subcloned into the pGEM-T vector from (Promega) and recombinant clones were sequenced using the T7 sequencing kit (Pharmacia). The clone pGEMT-1–75 was found to have certain degree of homology to the TRH receptor clone which we have designated TRHR-1, and was used to screen a rat brain stem-spinal cord cDNA library in AZAP II (Stratagene) in order to obtain full-length clone (hybridization: 2 × SSC, 5 × Denhardt’s solutions and 0.2% SDS, 65 °C, final wash: 5 mM EDTA, 0.2% SDS, pH 8.0, 65 °C). The TRH receptor cDNA was isolated and sequenced by the dideoxy sequencing method.

Binding of rTRHR2

The cloned cDNA was transiently expressed in the human kidney epithelial cell line HEK293. The cells expressing rTRHR2 receptor and GH4C1 neuronal cell line endogenously expressing the rat TRH-R1 receptor were fed 24 h prior to experimentation. On the day of the experiment, 10–12 million cells were centrifuged and gently resuspended in loading solution (loading solution for HEK293 cells: 11 ml of Dulbecco’s modified media 10% fetal bovine serum and 20% polyethylene glycol) and for GH4C1 cells: 11 ml of F-10 media 10% fetal bovine serum containing 42 ml of calcium green (8 ml final) and 42 ml of 20% polyvalent calcium and incubated at 37 °C for 1 h in a humidified chamber. Following the incubation step, cells were washed three times in 30 ml of Hank’s buffer (10 °C) and test compounds were added to the plates at a concentration of 100 μg/ml, 100 μg/ml, 10 μg/ml, 1 μg/ml, and 100 ng/ml. The plates were incubated for 90 min then vacuum-filtered through Packard GB/B Unifilter plates (preoaked for at least 2 h in 0.1% polyethyleneimine), washed three times with 1 ml each, of wash buffer (50 mM Tris, pH 7.0, 3 mM MgCl2, drier, and counted in the 2.9–35 KeV window in a TopCount (Packard) after adding 50 μl of MS-20 scintillation fluid/well.

**FLIPR Experiment**

A stable HEK293 clone expressing the TRHR2 receptor and GH4C1 neuronal cell line endogenously expressing the rat TRH-R1 receptor were fed 24 h prior to experimentation. On the day of the experiment, 10–12 million cells were centrifuged and gently resuspended in loading solution (loading solution for HEK293 cells: 11 ml of Dulbecco’s modified media + 10% fetal bovine serum containing 21 ml of Fluo3-AM (4 μl final) and 21 ml of 20% polyvalent calcium and for GH4C1 cells: 11 ml of F-10 media + 10% fetal bovine serum containing 42 ml of calcium green (8 ml final) and 42 ml of 20% polyvalent calcium and incubated at 37 °C for 1 h in a humidified chamber. Following the incubation step, cells were washed three times in 30 ml of Hank’s + 20% HEPES + 0.1% bovine serum albumin (pH 7.4) and resuspended in wash buffer. The cells were plated in 96-well poly-d-lysine coated black plates at a dilution of 100,000, 10,000, 1000, and 100 cells per well (100 μl), spun at 200 × g for 3 min, and analyzed using the FLIPR FLUORESCENT IMAGING PLATE READER (Argon Laser: 488 nm) to study calcium transients in response to TRH and related compounds. The data was analyzed using the Graph Prism nonlinear regression method to obtain maximum activation and EC50 values.

**Northern Blot Analysis**

A rat multiple tissue Northern blot (CLONTECH) containing 2 mg of rat poly(A)+ mRNA isolated from heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testes was used to study the distribution of rTRHR2 in various tissues. The blot was pre-hybridized at 42 °C for 2 h in a solution containing 50% formamide, 5 × SSPE, 10 × Denhardt’s solution, 100 mg/ml salmon sperm DNA, and 2% SDS. Hybridization with the full-length randomly primed cDNA (Ready-To-Go DNA labeling kit, Pharmacia) was carried out at 42 °C for 18 h in the solution described above and the final wash was 2 × SSC, 0.05% SDS at 60 °C. The blot was exposed at −80 °C for 7 days to Kodak Biomax film with intensifying screens.

In Situ hybridization

**Animals and Tissue Preparation—Adult male Sprague-Dawley rats (300 g; Charles River, St. Constant, Quebec) were sacrificed by decapitation. Brain, pituitary, and spinal cord were promptly removed, snap-frozen in isopentane at −40 °C for 20 s, and stored at −80 °C. Frozen tissue was sectioned at 14 mm in a Microm HM 500M cryostat (Germany) and thaw-mounted onto ProbeOn Plus slides (Fisher Scientific, Montreal, Quebec). Sections were stored at −80 °C prior to in situ hybridization.**
In Situ Hybridization—Sections were postfixed in 4% paraformaldehyde (BDH, Poole, United Kingdom) in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature and rinsed in 3 changes of 2× SSC. Sections were then equilibrated in 0.1 M triethanolamine, treated with 0.25% acetic anhydride in triethanolamine, rinsed in 2× SSC and dehydrated in an ethanol series (50–100%). Hybridization was performed in a buffer containing 75% formamide (Sigma), 600 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 1× Denhardt’s solution (Sigma), 50 mg/ml denatured salmon sperm DNA (Sigma), 50 mg/ml yeast tRNA (Sigma), 10% dextran sulfate (Sigma), 10 mM dithiothreitol, and [35S]UTP-labeled cRNA probes (1× 10^6 cpm/ml) at 55 °C for 18 h in humidified chambers. Following hybridization, slides were rinsed in 2× SSC at room temperature, treated with 20 mg/ml RNase IA (Pharmacia) in RNase buffer (10 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 7.5) for 45 min at room temperature, and washed to a final stringency of 0.1× SSC at 65 °C. Sections were then dehydrated and exposed to Biomax MR Kodak film for 10 days. Neuroanatomical structures were identified according to the Paxinos and Watson rat brain atlas (22).

RESULTS

Degenerated oligonucleotides were designed to the conserved regions of transmembrane domains 3 and 7 of several G protein-coupled receptors belonging to the adrenergic subfamily (23). In order to isolate novel G protein-coupled receptors expressed in the spinal cord, reverse transcriptase-PCR reactions were performed using rat spinal cord mRNA as a template. The amplified fragments whose sizes were between 500 and 800 bp were ligated to a cloning vector. After screening of the subcloned fragments by DNA sequence analysis, clone pGemT-1–75 showed marked sequence homologies with known G protein-coupled receptors. The most homologous sequence was the human and rat thyrotropin-releasing hormone receptors (TRHR) (3, 17, 24).

To obtain the full-length cDNA for this clone, a rat brain stem/spinal cord cDNA library was screened using the insert of pGemT-1–75 as a probe. DNA isolated from a positive phage by helper phage-induced excision and the cDNA insert of the resulting plasmid (pBS-TRHR2) was sequenced. An open reading frame of 352 amino acids was detected flanked by 3′-untranslated region and 5′-untranslated region of, respectively, 220 and 361 bp. The nucleotide sequence and predicted amino acid are displayed in Fig. 1. The relative molecular mass of the predicted protein is 39,500 daltons. Hydropathy analysis of the encoded protein is consistent with the putative topography of seven transmembrane domains, indicative of the G protein-coupled receptor family (23). In addition, sequence analysis revealed that the open reading frame of clone pBS-TRHR2 contains several conserved structural motifs and residues found among the members of the neuropeptide receptor family including an asparagine in TM1 (Asn-40), a leucine (Leu-64) and an aspartic acid (Asp-68) in TM2, and a serine (Ser-109), an arginine (Arg-120) and a tyrosine residue (Tyr-121) in TM3. Other motifs of the rTRHR2 receptor gene are the presence of a potential site for N-glycosylation in the amino terminus and the presence of several serines and threonines in the carboxyl terminus and the third intracellular.

![FIG. 1. Nucleotide sequence and deduced amino acid sequence of a novel rat TRH receptor (TRHR2; sequence numbers 1 and 2). Nucleotides are presented in the 5′ to 3′ orientation. Deduced amino acid sequence by translation of a long open reading frame is shown, along with the 5′ to 3′ untranslated sequences. Numbers above the sequence represent nucleotide (top line) and amino acid sequence (bottom line), using the single amino acid letter code.](image-url)
loop, which may serve as potential sites for phosphorylation by protein kinases.

An alignment of the deduced amino acid sequences of known TRH receptors is shown in Fig. 2. The overall amino acid sequence homology between rTRHR2 and the known rat TRH receptor is 50.6%. The homologies between the known rat TRH receptor (hereafter called rTRHR1) and rTRHR2 in TM1 to TM7 are 61, 80, 74, 58, 52, 77, and 71%.

When transiently transfected into HEK293 cells, the expression plasmid pcDNA3-TRHR2 generated the expression of specific [3H]TRH-binding sites. No specific [3H]TRH binding sites were generated by the transfection of the vector itself or a control pcDNA3 expression construct encoding the delta opioid receptor. To pharmacologically characterize the rTRHR2 receptor, we generated stable HEK293 cells expressing the rTRHR2 receptor by selecting pcDNA3-TRHR2 transfected cells using G418. Binding experiments were performed on a selected cell line called HEK293/TRHR2. A single class of saturable [3H]TRH-binding site was detected displaying an estimated Kd for [3H]TRH of 3.6 ± 0.6 nM and a Bmax of 126 ± 1 pmol/mg of proteins. Various TRH-related peptides were used in competition experiments. These experiments using [3H]TRH as a tracer revealed Ki values of 2.3 nM for pGlu-3-methyl-His-Pro-amide, 7.3 nM for TRH, 1507 nM for pGlu-His-Pro-Gly, and 10,000 nM for pGlu-Glu-Pro-amide (Table I).

In addition we compared the pharmacology of the novel TRHR2 receptor to that of TRHR1 receptors expressed in GH4C1 cells (25) by performing competition binding experiments. The K_i values measured using HEK293/TRHR2 or GH4C1 cells are summarized in Table I. The affinity of TRH is similar for TRHR2 and TRHR1. For both the TRHR1 receptor, present in GH4C1 cells, and for the TRHR2 receptor present in HEK293/TRHR2 cells, methyl-TRH (pGlu-3-methyl-His-Pro-amide) displays a slightly higher affinity than TRH. The ligand pGlu-His-Pro-Gly binds with a better affinity to the TRHR2 receptor (K_i = 1.4 μM) than to the TRHR1 receptor (K_i = 7.2 μM). This may indicate that TRHR2 is less susceptible than TRHR1 to NH2-terminal modification of the ligand.

The rTRHR1 receptor has previously been shown to couple to phospholipase C via a pertussis toxin-insensitive Gαq/α11 protein resulting in the mobilization of intracellular calcium stores. Based on these prior observations, we investigated if rTRHR2 could also activate the phospholipase C effector system. Using different peptidic TRH-derived compounds, the calcium signaling responses mediated by rTRHR1 endogenously expressed in GH4C1 cells and HEK293 stably transfected with rTRHR2 receptors were compared using the FLIPR system. Increasing concentrations of TRH, 3-CH3-TRH (pGlu-3-methyl-His-Pro-amide), pGlu-His-Pro-Gly, and pGlu-Glu-Pro-amide peptides were added to cells expressing either the rTRHR1 or the rTRHR2 receptors to measure calcium responses. As shown in Fig. 3, TRH and 3-CH3-TRH were equipotent at TRHR1 and TRHR2 receptors at stimulating increases in intracellular calcium, whereas pGlu-His-Pro-Gly was more potent at functionally activating the rTRHR2 receptors (EC50, 42 ± 22 nM) as compared with the rTRHR1 receptors (EC50, 1833 ± 223 nM). The pGlu-Glu-Pro-amide peptide, however, displayed no functional binding at either the TRHR1 and TRHR2 receptors. Hence, based on these observations the rTRHR2 receptor is a fully functional receptor coupled to the phospholipase C signaling pathway.

| TABLE I | K_i values (nM) for various ligands at the TRH receptors expressed in GH4C cells and the TRHR2 cloned receptor. |
|-----------------|---------------------------------|
| HEK293/TRHR2 | GH4C |
| TRH (pGlu-His-Pro-NH2) | 7.3 ± 0.6 | 7.6 ± 0.3 |
| pGlu-Glu-Pro-NH2 | >10,000 | >10,000 |
| pGlu-His-Pro-Gly | 1,507 ± 94 | 7,195 ± 688 |
| pGlu-3CH3-His-Pro-NH2 | 2.3 ± 0.2 | 1.4 ± 0.1 |

This table summarizes the Ki values for various ligands at the TRH receptors expressed in GH4C cells and the TRHR2 cloned receptor. Three independent experiments were performed each with duplicated data points.
Using Northern blot analysis of RNA isolated from heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testes, we detected expression of TRHR2 mRNA only in brain tissue (see Fig. 4). The apparent size of the mRNA is about 8 kilobases. Other tissues contained either no or insufficient amount of message to be detected after 1 week exposure under the conditions described.

The regional distribution of TRHR2 within the rat CNS and pituitary gland was further examined using in situ hybridization (Fig. 5, A-C). Film autoradiograms prepared from sagittal brain and spinal cord sections hybridized with 35S-labeled TRHR2 antisense riboprobe revealed a discrete and unique pattern of distribution. No signal was detected from sections hybridized with 35S-labeled TRHR2 sense probe (data not shown). In brain (Fig. 5A), the highest levels of TRHR2 mRNA were observed in the pontine nucleus, thalamus, and cerebellar cortex. Clearly, the pontine nucleus was the most intensely labeled structure in sagittal brain sections. A prominent TRHR2 hybridization signal was also present in thalamus where virtually every thalamic nucleus was either intensely or moderately labeled. Within the cerebellar cortex, intense TRHR2 labeling was detected in the lower tier of the molecular layer in all lobules. Moderate levels of TRHR2 mRNA were present in the anterior olfactory nucleus, in deeper layers of the neocortex, throughout the superior and inferior colliculi, in central gray as well as in several brainstem nuclei. Other cephalic areas such as the basal ganglia, hippocampus, and hypothalamus were generally devoid of TRHR2 labeling. Within the cerebral cortex, intense TRHR2 labeling was detected in the lower tier of the molecular layer in all lobules. Moderate levels of TRHR2 mRNA were present in the anterior olfactory nucleus, in deeper layers of the neocortex, throughout the superior and inferior colliculi, in central gray as well as in several brainstem nuclei. Other cephalic areas such as the basal ganglia, hippocampus, and hypothalamus were generally devoid of TRHR2 labeling. In spinal cord (Fig. 5B), expression of TRHR2 mRNA was detected exclusively within the dorsal horn. The labeling was moderate and uniformly distributed throughout the dorsal gray matter, spanning approximately laminae III to VI. In contrast to the CNS, no TRHR2 hybridization signal was detected in the pituitary gland (Fig. 5C).

For comparison, series of adjacent sections were probed in parallel with a TRHR1 riboprobe (Fig. 5, D and E). The level and pattern of TRHR1 mRNA expression in rat CNS and pituitary were entirely different from that of TRHR2. Overall, CNS levels of TRHR1 mRNA were low as compared with those of TRHR2. In brain (Fig. 5D), the highest levels of TRHR1 were observed in the accessory olfactory bulb. Moderate to weak TRHR1 labeling was present in the olfactory bulb, nucleus of the diagonal band of Broca, various hypothalamic nuclei, dentate gyrus, superficial gray layer of the superior colliculus, and in certain brainstem nuclei. In spinal cord (Fig. 5E), TRHR1 mRNA signal was observed predominantly over motoneurons of the ventral horn. In the pituitary gland (Fig. 5F), TRHR1 labeling was intense and selectively distributed over the anterior pituitary.

**DISCUSSION**

We have cloned a cDNA representing a novel rat thyrotropin-releasing hormone receptor (rTRHR2). Of all known G protein-coupled receptor sequences (EMBL/GenBank data base), the greatest homology was displayed between rTRHR2 and the human TRH receptor (3, 17, 24). In the TM regions, the sequence homology between rTRHR2 and this receptor ranges...
from 52 to 80%. The alignment of this rTRHR2 sequence, relative to other G protein-coupled receptors or other members of the neuropeptide receptor subfamily, indicates a unique sequence, proving rTRHR2 is a newly characterized receptor.

The transfection of HEK293 cells with a mammalian expression vector containing the rTRHR2 sequence resulted in the generation of high affinity TRH-binding sites. The $K_d$ values for TRH binding is in the low nanomolar range. The binding of labeled TRH was displaced by TRH analogues but not by TRH unrelated ligands. Pyroglutamylglutamylproline-amide, a peptide structurally related to TRH isolated from the rabbit prostate, does not bind to the rTRHR2 nor to the TRHR1 expressing cells ($K_d > 10,000 \text{ nM}$) (27). The rTRHR2 receptor evoked strong transient increases in $[Ca^{2+}]_i$, concentration in a dose-dependent fashion when activated by various concentrations of TRH, 3-CH$_3$-TRH, and pGlu-His-Pro-Gly but not by pGlu-Glu-Pro-amide. The pGlu-His-Pro-Gly peptide was the only ligand to selectively differentiate between the rTRHR1 and rTRHR2 subtypes since it functionally activated the rTRHR2 receptors more potently than the rTRHR1 receptors, and this difference was also reflected in binding affinities. These results demonstrate that the rTRHR2 gene encodes a functional receptor. Similar types of signaling responses were observed in cells expressing TRHR1 receptors (3, 28).

The rat TRHR2 receptor differs from the TRHR-1 in another aspect, i.e. the very limited distribution of its messenger RNA. The message for rat TRHR1 has been shown to be widely present both in periphery and in central nervous system as described by Northern analysis and reverse transcriptase polymerase chain reaction (29–31). The message for TRHR2, however, as assessed by Northern analysis appears to be restricted to or at least predominately expressed in brain. Under our conditions we could not detect any signal in any peripheral tissues included in our blots even after 7 days exposure. A single band between 8.0 and 8.5 kilobases was detected in the RNA from the brain. We have extended these studies of the presence of the TRHR2 in brain to include the regional distribution of the message in brain and spinal cord by in situ hybridization.

Our in situ hybridization studies indicate that distribution of TRHR2 mRNA in rat CNS is discrete and unique. A priori, the CNS pattern of TRHR2 expression suggests the involvement of at least two distinct modalities, somatosensory and motor. The discrete expression of TRHR2 mRNA in dorsal horn of the spinal cord, reticular formation, somatosensory nuclei of the thalamus (ventral posterolateral thalamic nucleus, ventral posteromedial thalamic nucleus), and neocortex is consistent with ascending pathways such as the spinothalamic and trigeminothalamic tracts (pain and crude touch) as well as the medial lemniscal system (discriminative touch). The presence of particularly high levels of TRHR2 in the pontine nucleus and the cerebellum is consistent with a role in motor control and/or proprioception, suggesting these receptors may also be the anatomical substrate for the previously described TRH effects on motor control (19).

Although both receptors subtypes are expressed in the rat brain, our in situ hybridization studies demonstrate the pattern of TRHR2 mRNA distribution to be entirely different from that of TRHR1. In fact, the CNS expression of TRHR2 and TRHR1 are virtually mutually exclusive, suggesting these receptors mediate quite different physiological roles. A clear example of this is the pituitary. Our findings as well as those of others (32) demonstrate that the anterior pituitary selectively expresses moderate levels of TRHR1 mRNA. In contrast, no detectable levels of TRHR2 expression were observed in the pituitary gland. Since TRH's neuroendocrine actions are predominantly associated with the anterior pituitary (8), these actions appear to be specific to TRHR1. Conversely, TRHR2, but not TRHR1, is selectively expressed in many key CNS regions associated with pain transmission, namely dorsal horn

![Fig. 5. In situ hybridization film autoradiograms showing localization of TRHR2 and TRHR1 mRNA expression in CNS and pituitary gland of the rat. Series of adjacent sagittal brain (A and D), spinal cord (B and E), and pituitary (C and F) sections were hybridized in parallel with $^{35}$S-labeled riboprobes directed to TRHR2 (A-C) and TRHR1 (D-F). The expression pattern of the novel TRHR2 in CNS and pituitary differs markedly from that of TRHR1. AL, anterior lobe (pituitary); AO, anterior olfactory nucleus; AOB, accessory olfactory bulb; CB, cerebellum; Cx, neocortex; DB, diagonal band of Broca; DG, dentate gyrus; DH, dorsal horn (spinal cord); Hypo, hypothalamus; IC, inferior colliculus; IL, intermediate lobe (pituitary); OB, olfactory bulb; PAG, periaqueductal gray; PL, posterior lobe (pituitary); Pn, pontine nucleus; SC, superior colliculus; SuG, superior gray layer of the superior colliculus; Th, thalamus; VH, ventral horn (spinal cord).](image-url)
of the spinal cord, thalamus, central gray and rostral ventral medulla, specifically implicating a role for TRHR2 in mediating TRH’s putative antinociceptive properties (11, 13).

Today, the development of TRH analogues for therapy is justified by the potential benefit of TRH agonists in improving motor neuron functions or other neurological functions, without triggering the endocrine effects evoked by TRH stimulation of the pituitary. In particular, TRH has been shown to improve the recovery following spinal cord injury (19). Importantly, TRH has also been involved in the modulation of spinal cord pain transmission (12, 33) and has been shown to act as an antinociceptive agent more potent than morphine when injected centrally (11). The regional distribution of the rTRHR2 message in the spinal cord and in the thalamus points to a potential role for rTRHR2 in the modulation of pain perception. rTRHR2 thus represents an important tool for the development of selective TRH-like compounds of potential use in neurobiology.

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