Pyroglutamyl Peptidase II Inhibition Enhances the Analeptic Effect of Thyrotropin-Releasing Hormone in the Rat Medial Septum

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ABSTRACT

Thyrotropin-releasing hormone (TRH; pGlu-His-Pro-NH₂) has multiple, but transient, homeostatic functions in the brain. It is hydrolyzed in vitro by pyroglutamyl peptidase II (PPII), a narrow specificity ectoenzyme with a preferential localization in the brain, but evidence that PPII controls TRH communication in the brain in vivo is scarce. We therefore studied in male Wistar rats the distribution of PPII mRNA in the septum and the consequence of PPII inhibition on the analeptic effect of TRH injected into the medial septum. Twelve to 14% of cell profiles expressed PPII mRNA in the medial-septum-diagonal-ban of Broca; in this region the specific activity of PPII was relatively high. Twenty to 35% of PPII mRNA-labeled profiles were positive for TRH-receptor 1 (TRH-R1) mRNA. The intramedial septum injection of TRH reduced, in a dose-dependent manner, the duration of ethanol-induced loss of righting reflex (LORR). Injection of the PPII inhibitor pGlu-Asn-Pro-7-amido-4-methylcoumarin into the medial septum enhanced the effect of TRH. The injection of a phosphinic TRH analog, a higher-affinity inhibitor of PPII, diminished the duration of LORR by itself. In contrast, the intraseptal injection of pGlu-Asp-Pro-NH₂, a peptide that did not inhibit PPII activity, or an inhibitor of prolyl oligopeptidase did not change the duration of LORR. We conclude that in the medial septum PPII activity may limit TRH action, presumably by reducing the concentration of TRH in the extracellular fluid around cells coexpressing PPII and TRH-R1.

Introduction

Thyrotropin-releasing hormone (TRH) is a small peptide (pGlu-His-Pro-NH₂) that in mammals regulates anterior pituitary secretions, including that of thyrotropin (TSH) and prolactin, as well as multiple homeostatic brain responses (Gary et al., 2003). These homeostatic effects are transient in part because TRH has an elimination half-life of a few minutes in vivo either in serum or in the brain (Jackson et al., 1979; Nagai et al., 1980; Spindel et al., 1981). There is no definitive consensus on the pathway of TRH inactivation in the brain extracellular space in vivo. Various peptidases have been proposed as critical. TRH is hydrolyzed in vitro to pGlu-His-Pro by prolyl oligopeptidase (POP; EC 3.4.21.26), an enzyme with multiple in vitro substrates that is localized in the cytosolic and synaptosomal membrane compartments in the brain (O’Leary and O’Connor, 1995). However, it is not yet known whether the membrane-bound form of POP is an ectoenzyme or what the biological role of POP is in the brain in vivo (Myöhänen et al., 2009). Another enzyme, pyroglutamyl peptidase II (PPII; EC 3.4.19.6; TRH-degrading ectoenzyme), hydrolyzes the pyroglutamyl-histidyl bond of TRH in vitro. PPII specificity is narrow, with TRH possibly its main substrate in vivo; its expression and activity are higher in the brain than in any other organ. PPII is an ectoenzyme enriched in synaptosomes, thus apparently adequately localized to hydrolyze an extracellular substrate (Charli et al., 1998; Heuer et al., 1998b).

The in vivo relevance of PPII has been evaluated in the context of the regulation of TSH secretion. The intraperitoneal injection of PPII inhibitors does not change basal serum levels of thyroid-stimulating hormone (TSH) in rats (Lindberg et al., 2001). This work was supported in part by the Consejo Nacional de Ciencia y Tecnología [Grant 61804] (to J.-L.C.); and the Dirección General de Asuntos del Personal Académico of the Universidad Nacional Autónoma de México [Grant IN221109] (to J.-L.C.). I.L. is a student in the Programa de Posgrado en Ciencias Biológicas de la Universidad Nacional Autónoma de México. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. http://dx.doi.org/10.1124/jpet.112.192278.

ABBREVIATIONS: TRH, thyrotropin-releasing hormone; ¥TRH, pGlu¥[P(O)(OH)]His-Pro-NH₂; TRH-R, TRH receptor; aCSF, artificial cerebrospinal fluid; DBB, diagonal band of Broca; hDBB, horizontal DBB; vDBB, vertical DBB; DMSO, dimethyl sulfosside; ISH, in situ hybridization; LORR, loss of righting reflex; MCA, 7-amido-4-methylcoumarin; MS, medial septum; PCR, polymerase chain reaction; POP, prolyl oligopeptidase; PPII, pyroglutamyl peptidase II; rPPII, recombinant PPII; RT, reverse transcription; TSH, thyrotropin; ZPP, N-benzyloxy carbonyl prolyl proline; JTP-4819, ((S)-2-[(S)-2-(hydroxyacetyl)-1-pyrrolidinyl]carboxy)-N-phenylmethyl)-1-pyrrolidinecarboxamide. 
both injected into the MS-DBB. The impact of PPII inhibitors on the analeptic effect of TRH, mined whether cells coexpress PPII and TRH-R1 mRNAs, effect of TRH involves cholinergic projection pathways, although PPII mRNA levels are relatively low in the MS-DBB lateral part of the MS-DBB, which generates cholinergic behavioral output of TRH activity (McCown et al., 1986). In this study, we tested the hypothesis that PPII controls TRH effects in the medial septum (MS)-diagonal band of Broca (DBB). We focused on the MS-DBB, part of the arousal circuits (Berridge 2008), because TRH injection in this region produces a reproducible analeptic effect that can be used as a behavioral output of TRH activity (McCown et al., 1986).

In the MS-DBB, TRH receptor 2 (TRH-R2) mRNA is concentrated in the medial part of the MS, which sends GABAergic projections to the hippocampus, whereas TRH receptor 1 (TRH-R1) mRNA levels are higher and concentrated in the lateral part of the MS-DBB, which generates cholinergic projections to the hippocampus (Heuer et al., 2000; O’Dowd et al., 2000). Levels of TRH binding sites are moderate and PPII mRNA levels are relatively low in the MS-DBB (Manaker et al., 1985; Heuer et al., 1998a). The analeptic effect of TRH involves cholinergic projection pathways, although other neurons may also be implicated (Horiga, 1998). To understand the role of PPII in the MS-DBB, we determined whether cells coexpress PPII and TRH-R1 mRNAs, because MS TRH seems to regulate cholinergic neurons and the impact of PPII inhibitors on the analeptic effect of TRH, both injected into the MS-DBB.

Materials and Methods

Animals. Adult male Wistar rats, weighing 270 to 350 g and raised at the animal facility of the Instituto de Biotecnología, Universidad Nacional Autónoma de México, were naïve to any previous treatment. Animals were maintained in standard environmental conditions (3–4 per cage; lights on between 7:00 AM and 7:00 PM; temperature 21 ± 2°C) and received ad libitum standard rat chow (2018S; Harlan, Indianapolis, IN) and tap water. Animal care and protocols were approved by the Animal Care and Ethics Committee of the Instituto de Biotecnología; animals were used according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

In Situ Hybridization Histochemistry. Serial 20-µm frozen brain coronal sections through the rostro-caudal extent of the septum were cut on a cryostat, adhered to slides treated with gelatin and poly-l-lysine, and stored at −70°C until prepared for in situ hybridization as described previously (de Gortari et al., 2006).

Sections were hybridized with a single-stranded [35S]UTP-labeled RNA probe complementary to the full-length rat TRH-R1 cDNA (Zhu et al., 2002) or a single-stranded [35S]UTP-labeled RNA probe complementary to the rat PPII cDNA (nucleotides 129–773; Schauder et al., 1994), each reduced to a 200- to 300-nucleotide size by alkaline hydrolysis. Hybridization was performed at 52°C as described previously (de Gortari et al., 2006). After posthybridization washes, sections were dipped into nitroblue tetrazolium autoradiography emulsion (Eastman Kodak Co., Rochester, NY) diluted 1:1 in distilled water. After 30 days of exposure at 4°C, silver grains were developed, and sections were counterstained with hematoxylin.

In some experiments, we used a single-stranded digoxygenin-UTP-labeled RNA probe complementary to the rat PPII gene (2 µg/ml). Digoxygenin detection was performed essentially as described previously (Sánchez et al., 1997), except that the antitoxin-oxyn- gen antibody was conjugated to alkaline phosphatase. Endogenous alkaline phosphatase was inactivated with 0.24 mg/ml levamisole (Sigma, St. Louis, MO), and a solution of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphatase was used to develop the color (Roche Diagnostics, Mannheim, Germany).

For double in situ hybridization, sections were hybridized with both a single-stranded [35S]UTP-labeled RNA probe complementary to TRH-R1 gene and a single-stranded digoxygenin-UTP-labeled RNA probe complementary to the rat PPII gene (2 µg/ml), each reduced in size by alkaline hydrolysis. After posthybridization washes, slides were treated to detect the digoxygenin-UTP-labeled RNA probe, dried, treated with 2% paraloid (Thermo Fisher Scientific, Waltham, MA), dissolved in acetone, and dipped into autoradiography emulsion.

Specificity of hybridizations was confirmed by various criteria. Sense probes generated only background signals (see, for example, Fig. 1). With antisense probes, whatever the labeling used, signal distributions were consistent with those of PPII and TRH-R1 mRNAs (Heuer et al., 1998a). We detected TRH-R1 mRNA in the dentate gyrus (granular layer), but not in the thalamic reticular, ventral, and central nuclei; PPII mRNA was detected in the CA2 region of the hippocampus and the piriform cortex, but not in the thalamic reticular paraventricular nuclei or layer 1 of the cerebro-

levels, but it increases (or extends) TRH- or cold stress-induced serum TSH levels (Scalabrino et al., 2007; Sánchez et al., 2009). Given that PPII activity is low in the anterior pituitary (Vargas et al., 1987), it is not expressed on thyro-}

hybridization as described previously (de Gortari et al., 2006).

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registered by the computer, and the number of cells for each category was counted. The resulting photographic images and maps were stored.

For single ISH with a [35S]UTP-labeled RNA probe, we ascribed silver grains to a hematoxylin-stained nuclear profile if they localized on the nucleus or in close proximity. For sections hybridized with a digoxigenin-UTP-labeled RNAprobe, labeled profiles were taken as positive if the blue purpura color was easily distinguished from the background. For double in situ hybridization, grains were ascribed to a color-labeled profile if they coincided with the color or were in close proximity. Profiles detected with a [35S]UTP-labeled RNA probe were considered positive if at least six grains were detected on the profile; they were separated in two populations: 6 to 10 grains (low levels of mRNA) or more than 10 grains (intermediate-high).

For each nucleus, we measured the percentage of profiles positive for PPII mRNA along the rostro-caudal extent of the septum, except for the septo-hippocampal nucleus in which we observed PPII mRNA-positive profiles but did not determine their proportion, because the number of sections was too small. Each septal nucleus was divided in 60 squares, and we counted all labeled profiles in 20% of the squares taken randomly. For double ISH, all of the squares dividing the MS-DBB, and in the septo-hippocampal nucleus for comparison, were used to determine the percentage of PPII mRNA-positive profiles expressing TRH-R1 mRNA. For other nuclei, the number of sections was too small to give a reliable value. For each nucleus, values are mean of values at three to seven antero-posterior levels.

Induction of Narcosis by Ethanol and Reversal by TRH and/or Enzyme Inhibitors. The injection of ethanol (Merck, Darmstadt, Germany) was performed during the light phase, between 10:00 AM and noon. The behavior of each animal was recorded on tape, and all assessments were based on this record. Time 0 is the time at which the intraperitoneal injection of ethanol terminated. Loss of righting reflex (LORR; animal in the prone position for more than 1 min) occurred in 2 to 3 min. We recorded the time from the termination of ethanol injection to the spontaneous recovery of the righting reflex (more than 15 s on the four legs); this was called the duration of LORR.

pGlu-His-Pro-NH₂ was from Bachem Biosciences (King of Prussia, PA), and pGlu-Asp-Pro-7-amido-4-methylcoumarin (MCA) (U.S. patent 7,378,397; Kelly, 2008) and pGlu-Asp-Pro-NH₂ were from Peptides International Inc. (Louisville, KY). Their purity (over 99%) was confirmed by reverse-phase high-performance liquid chromatography. The phosphinic analog of TRH [pGlu(P[O(OH)]His-Pro-NH₂)][†TRH] was synthesized as described previously (Matziari et al., 2008); its purity was 93% in reverse-phase high-performance liquid chromatography. N-benzoylcarbonyl prolyl prolinal (ZPP) was from Enzo Life Sciences, Inc. (Farmingdale, NY).

Intraperitoneal Injection of TRH. Rats were handled once daily for 3 days. To induce narcosis a 2 g/kg of ethanol (12% w/v in pyrogen-free saline solution; Sigma) was injected intraperitoneally, and rats were kept in individual cages. TRH (3–30 mg/kg; 5 mg/ml saline) or saline was injected intraperitoneally 25 min after ethanol injection.

Intracerebroventricular Injection of TRH and/or pGlu-Asp-Pro-MCA. One week before the experiment, under ketamine (100 mg/kg i.m.)/Anesket, Pia Agropecuaria, Mexico City, Mexico)/xylazine (Rompun, Bayer Health Care, Leverkusen, Germany) (10 mg/kg i.m.) anesthesia, rats were placed in a stereotoxic device (David Kopf Instruments, Tujunga, CA), and a 22-gauge stainless-steel cannula (Plastics One, Roanoke, VA) was implanted into the right lateral ventricle through a burr hole in the skull. Stereotaxic coordinates in reference to interaural were: antero-posterior, +9.2 mm; lateral, −1.1 mm; depth, −3.6 mm (Paxinos and Watson, 2005). The cannula was secured to the skull with two stainless-steel screws and dental cement and temporarily occluded with a dummy cannula. The skin incision was disinfected with 2% chlorhexidine digluconate (Maver Labs, Mexico City, Mexico). A total of 1,200,000 units of ampicillin were injected. Rats were housed in individual Perspex cages (47 × 26 × 20 cm) and handled once daily for 3 consecutive days before the experimental day to reduce stress. If an animal seemed sick (reduced activity), we did not test it. One week later, rats were transported to the nearly experimental room, and ethanol was injected (3 g/kg i.p.). Thirty minutes later, a 28-gauge stainless-steel tube was inserted into the external guide cannula; it extended 1 mm below the cannula and was connected with polyethylene tubing (Plastics One) to a 20-μl syringe (Hamilton Co., Reno, NV). Intracerebroventricular injections of TRH (1–25 μg) and/or pGlu-Asp-Pro-MCA (5 μg) in 2 μl of saline solution were made over 2 min by a microprocessor-controlled infusion pump (MD-1001; BAS Bioanalytical Systems, West Lafayette,
IN) via the indwelling cannula. The tip of the needle was left in place for 30 s before removal to prevent backward leakage.

**Intraseptal Injection of TRH, TRH-like Peptides, and Inhibitors.** Rats were implanted and treated as described above, except for the following differences. A 26-gauge guide cannula was implanted at: antero-posterior, +9.2 mm; lateral, −1.7 mm; depth, −2.6 mm, with a 14° angle between the vertical and cannula axes. Twenty minutes after an intraperitoneal ethanol injection (2.7 g/kg) a 33-gauge stainless-steel tube was inserted into the external guide cannula; it extended 4 mm away from the tip of the external guide cannula. Either TRH (1–25 μg), pGlu-Asn-Pro-MCA (5–45 μg), pGlu-Asp-Pro-NH2 (5 μg), pGlu-His-Pro-OH (1 μg), or ZPP (0.33 μg) or TRH combined with one of the inhibitors were injected in 2 μl of saline solution (0.5 μl/min) via the indwelling cannula. Where indicated, drugs were dissolved in artificial cerebrospinal fluid (aCSF: 140 mM NaCl, 3.35 mM KCl, 1.15 mM MgCl2, 1.26 mM CaCl2, 1.2 mM Na2HPO4, 0.3 mM NaH2PO4, and 0.1% bovine serum albumin, pH 7.4) or 10% DMSO in aCSF (DMSO-aCSF) instead of saline. To define the potential extent of drugs’ distribution, a few animals naive to any treatment were injected with 2 μl of pontamine blue (4% in water and 0.02% Tween 20; Sigma); the maximum spread from the injection site was 500 μm; however, this value may be an underestimate. The intraseptal injection of 2 μl of saline solution (at 0.5 μl/min; a slow rate) did not produce any tissue lesion. At the end of each experiment, animals were euthanized by decapitation. For intracerebroventricular and intraseptal injections, the brain was removed and rapidly frozen on powdered dry ice. Serial 20- to 25-μm frozen brain coronal sections through the rostro-caudal extent of the septum were cut on a cryostat (Brights, Huntington, UK); confirmation of cannula and microinjector position was made by observation of the tissue scar.

**Tissue Dissections for Biochemical and RT-PCR Analyses.**

The medial septum was obtained from a 0.8-mm (approximate thickness) frozen brain coronal slice, spanning a region from +9.2 to +10 mm [interaural coordinates (Paxinos and Watson, 2005)], using a sample corer (Fine Science Tools, Heidelberg Germany) with 0.5-mm internal diameter. Other brain regions (right and left lateral septum, frontal cortex, dorsal hippocampus, cerebellum, and ventral pons-medulla) were dissected by hand on the same or other coronal slices. Tissues were expelled into a microcentrifuge tube on dry ice.

**Peptidase Assays.** Tissue was homogenized in 50 mM NaPO4 buffer, pH 7.5 (buffer A). Membranes were collected by centrifugation (12,000 g; 15 min), the pellet was washed once with buffer A and 1 M NaCl, and centrifugation was repeated. Finally, the pellet was homogenized in buffer A and stored at −80°C until use. PPII activity was determined essentially as described previously (Vargas et al., 1994) except that no thyroid hormone or insulin was added. On day 5 in culture, cells were washed twice with Dulbecco’s minimum essential medium, and 500 μl of serum-free medium containing either buffer (Dulbecco’s minimum essential medium), TRH, pGlu-Asn-Pro-MCA, or 9TRH were added. At the time points stated in Results, culture medium was collected to measure TSH or prolactin release by radioimmunoassay with National Hormone and Pituitary Program reagents (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD).

**Data Analysis.** Results are presented as mean ± S.E.M. Data were compared by analysis of variance followed by Bonferroni-Dunn post hoc test or an unpaired t test where indicated. Values of p < 0.05 were considered statistically significant.

**Results**

**Expression of PPII in the Septum.** PPII mRNA-positive profiles were detected with a digoxigenin-UTP-labeled antisense RNA probe in various septal nuclei. Their density (number of PPII mRNA profiles per area) was higher in the various lateral septum subdivisions (data not shown) than in the MS and horizontal DBB (hDBB) where many profiles were detected (Fig. 1A). PPII mRNA-positive profiles were not detected with the sense probe (Fig. 1A).

Additional experiments, performed with a [35S]UTP-labeled antisense RNA probe, produced data consistent with those obtained with the digoxigenin-UTP-labeled antisense RNA probe. The highest density of PPII mRNA (number of silver grains per area) was detected in the lateral septum (mainly in the intermediate and ventral regions). Levels were medium to high in the septo-fimbrial and septo-hypothalamic nuclei. Lower levels were detected in the dorsal lateral septum, and levels were low in the DBB and very low in the MS and septo-hippocampal nucleus (data not shown). In most of the septal nuclei, including the MS-DBB, a small proportion of hematoxylin-labeled profiles (10–20%) was PPII mRNA positive. These profiles were generally labeled with low levels of PPII mRNA (Table 1).

In the brain, among distinct PPII mRNA species at least one of them encodes PPII while another encodes a truncated version (PPII*), which may function as a dominant-negative form. PPII* is codified by an mRNA that is probably the result of an exon extension at the border between exon 14 and intron 14 (Chávez-Gutiérrez et al., 2005). Because the hybridization probe we used is complementary to exon 1, it does not discriminate mRNAs that code for PPII from those that code for PPII*; cells detected by in situ hybridization may not code for active PPII. We thus determined by RT-PCR which of these mRNAs is expressed in the septum. Both mRNAs were detected in the brain, as well as in the lateral and medial septum (Fig. 2A).

Whether the medial septum contains PPII activity was previously unknown. The distribution of the activity of PPII in various brain regions was determined. Consistent with previous reports, frontal cortex activity was high, and ventral
pons-medulla activity was low (Vargas et al., 1987). Medial septum and lateral septum activities were intermediate between these values (Fig. 2B). Three independent experiments confirmed the value of medial septum PPII activity (data not shown).

### Distribution of TRH-R1 mRNA in the Septum

To determine the distribution of TRH-R1 mRNA, we used a [³⁵S]UTP-labeled antisense RNA probe. We detected a very high density of TRH-R1 mRNA-positive profiles in the hDBB, a high density in the medial septum (Fig. 1B), vertical DBB (vDBB), and septo-hippocampal nucleus, and a low density in the intermediate and ventral lateral septum, septo-fimbrial nucleus, and dorsal lateral septum (data not shown).

Colocalization of PPII and TRH-R1 mRNAs in the Septum. To analyze whether PPII and TRH-R1 mRNAs are coexpressed in the septum, sections were hybridized with both a [³⁵S]UTP-labeled RNA probe complementary to the TRH-R1 mRNA and a digoxigenin-UTP-labeled RNA probe complementary to the rat PPII mRNA. PPII mRNA colocalized with TRH-R1 mRNA in various regions of the septum, including the DBB (Fig. 3, A and B). The percentage of PPII mRNA profiles colabeled with TRH-R1 mRNA varied according to the septal nucleus, from undetectable to a maximum of 49% in the septo-hippocampal nucleus; it was 20% in the MS-vDBB and 35% in the hDBB. TRH-R1 mRNA levels in the PPII mRNA-positive profiles were generally low; in the

### TABLE 1

Quantitative analysis of PPII mRNA-positive profiles in various nuclei of the septum.

The percentage of hematoxylin-labeled cell profiles that were labeled for PPII mRNA, the proportion of PPII mRNA profiles with low levels of mRNA, the percentage of PPII mRNA profiles positive for TRH-R1 mRNA, and the proportion of double-labeled PPII/TRH-R1 profiles positive for low levels of TRH-R1 mRNA were determined in each nucleus as described under Materials and Methods. Profiles with low levels of mRNA had 6 to 10 grains. Mean values were obtained from various (3–7) sections distributed along the antero-posterior axis in each nucleus. The total number of cell profiles counted in each nucleus is shown in parentheses. 

<table>
<thead>
<tr>
<th>PPII mRNA-Positive Profiles</th>
<th>PPII mRNA Profiles with Low Levels of mRNA per Profile</th>
<th>PPII mRNA Profiles Positive for TRH-R1 mRNA</th>
<th>PPII/TRH-R1 Profiles with Low Levels of TRH-R1 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHN</td>
<td>Detected but not quantified⁴</td>
<td></td>
<td>49 (258)</td>
</tr>
<tr>
<td>DLS</td>
<td>10 (2529)</td>
<td>80</td>
<td>Not detected</td>
</tr>
<tr>
<td>ILS</td>
<td>14 (9068)</td>
<td>&gt;60</td>
<td>Detected but not quantified⁴</td>
</tr>
<tr>
<td>VLS</td>
<td>20 (1851)</td>
<td>50</td>
<td>Detected but not quantified</td>
</tr>
<tr>
<td>SFN</td>
<td>16 (1036)</td>
<td>&gt;70</td>
<td></td>
</tr>
<tr>
<td>SHyN</td>
<td>11 (2020)</td>
<td>70</td>
<td>Not detected</td>
</tr>
<tr>
<td>MS-DBB</td>
<td>12–14 (5788)</td>
<td>&gt;90</td>
<td></td>
</tr>
<tr>
<td>MS-vDBB</td>
<td>20 (1501)</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>MS-hDBB</td>
<td>35 (1404)</td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

SHN, septo-hippocampal nucleus; DLS, dorsal lateral septum; ILS, intermediate lateral septum; VLS, ventral lateral septum; SFN, septo-fimbrial nucleus; SHyN, septo-hypothalamic nucleus.

⁴ PPII mRNA-positive profiles were detected, but their proportion (in reference to total cell number) was not quantified.

⁵ Profiles positive for both PPII and TRH-R1 mRNAs were detected but their proportion (in reference to total cell number) was not quantified. For cells devoid of information, data were not or could not be determined.

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**Fig. 2.** PPII mRNAs and PPII activity are detected in the medial septum of Wistar rats. A, both mRNAs encoding PPII and PPII⁺ are expressed in the medium and lateral septum. Shown are the results of an electrophoretic analysis of mRNAs amplified with primers specific for PPII mRNAs (they target mRNAs that do not include the exon extension) or primers specific for PPII⁺ mRNAs (they target mRNAs that do include the exon extension). The top band corresponds to PPII⁺, and the bottom band corresponds to PPII cDNA. Lanes 2, 4, and 6 show amplification without RT. B, distribution of PPII enzymatic activity in various brain regions. Membrane PPII-specific activity was determined with 40 μM TRH-MCA as substrate. Data (pmoles of MCA per minute per milligram of protein) are mean ± S.E.M. (n = 3). Letters indicate significant differences between groups: a, c, f, h, and j, p < 0.001; d and i, p < 0.01; and b, e, and g, p < 0.05. Note that medial septum PPII activity was intermediate between the highest and lowest brain activities.
A section through the MS-DBB is shown in Fig. 3C. hDBB or MS-vDBB. A representative map of these profiles in values (Table 1). Profiles colabeled with both mRNAs in the hDBB, they were equally distributed between low and high activity by 44%. We also determined whether pGlu-Asn-Pro-MCA is an agonist of TRH receptors. In primary cultures of anterior pituitary cells, which express TRH-R1, TRH had a time-dependent (between 15 and 180 min) and dose-dependent (between $5 \times 10^{-10}$ and $10^{-7}$ M) stimulatory effect on the release of TSH or prolactin; there was no consistent effect of pGlu-Asn-Pro-MCA up to $5 \times 10^{-6}$ M (data not shown).

The intracerebroventricular injection of 5 μg of pGlu-Asn-Pro-MCA had no analeptic effect by itself and did not modify the analeptic effect of 5 μg of TRH (saline, 138 ± 19 min, n = 4; TRH, 85 ± 16 min, n = 5; pGlu-Asn-Pro-MCA, 141 ± 8 min, n = 5; TRH + pGlu-Asn-Pro-MCA, 102 ± 11 min, n = 3; *, p < 0.05 (unpaired t test)).

The injection of pGlu-Asn-Pro-MCA (5, 15, or 45 μg) into the MS did not produce an analeptic effect (Fig. 5, A and B), suggesting that the analeptic effect of TRH probably was not caused by an osmotic, but rather by a receptor-mediated effect. The duration of LORR was significantly reduced by the combined treatment of 5 μg of TRH and 5 μg of pGlu-Asn-Pro-MCA compared with saline solution alone or 5 μg of TRH alone. The effect of TRH alone was not significant (Fig. 5B).

The TRH-like peptide [Asp]$^2$-TRH does not inhibit PPII activity (Kelly et al., 2000). This was confirmed in brain membranes in vitro: control PPII activity, 100%; 1.28 mM [Asp]$^2$-TRH, 108%. The intraseptal injection of 5 μg of [Asp]$^2$-TRH increased slightly, but not significantly, the duration of LORR and did not enhance TRH action, whose effect was not significant (Fig. 5C).

ΨTRH, a phosphinic analog of TRH (Matziari et al., 2008), is a potent and specific PPII inhibitor (Cruz et al., 2008). At 2 μM it inhibited brain membrane PPII activity by 65%. We determined whether it is an agonist of TRH-R1 receptors. In primary cultures of anterior pituitary cells TRH had a stim-
Fig. 4. Analeptic effect of TRH in ethanol-intoxicated Wistar rats. A, effect of an intraperitoneal injection of saline or TRH (3, 10, or 30 mg/kg) on the duration of LORR induced by an intraperitoneal injection of 2 g/kg ethanol. TRH or saline was injected 25 min after the administration of ethanol. Data are mean ± S.E.M. (n = 4 for saline and n = 5 for TRH). *, p < 0.05 compared with saline group. B, effect of an intralateral ventricle injection of saline or 5 μg of TRH on duration of LORR induced by an intraperitoneal injection of 3 g/kg ethanol. TRH or saline was injected 20 min after the administration of ethanol. Data are mean ± S.E.M. (n = 4 for saline and n = 5 for TRH). *, p < 0.05 compared with saline group. C, effect of an intramedial septum injection of saline or 1 to 25 μg of TRH on duration of LORR induced by an intraperitoneal injection of 2.7 g/kg ethanol. TRH or saline was injected 30 min after the administration of ethanol. Data are mean ± S.E.M. (n = 4 for saline and n = 5, 3, and 4 for 1, 5, and 25 μg of TRH, respectively). *, p < 0.001 compared with saline group. D, positions of the tip of the cannula for the experiment shown in C.

Discussion

TRH has many pharmacological actions in brain and can restore homeostatic perturbations (Yarbrough et al., 2007). Its in vivo effects probably are limited by peripheral and central degradation. Although it is well established that TRH has a short half-life in serum, and degradation-stabilized analogs are more potent than TRH, the mechanism of TRH inactivation in the brain extracellular space has not been definitively established. Biochemical and cell biological results strongly suggest that TRH is inactivated by PPII (Charli et al., 1998; Heuer et al., 1998b), but competing alternatives have been put forward, in particular the involvement of POP. The data presented in this article demonstrate that in the septum PPII is expressed in multiple nuclei, with positive profiles were also TRH-R1 mRNA-positive. The data also suggest that PPII activity limits the analeptic action of TRH endogenously released from or injected into this region. Therefore, a pathway of TRH inactivation in the MS-DBB extracellular space may be its degradation by PPII, localized on the surface of TRH-R1 mRNA-positive cells.

A broad map of PPII mRNA distribution in the adult male Sprague-Dawley rat brain shows that levels are high in the lateral septum (especially in the dorsal part) but not in the medial septum-DBB, where a few scattered cells express low levels of PPII mRNA (Heuer et al., 1998a, 2000). Our data are in general agreement with these results, but we also show that in adult male Wistar rats the proportion of PPII...
mRNA-labeled profiles in the MS-DBB (12–14% of the hematoxylin-labeled profiles) is significant, although most have low levels of PPII mRNA. Because the focus of this study was not on profile numbers, we did not use unbiased stereology; thus, the values we report could have been affected by stereological factors. In addition, albeit unlikely, an unexpected cross-hybridization in the MS-DBB might have led to an overestimation of the number of PPII mRNA-labeled profiles. Furthermore, ambiguity in our and other PPII ISH data (Heuer et al., 1998a, 2000; de Gortari et al., 2006) stems from the fact that the probes used do not distinguish RNA species coding for full-length PPII from those coding for a truncated nonselective probe matches that of PPII activity (Heuer et al., 1998a). However, it remains possible that for some profiles the apparent double-labeling represents signals originating from two cells that might partially overlay each other. In addition, many PPII mRNA-labeled profiles are TRH-R1 mRNA positive, and 40 to 50% of these have intermediate to high levels of TRH-R1 mRNA. The double-labeled profiles are localized in two anatomically separated groups. Whether they correspond to functionally distinct cell types remains to be clarified. These data fit with indirect evidence that PPII is postsynaptic in the brain, because TRH and PPII mRNAs are differentially distributed in the lateral septum, the septo-hippocampal, and septo-fimbrial nuclei. In the MS-DBB and the hDBB 20 to 35% of PPII mRNA profiles are TRH-R1 mRNA positive, and 40 to 50% of these have intermediate to high levels of TRH-R1 mRNA. The acute injection of a large dose of ethanol affects the brain through multiple primary mechanisms, including enhancement of GABA-A receptor function, opening of G protein-activated inwardly rectifying K+ channels, inhibition of

![Intra medial septum injections](image)

**Fig. 5.** The intramedial septum injection of PPII inhibitors enhances the analeptic effect of TRH injected into the medial septum. A, effects of injection of 5 to 45 µg of pGlu-Asn-Pro-MCA on the duration of LORR induced by an intraperitoneal injection of 2.7 g/kg ethanol. pGlu-Asn-Pro-MCA or saline was injected 20 min after the administration of ethanol. Data are mean ± S.E.M. (n = 4 for saline and n = 4, 4, and 45 µg of pGlu-Asn-Pro-MCA, respectively). B, effects of injection of 5 µg of pGlu-Asn-Pro-MCA or 5 µg of TRH alone or combined on duration of LORR induced by an intraperitoneal injection of 2.7 g/kg ethanol. TRH, pGlu-Asn-Pro-MCA, or saline was injected 20 min after the administration of ethanol. Results from two independent experiments were pooled, because data were very similar. Data are mean ± S.E.M. (n = 4 for saline and n = 4, 4, and 5 for 5, 15, and 45 µg of pGlu-Asn-Pro-MCA, respectively). C, effect of an injection of 5 µg of pGlu-Asp-Pro-NH2 or 5 µg of TRH alone or combined on the duration of LORR induced by an intraperitoneal injection of 2.7 g/kg ethanol. TRH, pGlu-Asn-Pro-MCA, or saline was injected 20 min after the administration of ethanol. Data are mean ± S.E.M. (n = 9 for saline and n = 8, 10, and 9 for TRH, pGlu-Asn-Pro-MCA, and TRH + pGlu-Asn-Pro-MCA, respectively). ***, p < 0.0001 compared with the saline group. ***, p < 0.01 compared with the TRH group. ***, p < 0.0001 compared with the pGlu-Asn-Pro-MCA group. C, effect of an injection of 5 µg of pGlu-Asp-Pro-NH2 or 5 µg of TRH alone or combined on the duration of LORR induced by an intraperitoneal injection of 2.7 g/kg ethanol. The vehicle was aCSF. aCSF, TRH, and/or pGlu-Asn-Pro-MCA was injected 20 min after the administration of ethanol. Data are mean ± S.E.M. (n = 5 for aCSF and TRH + pGlu-Asn-Pro-MCA, n = 4 for TRH and/or pGlu-Asn-Pro-MCA). **, p < 0.01 compared with the aCSF group (unpaired t test). In agreement with the distribution of PPII and TRH-R1 mRNAs, we demonstrate that some PPII mRNA profiles are positive for TRH-R1 mRNA in the intermediate part of the lateral septum, the septo-hippocampal, and septo-fimbrial nuclei. In the MS-DBB and the hDBB 20 to 35% of PPII mRNA profiles are TRH-R1 mRNA positive, and 40 to 50% of these have intermediate to high levels of TRH-R1 mRNA. The acute injection of a large dose of ethanol affects the brain through multiple primary mechanisms, including enhancement of GABA-A receptor function, opening of G protein-activated inwardly rectifying K+ channels, inhibition of
Because Phe2-TRH and Tyr2-TRH, in vitro substrates of PPII/H9023, do not inhibit PPII activity, are unable to modify the analeptic response to hydrolysis by PPII for the exploration of central nervous system disorder therapies. These endogenous adjustments of TRH transmission may contribute to an exit from narcosis, because the exogenous application of TRH, either intraperitoneally, intracerebroventricularly, or into the medial septum, facilitates recuperation from ethanol-induced narcosis (McCown et al., 1986; Horita, 1998; our data). The cholinergic septo-hippocampal pathway may mediate the effect of TRH (Horita, 1998). Preliminary data suggest that some of the MS-DBB PPII cells are cholinergic (R. M. Uribe and J. L. Charli, unpublished work), in line with the cholinergic hypothesis. An alternative untested hypothesis is that TRH may activate the MS-DBB cholinergic cells that send projections to, and activate, a subpopulation of hypocretin/orexin neurons of the lateral hypothalamus, which are causal for awakening from sleep (Sakurai et al., 2005; Adamantidis et al., 2007).

A major finding is that the analeptic effect of exogenous TRH in the MS is potentiated by PPII inhibition. This conclusion is based on the following arguments: the PPII inhibitor ψTRH, which is not an agonist of TRH-R1, enhances the effect of TRH; and the analeptic effect of TRH is also enhanced by pGlu-Asn-Pro-MCA, a PPII inhibitor that is not an agonist of the TRH-R1 receptor. This inhibitor is also poor at displacing [3H]-3-methyl-His2-TRH (a TRH-R agonist) binding in rat cortical homogenates (Kelly et al., 2002), a region rich in TRH-R2 mRNA (Heuer et al., 2000). Finally, an analog of pGlu-Asn-Pro-MCA, pGlu-Asp-Pro-NH2, which does not inhibit PPII activity, is unable to modify the analeptic effect of TRH.

We also observed that the intramedial septum injection of ψTRH, a PPII inhibitor more potent than pGlu-Asn-Pro-MCA, significantly reverses the narcosis induced by ethanol. Because Phe2-TRH and Tyr2-TRH, in vitro substrates of PPII putatively present in brain, are not analeptic (Hinkle et al., 2002), this effect may be caused by an enhancement of endogenous TRH concentration in the MS-DBB extracellular space; additional experiments are required to confirm this interpretation.

Injection of TRH in other medial regions of the brain also produces analepsia, albeit with less potency than in the MS-DBB; this was clearly shown in the case of pentobarbital-induced narcosis (Kalivas and Horita, 1980). Because pontamine blue may underestimate the spread of injected drugs, it remains possible that TRH and PPII inhibitors diffused out of the MS and affected additional targets, but the significant levels of PPII activity in the MS, and failure of the intracerebroventricular injection of pGlu-Asn-Pro-MCA to enhance the analeptic effect of intracerebroventricular TRH, support the parsimonious interpretation that PPII inhibition in the MS may be sufficient to produce analepsia. Even if additional regions contributed to the effects of PPII inhibitors, our data support the concept that PPII activity controls TRH turnover in the brain extracellular space. This conclusion is consistent with a preliminary report that the intracerebroventricular injection of 5 μg of pGlu-Asn-Pro-MCA enhances the number of wet dog shakes induced by intracerebroventricular TRH (Kelly et al., 2001).

The spatial relationship between sites of peptide release, action, and inactivation is poorly understood, but is likely an important determinant of ectoepitopease capacity for regulating peptide action (see Introduction). We propose that in response to ethanol TRH release from the MS-DBB activates TRH-R1 receptors on the surface of cells expressing PPII. The proximity of PPII and TRH receptors may facilitate the capacity of PPII to limit TRH action and control TRH-R desensitization. It makes it more likely that PPII can control TRH action than if it is not expressed on TRH-R-positive cells. However, it remains possible that the colocalization of PPII with TRH-R1 (or TRH-R2) is not an essential prerequisite for the suggested role of PPII, and PPII expression by nearby cells that do not express TRH-R would be sufficient to control TRH action.

Although our data support the notion that PPII inactivates TRH once released into the brain extracellular space, there may be additional ways of efficient removal of TRH. Inhibition of POP enhances TRH levels in specific brain regions; this may be caused by a change in the intracellular turnover of TRH (Tenorio-Laranga et al., 2011). POP may hydrolyze TRH in the brain extracellular space because TRH injected intracerebroventricularly is rapidly degraded to pGlu-His-Pro (Spindel et al., 1981), and the oral administration of the POP inhibitor (S)-2-[(S)-2-(hydroxyacetyl)-1-pyrrolidinyl]carbonyl]-N-phenylmethyl]-1-pyrrolidinecarboxamide) (JTP-4819) together with subcutaneous TRH, at doses at which each agent alone has no effect, improves retention time in a passive avoidance test in scopalamine-treated rats (Toide et al., 1995). These data, however, contradict the evidence that the hydrolysis of exogenous TRH in hypothalamic slices produces a metabolite signature that is not consistent with POP but is with PPII activity (Méndez et al., 1999). Moreover, ZPP, a potent POP inhibitor, does not change the recuperation of TRH released from rat hypothalamic slices (Charli et al., 1987). Finally, membrane POP is possibly intracellular (Myöhänen et al., 2009). Because we observed that POP inhibition does not change narcosis time or TRH-induced recovery from narcosis, our data suggest that POP does not participate in TRH turnover in the medial septum extracellular space.

In conclusion, we have obtained data that suggest the analeptic effect of TRH is controlled by PPII activity in the medial septum-diagonal band of Broca. PPII activity may limit the capacity of TRH to activate TRH-R1 receptors involved in relaying homeostatic information to the circuits regulating exit from narcosis by controlling the extracellular turnover of TRH. PPII therefore seems relevant in both a neurohormonal (Sánchez et al., 2009) and neuromodulatory (this study) context. This result strengthens the rationale to develop PPII inhibitors or TRH-like receptor agonists resistant to hydrolysis by PPII for the exploration of central nervous system disorder therapies.
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**Authorship Contributions**

**Conducted research:** Joseph-Bravo and Charli.

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**Wrote or contributed to the writing of the manuscript:** Lazoano, Uribe, Joseph-Bravo, and Charli.

**Contributed new reagents or analytic tools:** Matziari.

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