TRH-R2 Exhibits Similar Binding and Acute Signaling but Distinct Regulation and Anatomic Distribution Compared with TRH-R1

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TRH (thyroliberin) is a tripeptide (pGlu-His-ProNH$_2$) that signals via G protein-coupled receptors. Until recently, only a single receptor for TRH was known (TRH-R1), but two groups identified a second receptor, TRH-R2. We independently discovered TRH-R2. Using an extensive set of TRH analogs, we found no differences in TRH-R1 and TRH-R2 binding or in acute stimulation of signaling. TRH-R2 was more rapidly internalized upon binding TRH and exhibited a greater level of TRH-induced down-regulation than TRH-R1. During prolonged exposure to TRH, cells expressing TRH-R2 exhibited a lower level of gene induction than cells expressing TRH-R1. TRH-R2 receptor mRNA was present in very discrete nuclei and regions of rat brain. A major mRNA transcript for TRH-R2 was seen in the cerebral cortex, pons, thalamus, hypothalamus, and midbrain with faint bands found in the striatum and pituitary. The extensive distribution of TRH-R2 in the brain suggests that it mediates many of the known functions of TRH that are not transduced by TRH-R1. The variations in agonist-induced internalization and down-regulation/desensitization, and anatomic distribution of TRH-R2 compared with TRH-R1, suggest important functional differences between the two receptors. (Molecular Endocrinology 14: 183–193, 2000)

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INTRODUCTION

TRH (thyroliberin) is a tripeptide (pGlu-His-ProNH$_2$) that functions as a hormone, a paracrine regulatory factor, and a neurotransmitter/neuromodulator. A review of the extensive actions of TRH was published in 1989 (1). It is known that TRH initiates some, if not all, of these effects by interacting with receptors on cell surfaces and that these receptors couple to G proteins (2, 3). Until recently, only a single G protein-coupled receptor (GPCR) for TRH was known (TRH-R1) (4). Two groups independently identified a second gene encoding another TRH receptor, TRH-R2, from a rat brain cDNA library (5) and a rat brain stem-spinal cord cDNA library (6). In a limited *in situ* hybridization study, Cao and colleagues (6) found that distribution of TRH-R2 mRNA in the central nervous system was distinct from that of TRH-R1 mRNA. We found that TRH-R2 exhibits higher basal signaling activity than TRH-R1 (7).

A preliminary pharmacological characterization of TRH-R2 was performed and the findings compared with TRH-R1. Both Itadani *et al*. (5) and Cao *et al*. (6) agreed that TRH-R1 and TRH-R2 bound TRH with equal affinity (5, 6). Cao and colleagues (6) presented data that rat TRH-R2 and TRH-R1 bound the

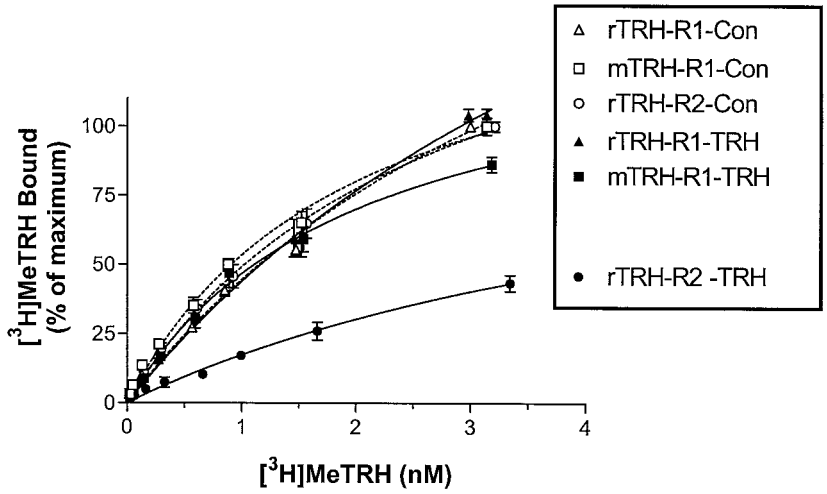

Fig. 1. Association Binding of [^{3}H]MeTRH to Control or TRH-Treated Cells Expressing mTRH-R1, rTRH-R1, or rTRH-R2

The experiments were performed as described in *Materials and Methods*. Control COS-1 cells were incubated in growth medium alone (Con) whereas TRH-treated cells were incubated in growth medium containing 1 μM TRH for 16–20 h (TRH). The points represent replicate data from two experiments.

Table 1. Binding Affinities of mTRH-R1 and rTRH-R2 for TRH Analogs

Analog	K_i (μM)	
	mTRH-R1	rTRH-R2
TRH	0.013 (0.010–0.015)	0.016 (0.013–0.020)
[DesazapGlu^1]TRH	1.4 (1.0–2.0)	3.5 (1.7–7.3)
[Pro^1]TRH	300 (65–1400)	630 (180–2300)
[Val^2]TRH	7.2 (4.8–11)	14 (9.1–22)
[Pyr^3]TRH	4.0 (2.9–5.6)	5.4 (3.6–8.2)
[CyclohexAla^2]TRH	3.8 (22–6.5)	4.2 (2.2–8.0)
αCH-TRH	75 (42–130)	150 (40–540)
βCH-TRH	2.0 (0.95–4.3)	1.3 (0.75–2.4)
pGlu-His-Pro-Gly-NH_2	0.048 (0.037–0.064)	0.048 (0.030–0.077)
pGlu-His-Pro-Gly	8.3 (3.9–17)	2.7 (1.8–3.9)

TRH analog MeTRH[1] with equal affinity but that TRH-R2 bound another TRH analog, pGlu-His-Pro-Gly, with higher affinity than rat TRH-R1. They concluded that these receptors exhibited different pharmacological characteristics. These data are too preliminary to justify such an important conclusion. It is noteworthy that, of four amino acid residues within the transmembrane helices and two within the extracellular loops of mouse TRH-R1 (8) that we identified as sites of direct interaction with TRH (4, 9), all six residues are conserved in rat (10, 11), human (12), and chicken (13) TRH-R1 and rat TRH-R2 (5, 6). This suggests that there is likely to be significant similarity between the binding of TRH analogs by these receptors although differences may be found.

[1] Abbreviations used: MeTRH = *N-t*-[methylHis]-TRH; CH-TRH = pGlu-cyclohexylAla-ProNH_2; αCH-TRH = (6S, 9S, 12S)-1-Aza-3-aminopyroglutamyl-4-cyclohexyl-9-carboxamide-2-oxo-bicyclo[4.3.0]non-2-ene; βCH-TRH = (6R, 9S, 12S)-1-Aza-3-aminopyroglutamyl-4-cyclohexyl-9-carboxamide-2-oxo-bicyclo[4.3.0]non-2-ene.

In this paper, we present the results of our independent discovery, extensive anatomic localization in rat brain, pharmacological characterization, and comparison of the cellular biology of rat TRH-R2 compared with TRH-R1. Our studies, using an extensive set of TRH analogs, revealed no differences in TRH-R1 and TRH-R2 binding or signaling. We have investigated the potential functional differences between TRH-R1 and TRH-R2 and have shown differences in agonist-induced internalization and desensitization/down-regulation of TRH-R2 compared with TRH-R1.

RESULTS

Cloning of a cDNA Encoding TRH-R2

A rat brain 5′-stretch cDNA library was amplified by PCR with a transmembrane helix 7 (TM7)-based degenerate primer (P1) paired with two primers specific for the 5′ (P2) and 3′ (P3) regions flanking the cDNA library inserts. One

Table 2. Potencies of mTRH-R1, rTRH-R1, and rTRH-R2 for TRH Analogs

Analog	EC_{50} (nM)		
	mTRH-R1	rTRH-R1	rTRH-R2
TRH	0.83 (0.61–1.1)	0.81 (0.53–1.2)	1.1 (0.53–2.3)
[DesazapGlu[1]]TRH	61[a]	48 (27–83)	63 (30–94)
[Val[2]]TRH	520[b]	780 (620–970)	1000 (490–2000)
[Pyr[3]]TRH	640[b]	980 (730–1300)	420 (140–1200)
pGlu-His-Pro-Gly-NH_2	2.5 (0.75–8.2)	nd[c]	1.1 (0.3–4.3)
pGlu-His-Pro-Gly	200 (160–280)	430 (260–700)	130 (31–270)

[a] (9).
[b] (15).
[c] nd, Not done.

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mTRH-R1(short)    KFRAAFRKLCNCKQKPTEKAANYSVALNYSVIKESDRFSTELEDITVTDTYVSTTKVSFDDTCLASEN*
mTRH-R1(long)     ------------------------------------------------------------------KNGLSSCAYSFSLTAKQERI*
rTRH-R1(long)     ----------------------------------------D-----------------------KNGPSSCTYGYSLTAKQERI*
rTRH-R1(short)    -------------------------------D-------RMDPVLVHMDIL*
rTRH-R2           ------L---W-RAAGPQRR-ARVLTS---AAQ-TSEGTEKM
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Fig. 2. Comparison of the Amino Acid Sequences of the Carboxyl Termini of mTRH-R1, rTRH-R1, and rTRH-R2.

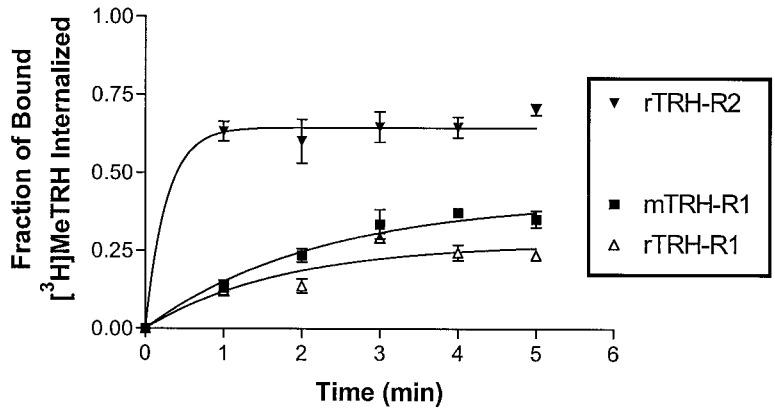

Fig. 3. TRH-Stimulated Internalization of MTRH-R1, rTRH-R1, and rTRH-R2

The experiments were performed as described in *Materials and Methods*. COS-1 cells were incubated in buffer containing 2 nM [^{3}H]MeTRH for the times indicated. The *points* represent replicate data from three experiments.

PCR product (~400 bp) was found to encode a novel GPCR from TM4 to TM7, sharing the greatest translated sequence identity of 44% to TRH-R1. This cDNA was labeled with [^{32}P]dCTP-α and used to probe the same rat brain cDNA library, which resulted in the isolation of two cDNA clones. These clones were amplified by PCR using primers P2 and P3 and the products subcloned into the pcDNA3 vector. Both cDNAs revealed identical sequences encoding the full-length receptor, which we named TRH-R2. TRH-R2 encoded a protein of 352 amino acids, which shared the greatest sequence identity of 68% in the TM domains with TRH-R1.

TRH-Binding Analysis

The affinities of mTRH-R1, rTRH-R1, and rTRH-R2 receptors for MeTRH were indistinguishable: dissociation constant (K_d) = 1.9 ± 0.10 nM for mTRH-R1, K_d = 3.8 ± 0.70 nM for rTRH-R1, and K_d = 4.6 ± 0.40 nM for rTRH-R2 (Fig. 1). In competition binding experiments, the calculated affinities of the three receptors for TRH were indistinguishable also: inhibition constant (K_i) = 13 ± 1.3 nM for mTRH-R1, K_i = 12 ± 1.9 nM for rTRH-R1, and K_i = 16 ± 1.8 nM for rTRH-R2. The binding affinities of a series of TRH analogs were tested. Because substitution of the side chain of each of the three residues of TRH has been shown to affect TRH binding to TRH-R1 (14), the analogs chosen were substituted in the first position (Pro-His-ProNH_2 and DesazapGlu-His-ProNH_2) (9), the second position (pGlu-Val- ProNH_2) (15), and the third position (pGlu-His-pyrrolidine) (15). Because we had shown that the conformation of analogs restricted in their rotation by

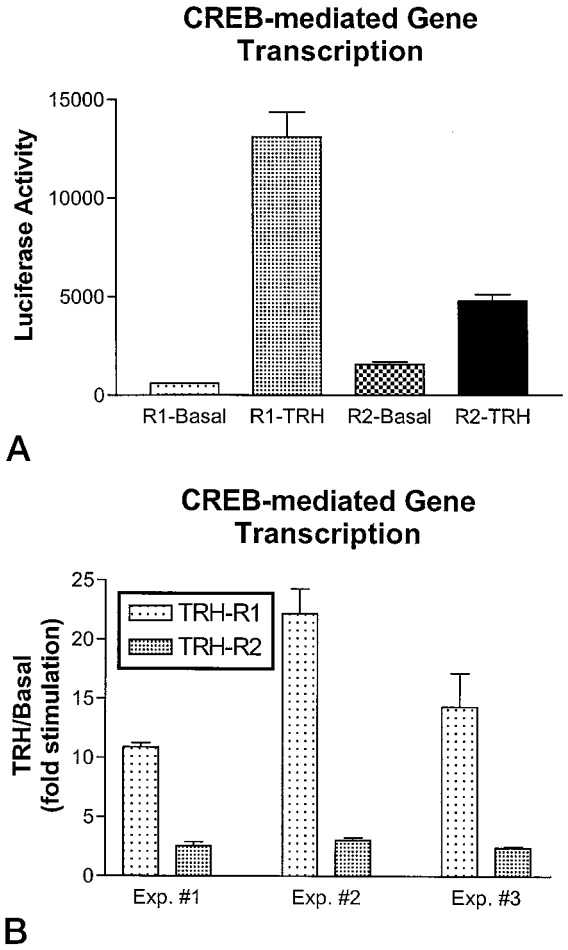

Fig. 4. Signaling Activity of TRH-R1 and TRH-R2 during Chronic Stimulation by TRH

Chronic signaling by 1 μM TRH was measured as activation of CREB-mediated luciferase activity (relative light units) in HEK 293 cells after 24 h. A, Absolute levels of basal and TRH-stimulated reporter gene induction in a representative sample of three experiments. B, Fold stimulation by TRH in the three experiments. *Bars* represent mean ± SD of triplicate samples.

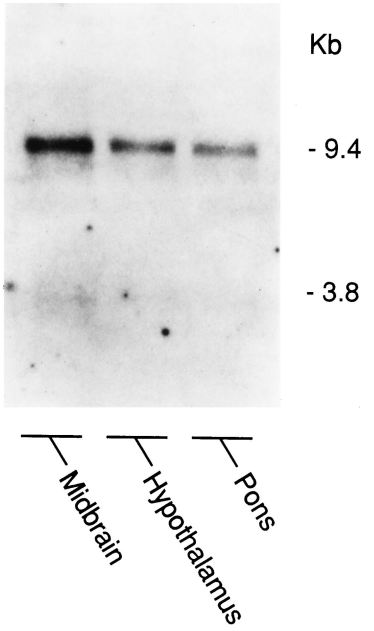

Fig. 5. Northern Blot Analysis of the Tissue Distribution of TRH-R2 in Rat Brain

Each lane contains 10 μg of poly(A)$^+$ RNA isolated from various rat tissues. The molecular size is indicated on the *right.*

creating a methylene bridge between residues at the 2- and 3-positions of a TRH analog exhibited stereospecific differences in binding to mTRH-R1 (16), we tested the binding of the two restricted analogs, αCH-TRH and βCH-TRH, and the freely rotatable parent analog, CH-TRH (pGlu-cyclohexylAla-ProNH$_2$). Lastly, because Cao and colleagues (6) reported that pGlu-His-Pro-Gly exhibited different affinities when binding to rTRH-R1 and rTRH-R2, we tested pGlu-His-Pro-Gly and pGlu-His-Pro-Gly-NH$_2$. Table 1 shows that all analogs tested bound to mTRH-R1 and rTRH-R2 with similar affinities.

Acute TRH-R Signaling

The potencies of TRH at mTRH-R1, rTRH-R1 and rTRH-R2 for acute stimulation of phosphoinositide hydrolysis were indistinguishable (Table 2), as were the maximal levels of TRH-stimulated second messenger formation (data not shown). Similar potencies with the three TRH-Rs were found for DesazapGlu-His-ProNH$_2$ ([DesazapGlu^1]TRH), pGlu-Val-ProNH$_2$ ([Val^2]TRH), pGlu-His-pyrrolidine ([Pyr^3]TRH), pGlu-His-Pro-Gly-NH$_2$, and pGlu-His-Pro-Gly.

Agonist-Induced Receptor Internalization And Down-Regulation

The rate of internalization of mTRH-R1 is affected by alterations in its carboxyl terminus (17, 18). mTRH-R1 (19) and rTRH-R1 (20) exhibit two alternative splice forms that affect their carboxyl termini, but no differences in internalization rates of these variants have been reported. A major difference between rodent TRH-R1s and rTRH-R2 is in the carboxyl termini (Fig. 2). The carboxyl terminus of TRH-R2 is shorter than both alternative splice variants of rat and mouse TRH-R1 and of the 42 amino acid residues in the carboxyl terminus of TRH-R2, only 15 are identical to those in TRH-R1. Figure 3 illustrates that there is a marked difference in the MeTRH-stimulated rate of internalization of rTRH-R2 compared with mTRH-R1 or rTRH-R1. Internalization stimulated by MeTRH was more rapid with rTRH-R2 ($t_{1/2}$ = 0.20 min) than with mTRH-R1 ($t_{1/2}$ = 1.6 min) or rTRH- R1 ($t_{1/2}$ = 1.2 min). Because agonist-stimulated receptor internalization may lead to receptor degradation, it was likely that prolonged exposure to TRH would cause a greater decrease in the levels of rTRH-R2 on the cell surface than

of the TRH-R1 receptors. Figure 1 illustrates the effects of exposure to 1 μM TRH for 16–20 h on TRH-R levels in cells expressing each of the three receptors. In these cells, TRH did not significantly decrease the levels of mTRH-R1 (88 $\pm$7% of control) or of rTRH-R1 (100 $\pm$ 17% of control) but caused an approximately 40% decrease in rTRH-R2 (63 $\pm$ 16% of control). Thus, rTRH-R2 exhibited more rapid agonist-stimulated internalization kinetics and a greater degree of down-regulation with prolonged agonist exposure than either rodent TRH-R1.

Chronic Stimulation By TRH

As shown previously (7), TRH-R2 causes a greater induction of reporter gene transcription than TRH-R1 in the absence of agonist (Fig. 4A); that is, TRH-R2 exhibits higher basal signaling activity than TRH-R1. In contrast, 24 h exposure to 1 μM TRH caused lesser induction of reporter gene transcription in cells expressing TRH-R2 than in cells expressing TRH-R1 (Fig. 4A). The fold stimulation for gene induction by TRH-R2 was between 2- and 3-fold, whereas that for TRH-R1 was between 10- and 22-fold (Fig. 4B). Thus, TRH-R2 was less effective in stimulating gene transcription than TRH-R1 during prolonged stimulation by TRH.

Tissue Distribution

Tissue distribution of TRH-R2 mRNA transcripts was obtained by Northern blot analysis using a cDNA fragment encoding TRH-R2 from TM4 to TM7 and poly(A)$^+$ RNA isolated from various rat tissues. In the brain, a major transcript of 9.4 kb (and a faint band of 3.8 kb) was seen in the pons, hypothalamus, and midbrain (Fig. 5). Faint bands of 9.4 kb were also found in the striatum and pituitary (data not shown).

TRH-R2 receptor mRNA distribution visualized by *in situ* hybridization histochemistry revealed abundant expression in very discrete nuclei and regions of rat brain (Figs. 6 and 7). There was extremely dense expression in frontoparietal cortex, particularly in the primary somatosensory and motor areas, and also in the primary visual area and primary olfactory cortex. Strong signals were also present in other areas of cortex, such as the anterior cingulate area, concentrated in the deeper rather than in the superficial layers of cortex. Further caudally, TRH-R2 mRNA expresssion was moderately dense also in the posterior cingulate area, retrosplenium, striate areas, and throughout the subiculum, in both dorsal and ventral portions.

Several thalamic nuclei displayed extremely dense labeling, such as the paraventricular, centromedial, anteroventral, and ventroposterior thalamic nuclei and the medial habenular nucleus. TRH-R2 mRNA was present less abundantly in other thalamic nuclei, such as the laterodorsal, lateroposterior, and ventromedial nuclei and the medial reuniens nucleus. In hypothalamus, TRH-R2 mRNA was most abundant in the anterior hypothalamic area and was also present in the medial preoptic and lateral hypothalamic areas, the paraventricular nucleus, and some of the mammillary nuclei. Moderate labeling was also seen in the bed nucleus of the stria terminalis, the nucleus of the diagonal band, some of the amygdaloid nuclei , and in the subthalamic nucleus. The geniculate nuclear complex contained very dense expression in the medial geniculate, in both the dorsal and ventral divisions of the nucleus, whereas in the lateral geniculate, moderate expression was observed largely in the dorsal division.

In midbrain, a punctate pattern of TRH-R2 mRNA expression was observed in the superior colliculus, periaqueductal gray, and the mesencephalic reticular nucleus. Small amounts of mRNA were present in the ventral tegmental area. The pontine gray expressed TRH-R2 mRNA very abundantly, and the central and rostral linear raphe nuclei showed moderately dense labeling as well. Lesser amounts were evident in the inferior colliculus, the nucleus sagulum, the pontine reticular nucleus, and the parabrachial nucleus. TRH-R2 receptor mRNA was detected in pituitary gland, as sections revealed a very small amount of labeling in the anterior lobe, whereas the neurointermediate lobe was devoid of any signal (Fig. 7).

DISCUSSION

A series of 11 TRH analogs were used to study the binding characteristics of TRH-R2 compared with TRH-R1. We found no significant differences in the affinities of binding by TRH-R1 and TRH-R2 of any analog. This may have been expected because the six residues in TRH-R1 (4, 9) that have been shown to directly interact with TRH are identical in TRH-R2. Six TRH analogs were used in studies of acute signaling potencies at TRH-Rs. There were no significant differences in the potencies of these analogs at the two TRH-Rs. We cannot explain the discrepancy between our data and that of Cao and colleagues (6), who reported that pGlu-His-Pro-Gly bound with higher affinity to and exhibited higher potency at TRH-R2 than at TRH-R1.

TRH stimulates TRH-R1 and TRH-R2 internalization and down-regulation (4) (Figs. 1 and 3). TRH-R1 internalization proceeds via clathrin-coated pits (21, 22) and likely leads to increased receptor degradation because receptors are targeted to lysosomes. This mechanism of TRH-induced TRH-R1 down-regulation is complemented by effects of TRH to decrease TRH-R1 gene transcription (23) and to increase TRH-R1 mRNA degradation (24). The rate of receptor internalization and the distribution within cells vary in different cell types (17, 25). In the series of experiments described herein, both mouse and rat TRH-R1 exhibited slower rates of internalization than TRH-R2. In concordance with the differences in their rates of internalization, TRH caused a much greater down-regulation of TRH-R2 than of TRH-R1. Because TRH-R internalization and down-regulation affect TRH

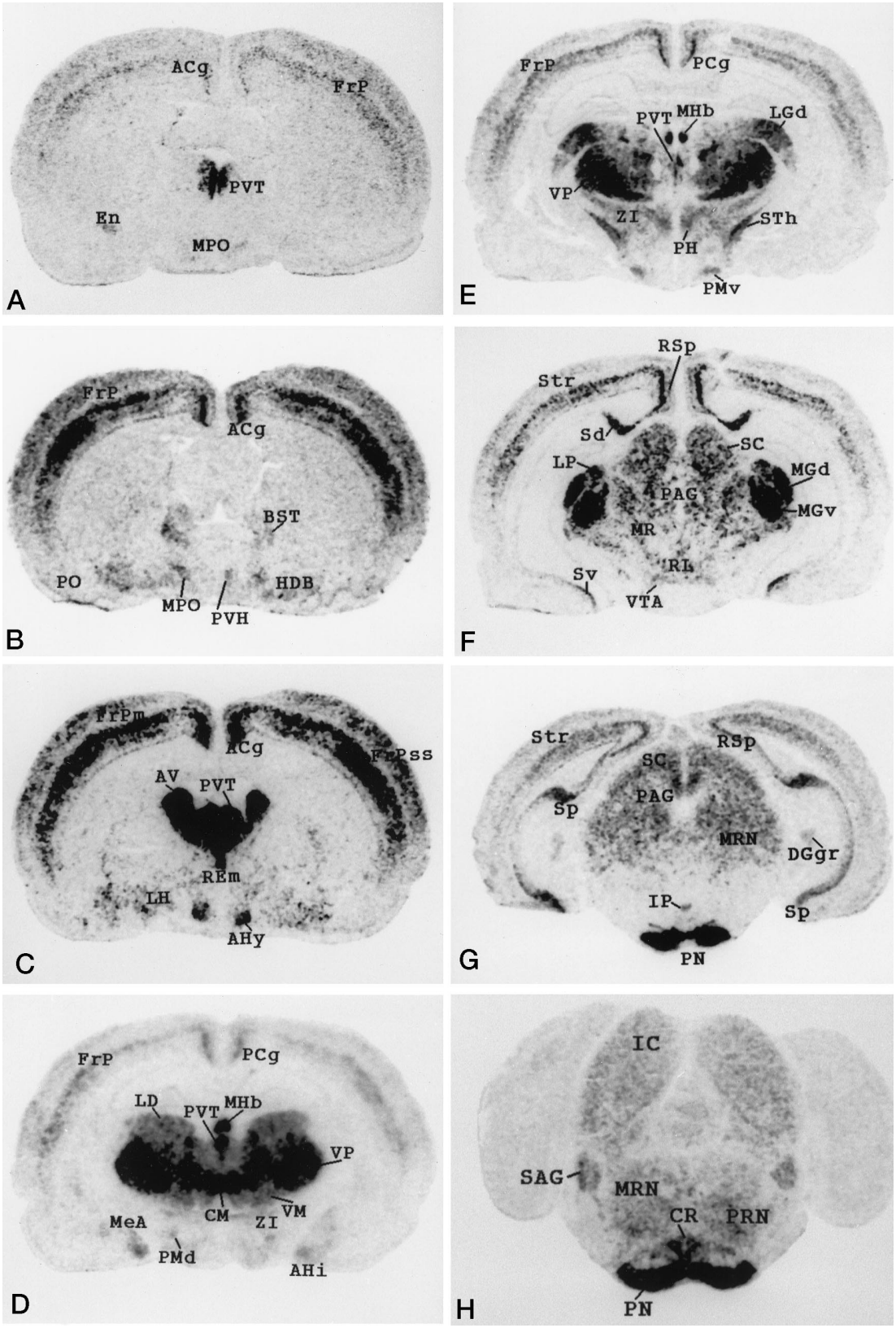

Fig. 6. *In Situ* Hybridization Studies of TRH-R2 mRNA

In situ hybridization studies were performed in coronal sections of rat brain using a ^{35}S-labeled cDNA probe. Representative coronal sections from a total of three separate experiments are shown. The sections are designated by the series of stereotactic coordinates derived from the rat brain atlas of Paxinos and Watson (34). A, Section −0.7 mm from bregma showing dense labeling

signaling (4, 18, 26), it was likely that these differences would lead to differences in TRH signaling mediated by TRH-R1 and TRH-R2 during prolonged TRH exposure. We measured the effect of prolonged stimulation by TRH on induction of transcription of a reporter gene and found that down-regulation of TRH-R2 was associated with a lesser degree of TRH-stimulated gene induction in cells expressing TRH-R2 than in cells expressing TRH-R1. Thus, it appears that TRH-R2 can be stimulated to signal acutely like TRH-R1 but exhibits greater down-regulation/desensitization when exposed to TRH chronically.

During the course of this work, the discovery of rat TRH-R2 was reported independently by two other groups (5, 6). Within the open-reading frame of TRH-R2, the published sequences varied by two single nucleotide differences located in the regions encoding the N-terminal portion of TM4 and the carboxy terminus. Specifically, Itadani *et al*. (5) reported an isoleucine and valine at amino acid positions 143 and 347, respectively, while Cao *et al*. (6) reported a methionine and glutamic acid at these respective positions. By comparison, our TRH-R2 sequence agreed with Ile^{143} in accordance with the report by Itadani *et al*. (5) but with Glu^{347} as reported by Cao *et al*. (6).

In situ hybridization analysis revealed that TRH-R2 mRNA exhibited a distinct brain distribution with especially abundant levels of expression in areas of the cortex, thalamus, and the pontine nucleus (Figs. 6 and 7). In addition, other areas of the midbrain including the medial and lateral geniculate nuclei, superior colliculus, periaqueductal gray, mesencephalic reticular nucleus, and central raphe nucleus displayed discrete levels of TRH-R2 mRNA expression. Together with strong distinct signals from various sensory and motor control areas in the cortex and thalamus (*e.g.* the striate areas of the primary visual cortex, the paraventricular, centromedial, anteroventral, and ventroposterior thalamic nuclei), TRH-R2 may play roles in nociception, motor control, and regulation of somatosensory transmission. Unlike the previous report (6), we found expression of small amounts of TRH-R2 mRNA in the anterior lobe of the pituitary (as seen clearly against the absence of signal in the neurointermediate lobe of the pituitary), suggesting possible roles for TRH-R2 in hormone regulation. Furthermore, dense labeling was seen in the hypothalamus in the anterior and lateral hypothalamic nuclei, as well as moderate levels of expression in the medial preoptic area, paraventricular nucleus, posterior hypothalamic nucleus, and the ventral and dorsal premammillary nuclei, suggesting that TRH-R2 may play a role in appetite regulation, motivation, or other hypothalamic functions. A comparison of the distributions of TRH-R1 and TRH-R2 mRNAs in rat brain (Table 3) shows that there are distinct distributions for the two TRH-Rs although there are some areas in which both receptors are expressed.

In summary, we have described a second GPCR for TRH. The extensive distribution of this receptor in the brain suggests that it mediates many of the known functions of TRH that are not transduced by

in paraventricular nucleus of thalamus (PVT). Signal is also seen in the deeper layers of frontoparietal cortex (FrP), in the primary somatosensory area, and in the anterior cingulate area (ACg). Some labeling is also visualized in the endopiriform nucleus (En) and the medial preoptic area of hypothalamus (MPO). B, Section −0.8 mm from bregma showing very dense signal in the deeper layers of the frontoparietal cortex (FrP) and the anterior cingulate area (ACg). Labeling is also seen in the bed nucleus of the stria terminalis (BST), the medial preoptic area (MPO), the nucleus of the diagonal band (HDB), the paraventricular nucleus of hypothalamus (PVH), and the primary olfactory cortex (PO). C, Section −1.3 mm from bregma showing dense expression in the frontoparietal cortex in the primary somatosensory (FrPss) and primary motor areas (FrPm), and in the anterior cingulate area (ACg). Very dense labeling is also seen in the anteroventral nucleus (AV) and paraventricular nucleus of thalamus (PVT) and in the nucleus reuniens, medial part (REm). Signal is also evident in the anterior hypothalamic nucleus (AHy) and in the lateral hypothalamic area (LH). D, Section −3.3 mm from bregma showing abundant expression in several thalamic nuclei, the venteroposterior nucleus of thalamus (VP) in the medial and lateral divisions, the central medial nucleus of thalamus (CM), the paraventricular nucleus of thalamus (PVT), and the medial habenular nucleus (MHb). Lesser signals were detected in the laterodorsal (LD) and ventromedial (VM) nuclei of thalamus and in the posterior cingulate area (PCg) and frontoparietal cortex (FrP). Labeling is also visualized in the zona incerta (ZI), dorsal premammillary nucleus (PMd), the amygdalohippocampal area (AHi), and the medial nucleus of amygdala (MeA). E, Section −3.9 mm from bregma showing labeling of the frontoparietal cortex (FrP), posterior cingulate area (PCg), and the ventral posterolateral and posteromedial nuclei of thalamus (VP), including the parvicellular portions of these nuclei. Signal is visualized also in the lateral geniculate nuclear complex, dorsal part (LGd), the medial habenular nucleus (MHb), paraventricular nucleus of thalamus (PVT), zona incerta (ZI), subthalamic nucleus (STh), posterior hypothalamic nucleus (PH), and ventral premammillary nucleus (PMv). F, Section −5.3 mm from bregma showing localization of mRNA in the deeper layers of cerebral cortex, in the striate areas (Str) of the primary visual cortex, the retrosplenium, and the subiculum, in both dorsal (Sd) and ventral (Sv) parts. Dense labeling was evident in the medial geniculate nuclear complex, in the dorsal (MGd) and ventral (MGv) parts. Labeling is also present in the superior colliculus (SC), lateral posterior nucleus of thalamus (LP), periaqueductal gray (PAG), mesencephalic reticular nucleus (MR), rostral linear raphe nucleus (RL), and the ventral tegmental area (VTA). G, Section −6.8 mm from bregma showing labeling of the striate areas of primary visual cortex (Str), the retrosplenial area (RSp), the subiculum, pyramidal layer of the ventral part (Sp), and the dentate gyrus crest (DGgr). Signal was also detected in superior colliculus (SC), periaqueductal gray (PAG), mesencephalic reticular nucleus (MRN), pontine nucleus (PN), and interpeduncular nucleus (IP). H, Section −7.9 mm from bregma showing signal in inferior colliculus (IC), nucleus sagulum (SAG), mesencephalic reticular nucleus (MRN), pontine reticular nucleus (PRN), central nucleus raphe (CR), and pontine nucleus (PN).

Table 3. Comparison of the Distributions of TRHR-1 and TRH-R2 in Rat Brain

Region	TRH-R1[a]	TRH-R2	Region	TRH-R1[a]	TRH-R2
Cortex			Hippocampus		
Rhinal cortex	+		Dentate gyrus	+	
Piriform cortex	+		Ammon's horn	+	
Frontoparietal cortex	+	+			
Primary visual cortex		+	Amygdala		
Primary olfactory cortex	+	+	Amygdaloid nuclei	+	+
Anterior cingulate area		+			
Posterior cingulate area		+	Septal region		
Retrosplenium		+	Stria terminalis	+	+
Striate areas		+	Medial septal nucleus	+	
Subiculum	+	+	Lateral septal nucleus	+	
			Septohippocampal nucleus	+	
Thalamus			Nucleus of the diagonal band	+	+
Paraventricular nucleus	+	+			
Centromedial nucleus		+	Corpus striatum		
Anteroventral nucleus		+	Caudate putamen	+	
Ventroposterior nucleus		+	Globus pallidus	+	
Posterior medial nucleus	+		Nucleus accumbens	+	
Laterodorsal nucleus		+			
Lateroposterior nucleus		+	Midbrain and hindbrain		
Ventromedial nucleus		+	Superior colliculus	+	+
Medial habenular nucleus		+	Inferior colliculus		+
Medial reuniens nucleus		+	Periaqueductal gray	+	+
Medial/lateral geniculate nucleus		+	Mesencephalic reticular nucleus		+
			Ventral tegmental area		+
Subthalamic area			Pontine gray		+
Subthalamic nucleus		+	Pontine reticular nucleus		+
			Central/rostral linear raphe nuclei		+
Hypothalamus			Periolivary nucleus	+	
Anterior hypothalamic area	+	+	Nucleus sagulum		+
Lateral hypothalamic area	+	+	Parabrachial nucleus	+	+
Posterior hypothalamic area	+		Motor trigeminal nucleus	+	
Medial preoptic area	+	+	Facial nucleus	+	
Lateral preoptic area	+		Hypoglossal nucleus	+	
Dorsomedial hypothalamic nucleus	+		Dorsal motor nucleus of vagus	+	
Paraventricular nucleus	+	+			
Periventricular nucleus	+		Pituitary		
Suprachiasmatic nucleus	+		Anterior lobe	+	+
Mamillary nucleus	+	+			

Expression of mRNA is indicated by a "+".
[a] TRH-R1 mRNA distribution as reported by Calza *et al.* (35) and Zabavnik *et al.* 1993 (36).

TRH-R1. In addition, the variations in agonist-induced internalization and down-regulation/desensitization of TRH-R2 compared with TRH-R1 suggest important functional and structural differences between the two receptors.

MATERIALS AND METHODS

Cloning of a cDNA Encoding TRH-R2

A rat brain 5′-Stretch cDNA library (CLONTECH Laboratories, Inc., Palo Alto, CA) was amplified by the PCR using *Pfu* polymerase (Stratagene, LaJolla, CA) and a degenerate oligonucleotide based upon the conserved TM 7 (P1: 5′-GAAGGCGTAGADBAEFGGHTT-3′; B = C or G, D = C or G or T, E = A or C or G or T, F = A or C or G, H = A or G) paired with two primers specific for the 5′ (P2: 5′-GGTGGCGACGACTCCTGGAGC-3′) and 3′ (P3: 5′-GACACCAGACCAACTGGTAAT-3′) regions flanking the cDNA library inserts. PCR conditions were as follows: denaturation at 94 C for 30 sec, annealing at 58 C for 40 sec, and extension at 72 C for 1 min, for 30 cycles, followed by a 7-min extension at 72 C. The PCR products were extracted with phenol/chloroform, precipitated with ethanol and electrophoresed on a low melting point agarose gel. PCR product bands were excised from the gel, ligated into the *Eco*RV site of pBluescript SK(-) (Stratagene), and sequenced. One insert appeared to encode a novel GPCR and was labeled with [^{32}P]dCTP-α (NEN Life Science Products, Boston, MA) by nick translation (Amersham Pharmacia Biotech, Arlington Heights, IL) and used to screen the same library amplified above as previously described (27). Positive phage clones were plaque purified and their inserts amplified by PCR using *Pfu* polymerase and primers P2 and P3. The PCR products were blunt-end ligated into the *Eco*RV site of pcDNA3 vector (Stratagene) and sequenced on both strands.

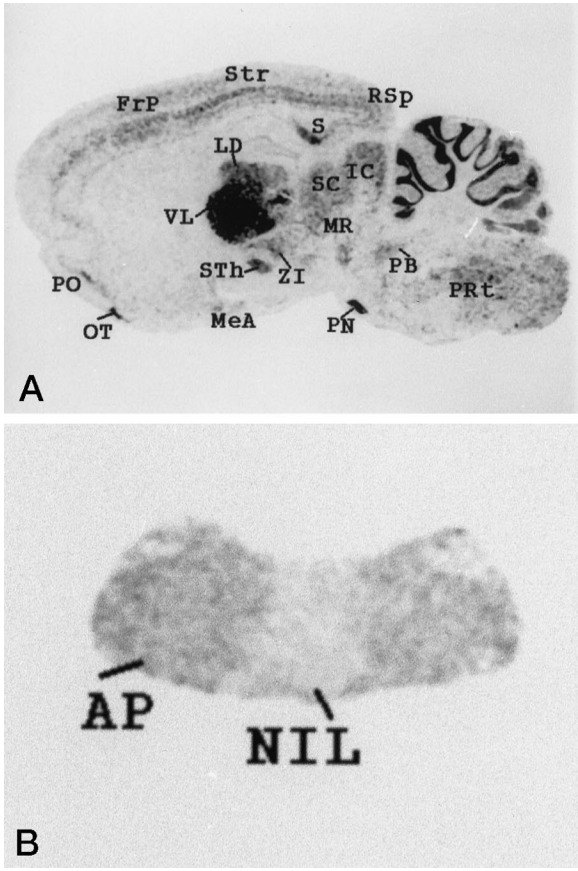

Fig. 7. *In Situ* Hybridization Studies of TRH-R2 mRNA

In situ hybridization studies were performed in sections of rat brain and pituitary gland, using a ^{35}S-labeled cDNA probe. Representative sections from a total of three separate experiments are shown. A, Sagittal section of rat brain 2.4 mm from midline showing signal in frontoparietal areas of cerebral cortex (FrP), and in the striate areas (Str), retrosplenial area (RSp), subiculum (S), and the primary olfactory area (PO). Labeling is also evident in the ventrolateral (VL) and laterodorsal (LD) nuclei of thalamus, the zona incerta (ZI), subthalamic nucleus (STh), olfactory tubercle (OT), and the medial amygdaloid nucleus (MeA). Expression of mRNA is also observed in the superior (SC) and inferior (IC) colliculi, mesencephalic reticular nucleus (MR), parabrachial nucleus (PB), pontine nucleus (PN), and pontine reticular nucleus (PRt). B, Section through pituitary gland showing faint expression of TRH-R2 mRNA in anterior lobe of pituitary (AP) and lack of signal in the neurointermediate lobe (NIL).

Cell Culture and Transfection

COS-1 cells were maintained and transiently transfected using the diethylaminoethyl-dextran method as described previously (28). In brief, cells were seeded 1 or 2 days before transfection at 0.7 to 1.5 $\times 10^6$ cells per 100-mm dish. After transfection, COS-1 cells were maintained in DMEM with 10% FCS for 1 day at which time cells were harvested and seeded into 24-well plates at 50,000 cells per well in DMEM with 5% FCS.

HEK 293 cells were grown in DMEM containing 10% FBS. On the day before transfection, the cells were seeded in 24-well dishes (30,000 cells per well). After 16 h, the medium was aspirated and the cells were transfected using calcium phosphate. The transfection cocktail contained 1 μg/ml receptor-encoding plasmid DNA, 1 μg/ml pFR-Luc, and 1 μg/ml pFA2-CREB (PathDetect *In Vivo* Signal Transduction Pathway *trans*-Reporting System, Stratagene)(7). [TRH-Rs signal via Ca^{2+}/calmodulin-dependent protein kinase (29) that may induce gene transcription via the transcription factor CREB (30).] Total DNA was kept constant by adding "empty" plasmid. "Mock" transfections were performed without receptor-encoding plasmid. The cells were exposed to the transfection cocktail for 6 h and then were incubated in DMEM containing 1% FBS for 16–24 h in the absence (basal) or presence of 1 μM TRH.

Receptor Binding

One day after reseeding into 24-well plates, association binding experiments with [^{3}H]MeTRH (0.1–10 nM) or competition binding experiments were carried out in HBSS, pH 7.4, using 2 nM [^{3}H]MeTRH and various concentrations of unlabeled analogs as described previously (31) with cells in monolayer for 2 h at room temperature. Equilibrium K_d values were derived from association experiments and equilibrium K_i values were derived from competition binding experiments for which curves were fitted by nonlinear regression analysis and drawn with the PRISM program (GraphPad Software, Inc.).

Internalization and Down-regulation

Internalization of TRH-Rs was measured as specifically bound [^{3}H]MeTRH that was resistant to acid wash (17). At the end of the incubation with 2 nM [^{3}H]MeTRH for the times shown, free ligand was removed by aspirating the binding buffer and washing the cells with 2 ml ice-cold buffer. Cells were then exposed to 1 ml 50 mM glycine, pH 3.5, 0.5 M NaCl for 1 min at 4 C, and then washed with 1 ml binding buffer. Acid-resistant [^{3}H]MeTRH was counted and compared with total specific binding. Internalized receptors are presented as the fraction of acid-resistant [^{3}H]MeTRH-bound receptors divided by total [^{3}H]MeTRH-bound receptors.

Down-regulation of TRH-Rs was measured as follows (24). Cells expressing TRH-Rs were incubated in growth medium containing 1 μM TRH for 16–20 h. Thereafter, the medium was aspirated and the cells were washed once with binding buffer, and then with 1 ml 50 mM glycine, pH 3.5, 0.5 M NaCl for 1 min (to remove TRH bound to receptors on the cell surface) and then again with 1 ml binding buffer. Total receptor number was then determined in association binding experiments with [^{3}H]MeTRH (0.1–10 nM).

Acute Signaling

TRH-R-mediated, stimulated phosphoinositide hydrolysis was measured in *myo*-[^{3}H]inositol-labeled cells as described (15).

Chronic Stimulation By TRH

Prolonged signaling by TRH was assayed using a firefly luciferase reporter gene under the control of CREB-responsive promoter in cells exposed to TRH for 24 h. Cells were transfected with plasmids encoding TRH-Rs and pFR-Luc and pFA2-CREB. After 24 h, cells in 24-well plates were washed with PBS and lysed with 0.5 ml of lysis buffer (25 mM GlyGly, pH 7.8, 15 mM $MgSO_4.6H_2O$, 4 mM EGTA, 1 mM dithiothreitol, 1% Triton X-100). Cell lysates (0.025 ml) were combined automatically with 0.125 ml reaction buffer (25 mM GlyGly, pH 7.8, 15 mM $MgSO_4.6H_2O$, 4 mM EGTA, 1 mM dithiothreitol, 15 mM KH_2PO_4, 2 mM ATP) and 0.025 ml luciferin (0.4 mM) in reaction buffer and the luminescence measured for 10 sec in a TR717 Microplate Luminometer (Tropix, Bedford, MA).

Northern Blot Analysis

mRNAs from several rat tissues were extracted as described previously (27). Briefly, total RNA was extracted by the method of Chomczynski and Sacchi (32) and poly $(A)^+$ RNA isolated using oligo(dT) cellulose spin columns (Pharmacia Biotech, Piscataway, NJ). RNA was denatured and size fractionated on a 1% formaldehyde agarose gel, transferred onto nylon membrane, and immobilized by UV irradiation. The blots were hybridized with a ^{32}P-labeled DNA fragment encoding TRH-R2 from TM4 to TM7, washed with 2× SSPE and 0.1% SDS at 50 C for 20 min and again with 0.1× SSPE and 0.1% SDS at 50 C for 2 h, and exposed to x-ray film at −70 C in the presence of an intensifying screen.

In Situ Hybridization Analysis

Preparation of rat brain sections and *in situ* hybridization procedures were done as previously described (33). Briefly, brains were removed from male Sprague Dawley rats (The Jackson Laboratory, Bar Harbor, ME) within 30 sec of decapitation, frozen, sectioned at 14 μm thickness using a microtome cryostat, thaw-mounted onto microscope slides and stored at −70 C. A cDNA fragment encoding the full length TRH-R2 was used as a probe for *in situ* hybridization and was labeled with [^{35}S]dCTP-α (NEN Life Science Products). The rat brain slices were incubated for 2 h in prehybridization solution, hybridized with the labeled probe (10^6 dpm/slice) for 16 h, and washed in conditions of increasing temperature and decreasing salt concentrations. The hybridized sections were dehydrated in a graded alcohol series and exposed to X-ray film (Dupont MRF-34) for 4–6 weeks at −70 C and developed. For use as controls, adjacent sections were hybridized after treatment with RNase, to confirm the specificity of hybridization.

Statistical Analysis

Statistical analysis was performed by *t test*.

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