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Evidence for interplay between thyrotropin-releasing hormone (TRH) and its structural analogue pGlu-Glu-Pro-NH_2 ([Glu^2]TRH) in the brain: An *in vivo* microdialysis study

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Abstract

Local perfusion of pGlu-Glu-Pro-NH_2, an endogenous peptide structurally related to thyrotropine-releasing hormone (TRH), via *in vivo* microdialysis into the rat hippocampus did not change the basal level of extracellular acetylcholine. However, co-perfusion of pGlu-Glu-Pro-NH_2 with TRH in equimolar concentrations yielded a significant attenuation of TRH-induced acetylcholine release. The results have supported the study's hypothesis that pGlu-Glu-Pro-NH_2 opposes the cholinergic effect of TRH in the mammalian central nervous system. The enantiomer pGlu-D-Glu-Pro-NH_2 affected neither basal extracellular nor TRH-induced increase of acetylcholine levels.

Keywords: TRH; TRH analogue; Acetylcholine release; Neuromodulator

Thyrotropin-releasing hormone (TRH; pGlu-His-Pro-NH_2) and [Glu^2]TRH (pGlu-Glu-Pro-NH_2) are structurally related endogenous peptides (Fig. 1) that are identified in neuronal and non-neuronal tissues [2,5,15,25,29]. As a hormone, TRH regulates endocrine functions mainly through the stimulation of synthesis and secretion of thyroid-stimulating hormone, growth hormone and prolactine in pituitary gland, and also acts independently as a neurotransmitter/neuromodulator in the central and peripheral nervous systems [2,15,25,41]. Recently, it has been suggested that the main neurobiological function of endogenous TRH is to promote homeostasis through neuronal mechanisms [8].

[Glu^2]TRH is known to be a fertilization-promoting peptide [1,4,12], but it also occurs in the brain, principally in the hippocampus, brain stem and dorsal colliculi [7]. [Glu^2]TRH is not derived from prepro-TRH [4], and binds to TRH receptors with very low affinity [14,29,40]. These observations suggest that pharmacological effects of [Glu^2]TRH are not related to TRH receptors [9,38]. In fact, a suppression of TRH-induced growth-hormone release from avian pituitary by [Glu^2]TRH has been observed [13]. Exogenously administered [Glu^2]TRH manifests analeptic, neuroprotective, antidepressant, anticonvulsant and many other activities in mammals [3,14,20,21,28,29]. However, this structural analogue lacks the endocrine effect of TRH (TSH release) and, thus, does not elevate thyroid-hormone levels [21]. TRH and its analogues have been considered lead compounds [31] for the development of agents potentially useful for the treatment of several CNS diseases including Alzheimer's disease, amyotrophic lateral sclerosis, epilepsy, mood disorders and depression [8,10,21,39]. Effects after [Glu^2]TRH administration are usually more robust than those of TRH, because [Glu^2]TRH is not hydrolyzed efficiently by serum thyroliberinase, the soluble pyroglutamyl aminopeptidase (PAP I, EC 3.4.19.3) and the CNS ectoenzyme pyroglutamyl aminopeptidase (PAP II, EC 3.4.19.6) responsible for the rapid degradation of TRH *in vivo* [6].

Many CNS-effects of TRH are mediated via neurotransmitters [15]. A robust effect of TRH on the stimulation of acetylcholine (ACh) synthesis in the CNS and, therefore, the subsequent increase of the extracellular ACh level are well known [11,18,24,36], and a vast array of its CNS-actions such as analeptic activity [30] is believed to occur primarily through

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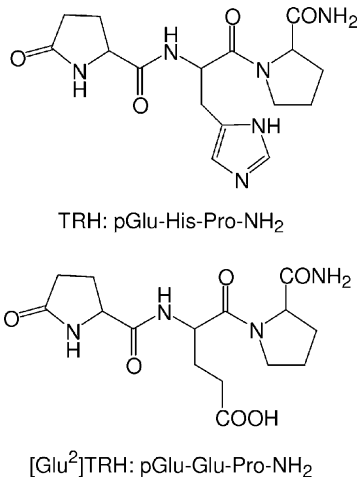

Fig. 1. Structure of TRH and [Glu^2]TRH.

a cholinergic mechanism [23]. Potential therapeutic effects of TRH and its analogues extend far beyond their analeptic activity, because the cholinergic system is affected in a wide array of CNS disorders. For example, cholinergic changes are manifested in neurodegenerative diseases such as Alzheimer's disease [37] and, also, during normal aging [22]. Analeptic effect of [Glu^2]TRH has been demonstrated in rats upon intracisternal [14] and in mice upon intravenous administration [34], respectively. However, a link between pharmacological activities exerted by [Glu^2]TRH and potential stimulation of ACh release in the mammalian brain has not been shown. In our preliminary experiments [33], we found that [Glu^2]TRH actually attenuated TRH-induced ACh release in the rat hippocampus, which would argue for an interplay between these two structurally related peptides in the mammalian central nervous system (CNS) similar to that observed in the avian pituitary regarding growth-hormone release [13]. We selected intracerebral microdialysis in rats for the *in vivo* testing of this hypothesis. The hippocampus was targeted by our experiments, because TRH and its analogues have shown the most robust ACh-releasing effect in this area of the brain [16,17,35,39]. In addition to [Glu^2]TRH, we have also tested its enantiomer [D-Glu^2]TRH.

TRH and [Glu^2]TRH were purchased from Bachem BioSciences (Torrance, CA). The enantiomer pGlu-D-Glu-Pro-NH_2 ([D-Glu^2]TRH) was synthesized in our laboratory by semi-automated solid-phase peptide synthesis (SPPS) using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry.

Male Sprague–Dawley rats (250–300 g body weight; three per treatment group in between subjects design) were used for the brain microdialysis experiments. They were housed two per cage in an environmentally controlled room on a 12-h light:12-h dark cycle, and were maintained *ad libitum* on water and rodent chow. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Florida before initiation of the study.

The implantation of a guide cannula (CMA/Microdialysis Inc., Acton, MA) was done stereotaxically [27] under aseptic conditions by using a frame equipped with a micromanipulator after the rats had been completely anesthetized (sodium pentobarbital, 60 mg/kg i.p.). The top of the head was shaved and a midline cut was made through the skin. Fascia over the skull was scraped away, and the cut was protracted. With a hand-held dental drill, one hole was drilled through the skull to place a guide cannula at into the brain. Two additional holes were made anterior and posterior to this hole for the anchor screws. The guide cannula was inserted through the hole into the hippocampus (based on its location at anterior to bregma, A: −5.6 mm; lateral to midline, L: −4.8 mm; vertical, V: −4.0 mm [27]), and held permanently in place with cranioplastic cement filled into the protracted area. The actual process of insertion of the probe into the hippocampus started after allowing 7 days for the wound to heal following the implantation of the guide. After connecting the tubing for the influx and efflux of the perfusion fluid to the microdialysis probe and then inserting the probe into the cerebral guide cannula, the animal was placed into the clear plastic bowl of an animal handling unit (Stand-alone Raturn, BASi, West Lafayette, IN) for sample collection. The rat was sacrificed after the microdialysis experiment, and the placement of the probe was verified by histological observation.

Concentric microdialysis probes (CMA/12, CMA/Microdialysis Inc.) with a 4 mm long polycarbonate membrane (cutoff molecular weight 20,000 Da) were used. An artificial cerebrospinal fluid (purchased from Harvard Apparatus, Holliston, MA) containing 2 μM neostigmine was employed as a perfusion medium. Syringe pumps (1 mL BeeStinger), their controller (BeeKeeper) and a refrigerated fraction collector (HoneyComb) used throughout the experiments were purchased from BASi. The system was first equilibrated for 1 h with artificial CSF only. Then after a 2 h perfusion of the probes at 2 μL/min flow rate with the artificial CSF containing the 2 μM neostigmine, the peptide (TRH, [Glu^2]TRH or [D-Glu^2]TRH) or a mixture of peptides (TRH plus [Glu^2]TRH or TRH plus [D-Glu^2]TRH) was dissolved in the same perfusion fluid at 1 nmol/μL concentration each, and the probe placed in the hippocampus was perfused with this solution, respectively, for 4 h while collecting microdialysates in 20 min fractions. A manual valve (CMA/110, CMA/Microdialysis Inc.) was used for switching perfusion solutions without the interruption of the flow through the probe.

The system for the analysis of ACh consisted of an MF-9089 solvent degassing unit, a PM-80 pump, a CC-5 injector with a 5 μL loop, a 5.5 cm × 1.0 mm MF8907 pre-column choline oxidase/catalase IMER column for choline degradation, a 50 cm × 1.0 mm i.d. MF-8904 microbore ion-exchange chromatography (IEC) column, a 2 cm × 1.0 mm MF 8903 microbore immobilized enzyme reactor (IMER), and an LC-4C electrochemical (amperometric) detector (all purchased from BAS Inc., West Lafayette, IN). A CC-5 flow cell containing a peroxidase redox polymer coated on the glassy carbon electrode was used, and the working potential against the Ag/AgCl reference electrode was +100 mV. The mobile phase (50 mM K_2HPO_4 buffer, pH 8.5, containing 0.005% Kathon CG) was delivered at a flow rate of 140 μL/min. A PeakSimple chromatography data system (SRI, Menlo Park, CA, USA) was used for data acquisition and processing. Basal spontaneous release of the neurotransmitter was obtained as the mean of the ACh concentrations measured in the four consecutive 20 min fractions

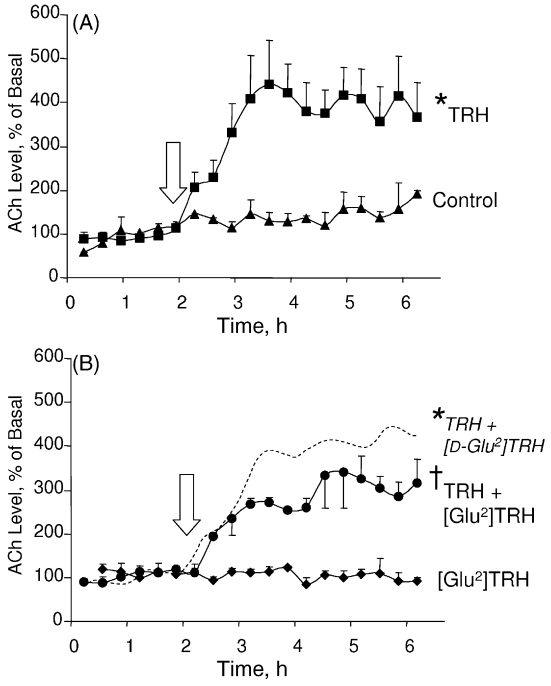

Fig. 2. Extracellular ACh level in the rat hippocampus measured via *in vivo* microdialysis upon perfusion of the probes at 2 μL/min with: (A) vehicle (control, triangles), and a solution of TRH (1 nmol/μL, squares), and (B) [Glu^2]TRH (1 nmol/μL, diamonds) and a mixture of TRH and [Glu^2]TRH (1 nmol/μL each, circles) solutions. Dashed line summarizes an experiment involving the perfusion of a mixture of TRH and [D-Glu^2]TRH (1 nmol/μL each). Data are given relative to the basal ACh levels measured prior to the perfusion of the compound(s). Time for introduction of the test agent(s) is indicated by the block arrow. Error bars indicate S.E.M. ($n = 3$), asterisks denote statistically significant differences ($p < 0.05$) from the control group (no perfusion with peptide, triangles), and dagger indicates statistically significant difference from both the control and TRH groups (repeated measures ANOVA followed by *post hoc* Student–Newman–Keuls test, $p < 0.05$).

preceding the perfusion of the experimental agent and the percentage of this basal spontaneous release was determined from the ACh concentrations measured in the consecutive fractions. Each animal served as its own reference upon expressing ACh levels as percentages of basal release. Data were reported as mean ± standard error of the mean (S.E.M.). Statistical evaluation was done by repeated measures analysis of variance (ANOVA) followed by *post hoc* Student–Newman–Keuls test. Differences were considered significant with $p < 0.05$.

Typical basal ACh concentrations in rat hippocampal microdialysates during the *in vivo* perfusion by 2 μM neostigmine solution were between 40 and 80 nM [32]—values similar to those reported by others [11,26]. As shown in Fig. 2A, the profiles showed a typical evolution of the response upon switching the probe to the continuous perfusion of TRH (1 nmol/μL) and TRH considerably increased ACh-release in the rat hippocampus [11]. The increase of ACh-levels also manifested variation due to circadian rhythm [19]. However, [Glu^2]TRH had no significant effect on the concentration of this neurotransmitter compared to the control experiment (i.e., perfusion with solution that did not contain peptide). On the other hand, a statistically significant attenuation of TRH-induced ACh-release was observed in the first 2 h of the experiments, when TRH and [Glu^2]TRH (1 nmol/μL each) were co-perfused (Fig. 2B). This attenuation appeared to diminish with prolonged perfusion, which might indicate desensitization to [Glu^2]TRH-mediated anticholinergic pathways upon continuous stimulation by this peptide. Dose–response relationships of this neurochemical effect have been beyond the scope of the study. A pharmacological paradigm will be employed to address whether [Glu^2]TRH is a competitive modulator of TRH in the CNS, and the studies will be reported in a separate publication.

Like [Glu^2]TRH, the enantiomer [D-Glu^2]TRH had no statistically significant effect on the concentration of this neurotransmitter (data not shown) compared to the control experiment (i.e., perfusion with solution that did not contain peptide). However, co-perfusion of [D-Glu^2]TRH with TRH did not result in the attenuation of the TRH-induced increase in ACh-levels (Fig. 2B, error bars are not shown to avoid clutter). This observation indicates that the modulating effect [Glu^2]TRH is stereospecific.

In conclusion, our studies have shown that [Glu^2]TRH does not influence ACh release upon its local perfusion into the hippocampus. Therefore, this peptide does not exert its various CNS effects directly though a cholinergic mechanism. Moreover, [Glu^2]TRH moderately attenuates ACh release induced by TRH, which indicates that it opposes the effect (i.e., it is a negative modulator) of TRH in the CNS, and may not be implicated as a therapeutic lead to ameliorate CNS maladies involving cholinergic hypofunction such as cognitive deficits [15]. On the other hand, [Glu^2]TRH may selectively stimulate antidepressant or neuroprotective pathways [20,21] that are not directly associated with the ACh-releasing action of TRH [11] and its endogenous [28] or synthetic TRH analogues [26,39].

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