highly responsive, genetically inbred mice. Cultured parasites may be more susceptible to inhibitory agents than parasites developing in vivo. Since a single developing schizont is sufficient to induce a blood infection, and since some sporozoites manage to develop even in the presence of antibodies, the humoral protection resulting from immunization may vary from one individual to the other and may depend in part on the size of the challenge inoculum.

In our culture assay, only diluted sera could be tested. Since a positive relation was found between the degree of invasion inhibition and the level of antibody, a correspondingly greater effect might be expected in vivo, where the antibody would be undiluted. Antibodies may also cooperate with other components of the immune system, such as macrophages and Kupffer cells, particularly if sporozoites have to transit these cells in vivo to reach the hepatocyte (*14*). Sensitized T lymphocytes could also play a toxic role or interfere indirectly by releasing products such as gamma interferon.

Finally, it is encouraging that alum, which is licensed for use in man, was nearly as effective as CFA in inducing protective antibodies.

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Thyrotropin-Releasing Hormone Precursor: Characterization in Rat Brain

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To characterize the precursor of mammalian thyrotropin-releasing hormone (TRH), a rat hypothalamic λgt11 library was screened with an antiserum directed against a synthetic peptide representing a portion of the rat TRH prohormone. The nucleotide sequence of the immunopositive complementary DNA encoded a protein with a molecular weight of 29,247. This protein contained five copies of the sequence Gln-His-Pro-Gly flanked by paired basic amino acids and could therefore generate five TRH molecules. In addition, potential cleavage sites in the TRH precursor could produce other non-TRH peptides, which may be secreted. In situ hybridization to rat brain sections demonstrated that the pre-proTRH complementary DNA detected neurons concentrated in the parvocellular division of the paraventricular nucleus, the same location as cells detected by immunohistochemistry. These findings indicate that mammalian TRH arises by posttranslational processing of a larger precursor protein. The ability of the TRH prohormone to generate multiple copies of the bioactive peptide may be an important mechanism in the amplification of hormone production.

THYROTROPIN-RELEASING HORMONE (TRH, pyroGlu-His-ProNH_2) has a central role in the regulation of the hypothalamic-pituitary-thyroid axis (*1*). Although TRH was the first hypophysiotropic peptide to be characterized structurally (*2*), the mechanism of its biosynthesis has been controversial. The initial hypothesis that TRH was synthesized by a nonribosomal mechanism (*3*) has not been confirmed (*4*). The alternative view, that TRH arose by a messenger RNA (mRNA)–directed ribosomal mechanism, was suggested by several subsequent studies (*5*) and is supported by the isolation of a complementary DNA (cDNA) encoding a portion of the TRH precursor from frog skin (*6*), a tissue that has a relative abundance of TRH (*7*).

To determine whether TRH in the mammalian hypothalamus also arises from the posttranslational cleavage of a larger precursor protein, we raised an antiserum (No. 342) against a synthetic peptide hypothesized to represent a portion of the mammalian TRH prohormone (*8*). Immunocytochemical studies indicated that this antiserum recognizes the rat TRH prohormone rather than the fully processed peptide (*8*). This provided us with the means to identify a cDNA that encodes the TRH precursor from a rat hypothalamic λgt11 bacteriophage expression library based on methods described by Young and Davis (*9*). Antisera against TRH itself cannot be used to screen such libraries because of the extensive posttranslational processing undergone by this peptide. Because of the small size of the TRH sequence and the degeneracy of the codons representing the three amino acids, the application of conventional hybridization techniques to identify a cDNA encoding mammalian hypothalamic TRH would have presented several problems. Furthermore, it seemed likely that the nucleotide sequences encoding the amphibian and mammalian TRH precursors have diverged.

The expression library was prepared by isolating polyadenylated RNA from 65 adult Sprague-Dawley rat hypothalami to generate double-stranded cDNA (*10*). Approximately 3×10^7 recombinant phage with an average insert size of 500 base pairs (bp) were generated from 1 μg of insert cDNA. The library was plate-amplified to a titer of 10^{10} plaque-forming units per milliliter. Plaques (7.5×10^5) from the rat hypothalamic λgt11 library were screened with the proTRH antiserum. Of these, eight immunopositive bacteriophage clones were identified and purified by sequential low-density plating (Fig. 1). The largest of the cDNA's (pLW 4-2, 1322 bp) was subcloned into the plasmid pUC-12 for further study.

The restriction map and sequencing strategy used to characterize pLW 4-2 are illustrated in Fig. 2A. The nucleotide and corresponding amino acid sequence are depicted in Fig. 2B. This cDNA includes an open

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reading frame of 765 bp flanked by 102 and 454 bp of 5′ and 3′ untranslated sequences, respectively. Using an antisense RNA probe prepared from pLW 4-2 for in situ hybridization histochemistry of rat brain, we observed silver grains densely concentrated over cell bodies in the anterior and medial parvocellular divisions of the paraventricular nucleus as well as in other locations in the hypothalamus (Fig. 3). These regions contain neurons that are immunoreactive for both TRH (*11*) and the TRH prohormone (*8*), demonstrating that pLW 4-2 represents an mRNA in TRH-producing neurons. Blot hybridization analysis (*12*) of mRNA isolated from rat hypothalamus demonstrated that the pre-proTRH mRNA is approximately 1700 nucleotides in length, indicating that pLW 4-2 is nearly full-length.

The cDNA sequence of the TRH precursor encodes a protein of 255 amino acids (molecular weight, 29,247) (Fig. 2B). The amino terminus is rich in hydrophobic amino acids characteristic of a signal region (*13*). The sequence Gln-His-Pro-Gly occurs five times in the deduced polypeptide. In each instance, this sequence is flanked by paired basic residues, suggesting that five TRH molecules could be generated from the precursor.

In addition to TRH, posttranslational processing of the prohormone at paired basic residues could produce seven other peptides ranging in size from 10 to 49 amino acids. Two of these peptides may

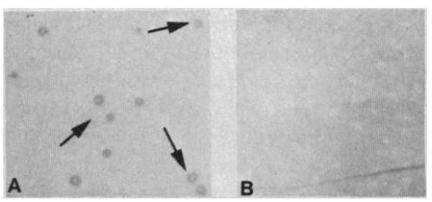
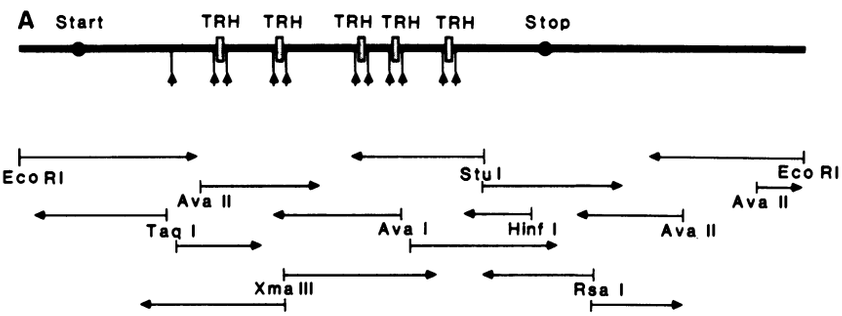


Fig. 1. (A) Appearance of immunopositive plaques identified with an antiserum against proTRH. An aliquot of amplified λgt11 hypothalamic library was adsorbed onto *Escherichia coli* (Y1090) and plated at a density of 20,000 plaques per 150 mm of enriched LB-ampicillin plate. The bacteriophages were grown at 42°C for 4 hours to induce lysis. Nitrocellulose filters precoated with 10 m*M* isopropylthio-β-D-galactoside (IPTG, Bethesda Research Laboratories) were overlaid on the plaques and incubated for 2.5 hours at 37°C to induce fusion protein expression. After marking the position of the filters with a needle, the filters were removed and washed in 30 ml of 0.05*M* tris-buffered saline (TBS) (*p*H 7.4) for 10 minutes. Filters were subsquently incubated for 60 minutes in 30 ml of TBS containing 10 percent normal goat serum (NGS) and 1 percent bovine serum albumin (BSA) to prevent nonspecific binding of immunoglobulins. Antiserum to proTRH (No. 342), preadsorbed with a lysate of *E. coli* to diminish background staining, was diluted 1:500 in TBS containing NGS and 0.5 percent BSA and incubated with the filters overnight at room temperature on a rotary shaker. After being washed in TBS, the filters were incubated for 4 hours at room temperature on a rotary shaker with goat antibody to rabbit immunoglobulin G conjugated to alkaline phosphatase (Miles Scientific) diluted 1:400. After being washed in TBS containing 1 percent sodium deoxycholate and 1 percent Triton X-100, the filters were reacted with 0.04 percent naphthol AS-MX phosphate (Sigma) and 0.5 percent fast red TR salt (Sigma) for 1 hour in the dark. (B) Control nitrocellulose disk showing the absence of reaction product when antiserum 342 had been preadsorbed with 10^{-6}*M* of the synthetic decapeptide (proTRH-SH) 18 hours before the immunochemical procedure was initiated.



B

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                                                        TCCTTGGATTCGGGAGTATTGCAAACTCTAC
CCAGCCAGTTTGCACTCTTCAGCTCAGCATCTTGGAAAGCTCTGCAGAGTCTCCACTTCGCAGACTCCAGG 102

ATG CCG GGA CCT TGG TTG CTG CTG GCT CTG GCT TTG ATC TTC ACC CTA ACT GGT
 1 Met-Pro-Gly-Pro-Trp-Leu-Leu-Leu-Ala-Leu-Ala-Leu-Ile-Phe-Thr-Leu-Thr-Gly

ATC CCT GAA TCC TGC GCC TTG CCG GAG GCA GCC CAG GAG GAA GGT GCA GTG ACT 210
 Ile-Pro-Glu-Ser-Cys-Ala-Leu-Pro-Glu-Ala-Ala-Gln-Glu-Glu-Gly-Ala-Val-Thr

CCT GAC CTT CCT GGC CTG GAG AAT GTT CAG GTC CGG CCA GAA CGT CGA TTC TTG
37 Pro-Asp-Leu-Pro-Gly-Leu-Glu-Asn-Val-Gln-Val-Arg-Pro-Glu-Arg-Arg-Phe-Leu

TGG AAA GAC CTC CAG CGG GTG AGA GGG GAC CTC GGT GCT GCC TTA GAC TCC TGG 318
Trp-Lys-Asp-Leu-Gln-Arg-Val-Arg-Gly-Asp-Leu-Gly-Ala-Ala-Leu-Asp-Ser-Trp

ATC ACA AAA CGC CAG CAT CCA GGC AAA AGG GAG GAG GAG GAA AAA GAC ATT GAA
73 Ile-Thr-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Glu-Glu-Glu-Glu-Lys-Asp-Ile-Glu

GCT GAA GAG AGG GGA GAC TTG GGA GAA GGG GGA GCC TGG AGA CTC CAC AAA CGA 426
Ala-Glu-Glu-Arg-Gly-Asp-Leu-Gly-Glu-Gly-Gly-Ala-Trp-Arg-Leu-His-Lys-Arg

CAG CAC CCC GGC CGA CGT GCC AAC CAG GAC AAG TAT TCA TGG GCA GAT GAG GAG
109 Gln-His-Pro-Gly-Arg-Arg-Ala-Asn-Gln-Asp-Lys-Tyr-Ser-Trp-Ala-Asp-Glu-Glu

GAC AGT GAC TGG ATG CCA CGG TCC TGG TTA CCA GAT TTC TTT CTG GAT TCC TGG 534
Asp-Ser-Asp-Trp-Met-Pro-Arg-Ser-Trp-Leu-Pro-Asp-Phe-Phe-Leu-Asp-Ser-Trp

TTC TCA GAT GTC CCC CAA GTC AAG CGG CAG CAC CCT GGC AGG CGA TCC TTC CCC
145 Phe-Ser-Asp-Val-Pro-Gln-Val-Lys-Arg-Gln-His-Pro-Gly-Arg-Arg-Ser-Phe-Pro

TGG ATG GAG TCT GAT GTC ACC AAG AGG CAA CAT CCA GGC CGG AGG TTC ATA GAT 642
Trp-Met-Glu-Ser-Asp-Val-Thr-Lys-Arg-Gln-His-Pro-Gly-Arg-Arg-Phe-Ile-Asp

CCC GAG CTC CAA AGA AGC TGG GAA GAA AAA GAG GGA GAG GGT GTC TTA ATG CCT
181 Pro-Glu-Leu-Gln-Arg-Ser-Trp-Glu-Glu-Lys-Glu-Gly-Glu-Gly-Val-Leu-Met-Pro

GAG AAA CGC CAG CAT CCT GGC AAA AGG GCA TTG GGT CAT CCC TGT GGG CCC CAG 750
Glu-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Ala-Leu-Gly-His-Pro-Cys-Gly-Pro-Gln

GGG ACT TGT GGT CAA ACA GGC CTG CTC CAG CTT CTA GGT GAC CTG AGC AGG GGT
217 Gly-Thr-Cys-Gly-Gln-Thr-Gly-Leu-Leu-Gln-Leu-Leu-Gly-Asp-Leu-Ser-Arg-Gly

CAG GAG ACC CTG GTG AAG CAA AGC CCA CAA GTG GAA CCC TGG GAC AAG GAA CCT 858
Gln-Glu-Thr-Leu-Val-Lys-Gln-Ser-Pro-Gln-Val-Glu-Pro-Trp-Asp-Lys-Glu-Pro

CTG GAG GAG TAAGGCCAGAGTCAGGCTTTAGGTCTAGGATGATGTAAGCCCTGTATTCCCTATCCTGT
253 Leu-Glu-Glu ***

TCCCTTCACTAGCTGTCCTCTCTTAGATGCTAACCCTGGGCCCTCTGTACATCGTCCACCCAAACCCCTTC 997
CTTACCGACTTCAGAGACTTTAGAAAGCCAGTCAGGAAGTTAAAACCCTACTTATCCCTTCAAGCAAGGGG
GTGGGAGTCACACCCCTTCAGCACTGGCCAAGATGGTTCTTTCACACCTTCTAAGGGACCTCCTCAGAAAG
GAAGGGTAGAATTGAAATGTTTTGGTGTTAAAACTTCTGTAATCTGCCCCATGTGGTAAGACTGACCTGGT
ATAGCTTCAGCGCATCCTCCAAGGTTGGGGTCCCTGAGCAGTTTGGGAGATGTTTAGATATGTCCTTGGGG 1281
TGGGGGGTCGCATCTTGTATATAACCCTGCTCTTGGGAAT
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Fig. 2. (A) Restriction map and sequencing strategy used in characterizing the pre-proTRH immunoreactive clone, pLW 4-2. Horizontal arrows indicate the direction of sequencing and the location and length of sequenced regions. DNA fragments were labeled at the 5′ end with γ-[^{32}P]adenosine triphosphate and polynucleotide kinase and were sequenced by the method of Maxam and Gilbert (*19*). Location of paired dibasic residues in the deduced TRH prohormone is denoted by upward arrows. (B) Nucleotide sequence of the cDNA insert from clone pLW 4-2 and the predicted amino acid sequence of pre-proTRH. The Arg-Arg residues contained in the amino terminal portion of the prohormone and the repeating sequences of TRH and their flanking dibasic amino acids are underlined. The stop codon is designated by three asterisks. The region underlined by dashes designates the predicted signal region (*20*). Amino acids are numbered on the left and nucleotides on the right.

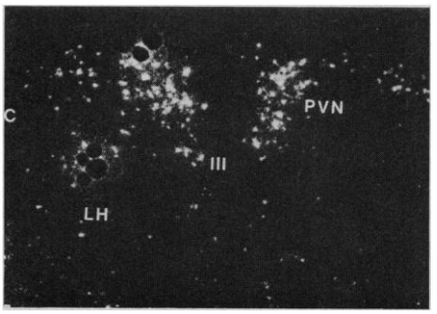

Fig. 3. In situ hybridization histochemistry (dark-field photomicrograph) of pre-proTRH mRNA in a 20-μm coronal section through the hypothalamus of rat brain fixed with 4 percent paraformaldehyde. A Pst I–Eco RI fragment (1241 bp) of pLW 4-2 was inserted into the expression vector pSp65 in reverse orientation and used to generate a [^{32}P]guanosine 5′-triphosphate–labeled, antisense, single-stranded RNA probe. The tissue was hybridized for 16 hours at 43°C in a buffer containing 2× SSC, 250 m*M* tris (*p*H 7.5), 0.5 percent sodium pyrophosphate, 0.5 percent SDS, 10 percent dextran sulfate, 0.25 percent PVP 360, 50 percent formamide, 0.25 percent bovine serum albumin, 0.25 percent Ficoll 400, denatured salmon sperm DNA (250 μg/ml), and the radiolabeled (5 × 10^5 count/min per 10 μl) antisense RNA probe (*21*). Silver grains are seen in the autoradiogram after a 6-day exposure over neurons in the paraventricular nucleus (PVN) and in the lateral hypothalamus (LH). Original magnification ×40.

arise from the amino terminal portion of the molecule after cleavage of an Arg-Arg sequnce 22 amino acids upstream from the first TRH sequence (Fig. 2, A and B). Some of these non-TRH peptides may be secreted. This possibility is supported by the observation that an epitope that we have immunologically identified in dense core vesicles in axon terminals in the median eminence (*14*) also appears to be present in the fusion protein produced by the proTRH immunopositive bacteriophage (*15*). We previously speculated that this epitope may represent part of the TRH precursor molecule (*14*). In addition, the presence of immunoreactive proTRH (*16*) and TRH mRNA (*17*) in regions of the central nervous system in which the tripeptide TRH has not been identified (*12, 14*) suggests that processing of the TRH prohormone to peptides other than TRH may occur in certain brain regions.

These findings establish that the mode of TRH biosynthesis in the mammalian brain is by posttranslational cleavage of a larger precursor protein. Like the enkephalin precursor (*18*), processing of proTRH could yield several copies of the biologically active peptide but may also generate other peptides of potential importance. Only the repeated TRH coding units dispersed throughout the precursor, however, are maintained between the amphibian (*6*) and mammalian prohormones. Conservation of this pattern throughout evolution suggests that the ability of a precursor to generate multiple bioactive peptides may be an important mechanism in the amplification of hormone production.

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12. Polyadenylated mRNA was isolated from rat hypothalamus, electrophoresed on a denaturing 1 percent agarose gel, and transferred to Zetabind (AMF) by electroblotting. The blot was prehybridized for 3 hours at 42°C in 6× standard saline citrate (SSC), 5× Denhardt's reagent, 50 percent formamide containing salmon sperm DNA (500 μg/ml), and hybridized for 48 hours at 68°C in the same buffer containing antisense RNA probe (4 × 10^6 cpm/ml). The antisense RNA probe was generated by inserting a Pst I–Eco RI fragment of pLW4-2 into the expression vector pSp65 (Promega Biotech). The blot was washed two times at 68°C in 2× SSC and 0.1 percent sodium dodecyl sulfate (SDS) and then twice in 0.2× SSC and 0.1 percent SDS at the same temperature. The autoradiogram was developed after exposure to x-ray film for 30 minutes at −70°C with an intensifying screen.
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Systemic Ethanol: Selective Enhancement of Responses to Acetylcholine and Somatostatin in Hippocampus

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In rat hippocampal pyramidal cells tested in situ by iontophoresis of several neurotransmitters, ethanol significantly enhanced excitatory responses to acetylcholine and inhibitory responses to somatostatin-14 but had no statistically significant effect on excitatory responses to glutamate or inhibitory responses to γ-aminobutyric acid or, in preliminary tests, to norepinephrine or serotonin. The effects of ethanol on responses to acetylcholine and somatostatin-14 may provide insight into synaptic mechanisms underlying the behavioral consequences of ethanol intoxication.

ETHANOL HAS PRONOUNCED EFfects on human behavior and a wide variety of effects on neuronal activity (*1, 2*). However, in spite of considerable research, the basic neuronal mechanisms underlying ethanol intoxication, tolerance, and dependence remain to be elucidated. Previously, we (*3*) evaluated the effects of ethanol on synaptic transmission in the rat hippocampus, because this aspect of neuronal activity is highly sensitive to the action of psychoactive drugs (*4*), including ethanol (*2, 5*). Indeed, systemic application of ethanol at doses associated with behavioral intoxication increased the excitatory and in-

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