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25. Total cell poly(A)-rich RNA was prepared from normal rat thyroid, trigeminal ganglia, and a series of brain regions, as previously described (*21*). Aliquots (5 μg) were denatured and subjected to electrophoresis on 1.5 percent agarose-formaldehyde gels. RNA was transferred to nitrocellulose, washed in prehybridization buffer, and hybridized to clonal probes nick-translated to a specific activity of 1×10^8 to 3×10^8 cpm per microgram of DNA, with [α-^{32}P]dCTP as the labeled nucleotide. A p-α-CGRP$_1$ 3′ Hae III–Dde I fragment is specific for the noncoding region of α-CGRP mRNA (designated d in Fig. 2); a p-β-CGRP$_1$, Hpa II–Nco I fragment corresponding to 5′ noncoding information, and a Hpa II–Alu I fragment of p-β-CGRP$_2$ corresponding to the 3′ noncoding region of β-CGRP mRNA provided the β-CGRP mRNA-specific probes. The 5′- and 3′-noncoding β-CGRP probes gave identical results; the 5′ noncoding probe was used in the data shown in (B). Size standards were provided by migration of calcitonin and CGRP mRNA species from rat medullary thyroid tumors (*2*).
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27. Tissue was fixed by vascular perfusion and frozen sections 25 μm thick were cut on a sliding microtome (*28*). Sections were mounted on poly-L-lysine–coated slides and air-dried. Pretreatment, hybridizations, and washing conditions have been described for RNA probes (*29*). Briefly, sections were digested with proteinase K (10 μg/ml, 37°C, 30 minutes), acetylated, and dehydrated. After thorough drying, 50 μl of hybridization mix containing ^{32}P-labeled probe (10^8 cpm/ml) was spotted on each slide. Slides were incubated at 50°C for 16 hours. Slides were rinsed, digested with ribonuclease (RNase A, at 20 μg/ml 37°C, 30 minutes) and washed in 0.1× SSC for 30 minutes at 53°C. After dehydration, the sections were exposed to Cronex 4 film (DuPont) for 15 days at 4°C. Specific RNA probes were generated with in vitro transcription vectors. An α-CGRP vector was produced by cloning the 450-bp Taq I–Sau 3A α-CGRP genomic fragment (f in Fig. 2) into pSP64. This fragment contains 170 bp of single-copy 3′ noncoding sequence and 280 bp of single-copy 3′ flanking sequence. The β-CGRP vector contains a single-copy 220-bp Nco I–Alu I cDNA insert (probe e in Fig. 2) cloned into pGEM-1 (Promega). After the vectors were linearized, runoff transcripts were produced with SP6 polymerase (α probe) or T7 polymerase (β probe) in standard reactions in which only ^{32}P-labeled UTP (600 Ci/mmol was used. Specificity of the α-CGRP and β-CGRP probes was confirmed by Northern blot RNA analysis [see (*30*)].
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31. We thank P. Sawchenko and L. Stolarsky for their advice and discussion of data in manuscript and M. Richards for its preparation. The work was supported by grants from the American Cancer Society and the National Institutes of Health. S.G.A. was supported by a Schering-Plough Foundation Fellowship from the Life Sciences Research Foundation. R.M.E., L.S., M.G.R., and S.L. acknowledge support from the Matthew, Clayton, and McKnight Foundations and the American Cancer Society, respectively. J.L.A. is a predoctoral trainee in the Department of Biology, University of California, San Diego.

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Immunohistochemical Localization in the Rat Brain of the Precursor for Thyrotropin-Releasing Hormone

Abstract. *A rabbit antiserum to a peptide sequence present in the precursor for thyrotropin-releasing hormone (proTRH), deduced from cloned amphibian-skin complementary DNA, was raised by immunization with the synthetic decapeptide Cys-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Cys (proTRH-SH). Immunohistochemical studies on rat brain tissue showed staining of neuronal perikarya in the parvicellular division of the paraventricular nucleus of the hypothalamus and the raphe complex of the medulla, identical to that already described for thyrotropin-releasing hormone (TRH). Immunostaining was abolished by preincubation with proTRH-SH (10^{-6}M) but not TRH (10^{-5}M). Both TRH precursor and TRH were located in neurons of the paraventricular nucleus. However, in contrast to the findings for TRH, no staining was observed in axon terminals of the median eminence. These results suggest that a TRH precursor analogous to that reported in frog skin is present in the rat brain and that TRH in the mammalian central nervous system is a product of ribosomal biosynthesis.*

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The long-standing controversy (*1*) concerning the mode of biosynthesis of thyrotropin-releasing hormone (TRH) has recently been resolved. It is now clear that TRH, like other hypothalamic releasing factors such as luteinizing hormone–releasing (LH-RH) (*2*), arises from the post-translational cleavage of a large precursor protein (*3*) and not by soluble nonribosomal enzymatic mechanisms such as those that produce the small neural peptide carnosine (*4*). On the basis of studies reporting large quantities of TRH in amphibian cutaneous tissue (*5*), Richter *et al.* (*6*) isolated messenger RNA from the skin of the frog *Xenopus laevis* and were able to obtain a complementary DNA (cDNA) clone with an insert of 478 nucleotides coding for a portion of the preprohormone precursor of TRH (preproTRH). The deduced TRH precursor of 123 amino acids contains three copies of the sequence Lys-

Arg-Gln-His-Pro-Gly-Lys/Arg-Arg and a fourth incomplete copy. The paired flanking basic amino acids are potential cleavage sites in peptide biosynthesis (*7*). TRH (pGlu-His-ProNH_2) would then arise after enzymic amidation at the carboxyl terminus with the glycine residue acting as an amide donor (*8*) while glutamine would undergo cyclization to form the mature hormone.

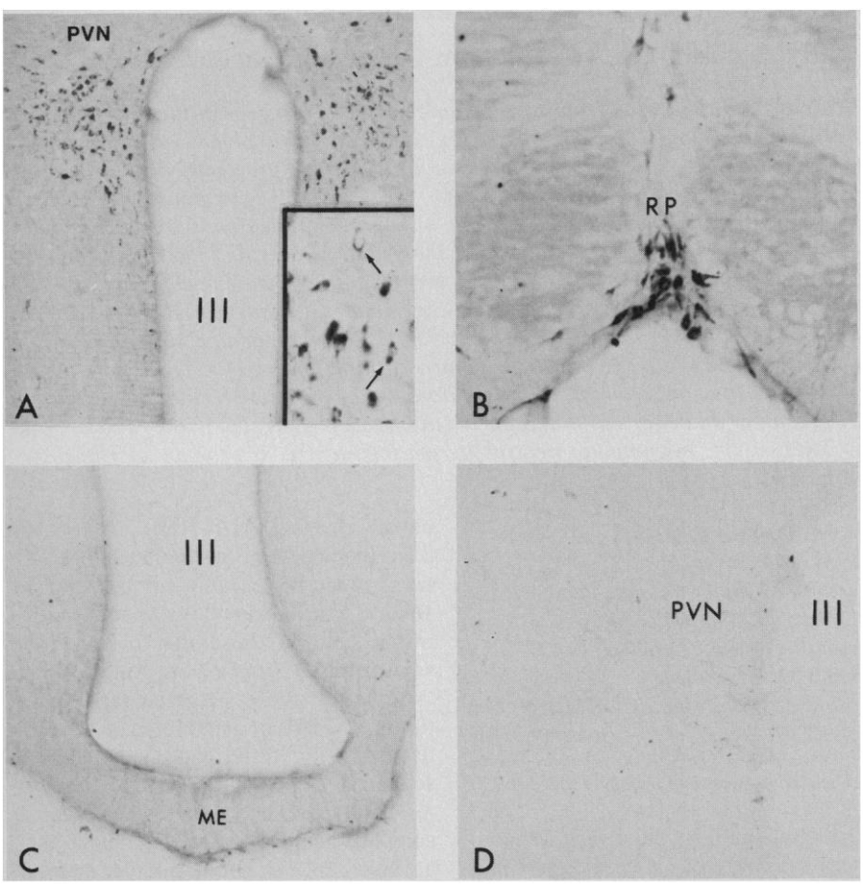
Earlier studies on the TRH precursor have been hampered by the small size of the TRH molecule. Antisera raised against the tripeptide have required that the NH_2-terminal pyroglutamyl ring and the COOH-terminal amide be intact for immunologic reactivity (*9*). Consequently, such antibodies do not recognize COOH-terminal or NH_2-terminal extensions of the TRH sequence as would be present in a TRH precursor.

It is not known whether the mechanism of TRH biosynthesis in the mammalian hypothalamus is similar to that in frog skin. To study this further, we immunized New Zealand White rabbits with the artificial peptide Cys-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Cys (proTRH-SH) (*10*) coupled to bovine thyroglobulin through a lysine residue with glutaraldehyde (*11*) and emulsified in Freund's adjuvant. The terminal cysteinyl residues in this synthetic decapeptide are available for oxidative cyclization (*12*), thereby increasing the probability of generating an antibody directed against the mid-region of the molecule and its extended counterpart sequences in native proTRH.

After 3 months, one antiserum (342) bound ^{125}I-labeled proTRH-SH. The binding was inhibited by synthetic proTRH-SH but not by TRH, various TRH metabolites, or other hypothalamic and neural peptides (*13*).

To determine whether the TRH precursor could be identified in the mammal, we prepared brain tissue from Sprague-Dawley rats (Charles River) fixed by intracardiac perfusion with either 4 percent paraformaldehyde or Bouin's solution. Some animals received stereotactic injections of colchicine (75 μg in 10 μl of saline) into the lateral ventricle 48 hours before fixation so as to improve visualization of immunoreactive neuronal perikarya (*14*). Vibratome sections through the forebrain, medulla oblongata, and spinal cord were prepared for immunocytochemistry by the peroxidase-antiperoxidase (PAP) technique (*15*). Antiserum 342 was used at a titer of 1:750 diluted in 0.05*M* tris-buffered saline, *p*H 7.6, containing 0.2 percent Triton X-100.

Immunoreactivity was localized to neuronal perikarya within the anterior and medial parvicellular division of the paraventricular nucleus (PVN) in the hypothalamus, in the raphe complex of the medulla (Fig. 1, A and B), and in other regions of the forebrain. This distribution is identical to what we have demonstrated as typical for TRH-containing perikarya (*14*). The character of immunostaining with antiserum 342 was distinct, with immunoperoxidase staining present in the cytoplasm near the nucleus (Fig. 1A). No immunoreactive material was seen in axons or axon terminals in the median eminence (Fig. 1C) or in the spinal cord, even in animals that had not been treated with colchicine. In addition, reaction product was rarely seen in first-order dendrites, and



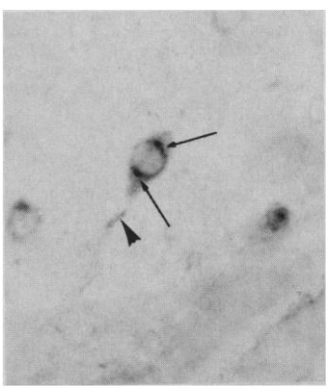


Fig. 1 (left). Coronal vibratome sections (50 μm) through the rat hypothalamus and the medulla, showing the presence of immunoreaction product (PAP technique) in the cytoplasm of parvicellular neurons in the paraventricular nucleus (PVN) (A) and in the raphe pallidus (RP) (B), using an antiserum (342) raised against a synthetic sequence of the frog-skin precursor of proTRH. The reaction product tends to encircle the nucleus (arrows in inset) and is absent from axon terminals in the median eminence (ME) (C). Control section (D) shows the absence of immunoreaction product in the PVN when the antiserum was preadsorbed with the synthetic decapeptide Cys-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Cys; III, third ventricle. Magnification ×128 (A, B, and C); ×320 (inset in A and D). Fig. 2 (right). Sequential immunostaining (PAP technique) of the same paraventricular neuron with antiserum 342 directed against proTRH-SH and with an antibody raised against synthetic TRH. Diaminobenzidine (brown reaction product) was used as the chromagen to localize immunostaining with antiserum 342, and 4-chloro-l-naphthol (blue reaction product) was used to localize immunostaining of the tripeptide TRH. Note intense immunoreactivity encircling the nucleus with antiserum 342 (arrows) in contrast to the delineation of the cell soma and processes with antiserum to TRH (arrowhead). Magnification ×200.

then only in colchicine-treated animals. This is in contrast to the findings with the tripeptide TRH, which fills the cell cytoplasm, dendrites, and axons and is densely concentrated in the external zone of the median eminence (*14*) and the interomediolateral column of the spinal cord (*16*).

We further demonstrated the distinct compartmentalization of immunostaining within neurons in the PVN by sequential immunochemistry (*16*), using antiserum 342 and then antiserum to TRH (*14*) in colchicine-treated animals fixed with acrolein (*17*). Evidence for the presence of the two antigens was seen in parvicellular neurons of the PVN, with antiserum 342 reacting with cytoplasmic components encircling the nucleus and antiserum to TRH delineating the entire cell soma and processes (Fig. 2).

Immunoreactivity in control sections was abolished by preincubating antiserum 342 with $10^{-6}M$ proTRH-SH (Fig. 1D) but not with $10^{-5}M$ synthetic TRH (Bachem). We prevented TRH immunostaining by pretreating TRH antiserum with $10^{-5}M$ synthetic TRH.

These results show that an antiserum (342) raised against a synthetic sequence of the frog-skin TRH precursor (proTRH) immunostains neurons in the rat brain that also contain the tripeptide TRH. Because of the distinct compartmentalization of immunostaining with antiserum 342 in the cell somata, highly suggestive of an association with the Golgi apparatus (*18*), we propose that this antibody reacts specifically with the rat proTRH. Further, since immunoreactivity is confined to the cell body and is not present in axon terminals as is the tripeptide TRH, it appears that processing of the proTRH does not occur during axonal transport as described for propressophysin, the precursor to vasopressin (*19*). Rather, the data indicate that the proTRH is rapidly converted to TRH within the neuronal perikaryon, perhaps even before leaving the Golgi apparatus, in a manner that may be similar to the processing of the enkephalin precursor (*20*).

The findings suggest that a TRH precursor analogous to that reported in amphibian skin is present in the rat brain and that TRH biosynthesis in the mammalian central nervous system occurs by post-translational cleavage of a precursor protein rather than by a nonribosomal enzymic process. The availability of an antibody that specifically recognizes the rat hypothalamic TRH precursor has recently enabled our group to elucidate its cDNA sequence, which encodes a protein with a molecular size close to 30,000 daltons (*21*). These developments confirm the validity of the prohormone antiserum and shed light on the factors involved in the regulation of TRH biosynthesis in the mammalian brain.

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Enhanced Metabolism of *Leishmania donovani* Amastigotes at Acid *p*H: An Adaptation for Intracellular Growth

Abstract. *Amastigotes (tissue forms) of* Leishmania donovani *isolated from infected hamster spleens carried out several physiological activities (respiration, catabolism of energy substrates, and incorporation of precursors into macromolecules) optimally at* p*H 4.0 to 5.5. All metabolic activities that were examined decreased sharply above the optimal* p*H. Promastigotes (culture forms), on the other hand, carried out the same metabolic activities optimally at or near neutral* p*H. This adaptation to an acid environment may account in part for the unusual ability of amastigotes to survive and multiply within the acidic environment of the phagolysosomes in vivo.*

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Leishmania donovani, the agent of visceral leishmaniasis, is a dimorphic protozoan parasite. In tissues of infected hosts, amastigote forms of the organism establish residence within macrophages of reticuloendothelial organs by phagocytic engulfment. Macrophages of human and animal reservoirs serve as obligatory host cells that permit the survival and intracellular multiplication of leishmania amastigotes. *Leishmania donovani* and other leishmanial species do not inhibit phagosome-lysosome fusion after ingestion by phagocytic cells (*1–5*), as do several other intracellular pathogens (*6–8*). Thus leishmanias have mechanisms that permit survival, growth, and cell division in an environment that is inimical to most life forms. Unlike the rickettsiae (*9*) and *Trypanosoma cruzi* (*10*), both of which escape from phagocytic vesicles into the cell