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Synthesis and behavioral evaluation of a chemical brain-targeting system for a thyrotropin-releasing hormone analogue

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Abstract – A chemical brain-targeting system, in which a redox 1,4-dihydropyridine <=> pyridinium function serves as a targeting moiety and a cholesteryl ester contributes to the improved penetration across the blood–brain barrier, was synthesized for a centrally active TRH analogue, pGlu-Leu-Pro-NH_2. Our retrometabolic design was also based on the progenitor sequence, Gln-Leu-Pro-Gly, where the C-terminal glycine (Gly) functions as an amide precursor via peptidyl glycine α-amidating monooxygenase (PAM) and glutamine (Gln) is the precursor of the N-terminal pyroglutamyl (pGlu) by glutaminyl cyclase. The molecular design included an endopeptidase (post-proline cleaving enzyme) cleavable spacer function. Treatment with the chemical targeting system significantly improved memory-related behavior, without altering thyroid function, in a passive avoidance paradigm in rats bearing bilateral fimbrial lesions. © Elsevier, Paris

TRH analogue / brain-targeting / retrometabolic design / memory improvement

1. Introduction

Besides its role in the regulation of endocrine function, thyrotropin-releasing hormone (TRH) has been long recognized as a modulatory neuropeptide in the central nervous system (CNS) [1]. Extrahypothalamic TRHergic pathways function independently from the thyroid system. TRH receptors and immunopositive TRH-like material in several medullary and forebrain areas are involved in the central control of cardiorespiratory functions, which further supports the involvement of TRH in the central regulation of autonomic functions [2, 3]. At the receptor level in the CNS, different TRH receptor subtypes or (high- and low-affinity) binding sites appear to exist [4]. TRH also has been shown to exert a variety of extrahypothalamic effects in animals. The best documented effect of this neuropeptide is its analeptic action [5–10]. The analeptic effects of TRH appear to be mediated by a cholinergic mechanism [5–7] and may involve an interaction with low affinity binding sites [4].

Cholinergic dysfunction is intimately associated with the symptoms of Alzheimer's disease (AD) [11, 12]. Based on the positive effects of TRH and its synthetic analogues on drug-induced cholinergic hypofunction [5–10], these compounds have been implicated in current approches to AD drug therapy [13–17]. The potential use of TRH as an anti-AD agent is, however, hampered by several factors. TRH has short half-life after systemic administration [18], does not effectively penetrate the blood–brain barrier (BBB) [19], and at doses causing significant cognitive improvement its endocrine effect is usually manifested. The poor access of TRH to the CNS is attributable to the low distribution coefficient ($K_D < 0.005$ between n-octanol and water) preventing transport through the BBB [20]. There have been numerous attempts to modify the structure of TRH to obtain metabolically stable analogues and/or to improve potency and selectivity by dissociating the endocrine and CNS function [21–25]. While these analogues improve meta-

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bolic instability and lipid-solubility of TRH, their peptide character prevents substantial brain delivery.

A chemical targeting system based on a peptide progenitor for a TRH analogue, pGlu-Leu-Pro-NH_2 or [Leu^2]TRH (**1**), has been developed, and a significant improvement in the analeptic effect has been reported in animals, compared to the unmodified peptide [26]. The rationale for the selection of this compound as a lead is justified by its increased CNS activity, together with the minimized or absent systemic effects, compared to TRH [25]. Based on C-terminal glycine (Gly) as an amide precursor via peptidyl glycine α-amidating monooxygenase (PAM) and glutamine (Gln) serving as a precursor of the N-terminal pyroglutamyl (pGlu) for glutaminyl cyclase, a Gln-Leu-Pro-Gly progenitor sequence was used in our method involving retrometabolic design [27]. In this paper, we report the design and synthesis of a brain-targeting system for [Leu^2]TRH, along with its comparative evaluation in memory-related effects in rats bearing bilateral fimbrial lesions as a model for AD.

2. Chemistry

2.1. Design

In this chemical brain-targeting approach shown in *figure 1*, specific lipophilic groups are applied to assure

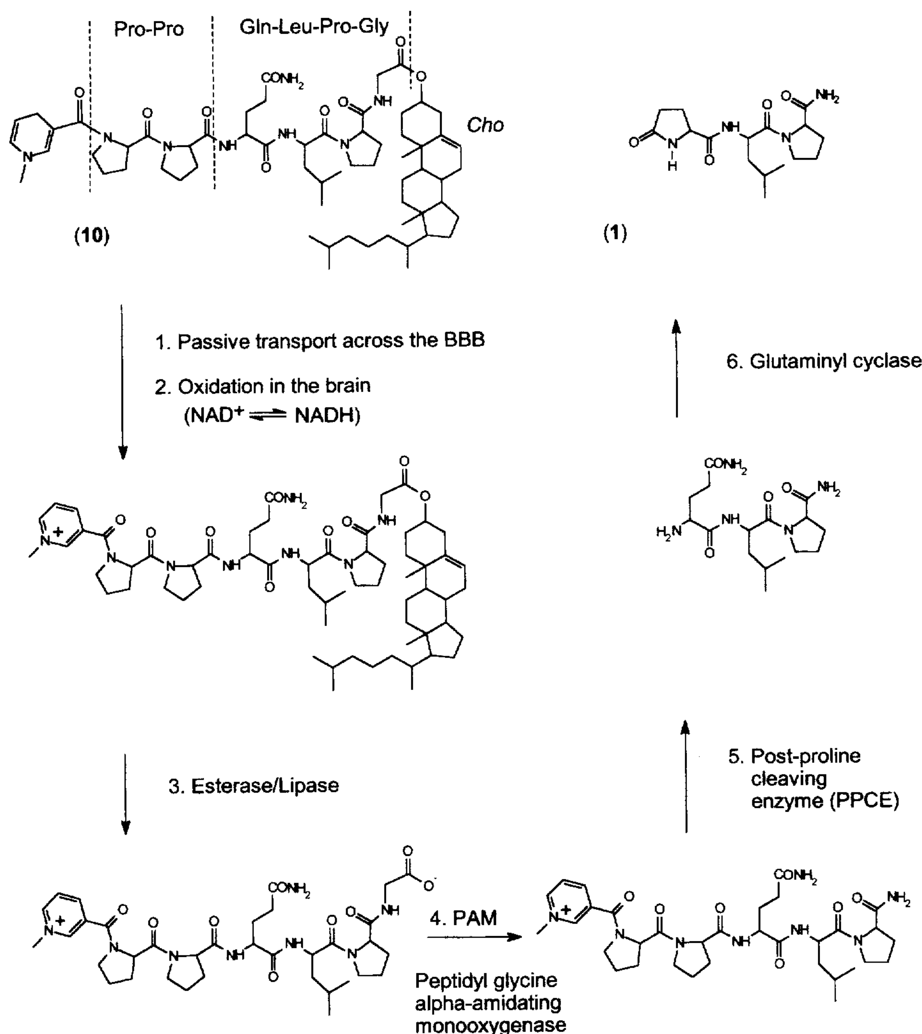

Figure 1. Brain-targeting of [Leu^2]TRH by covalent modification and sequential metabolism.

BBB penetration, and they also prevent the peptide segment from recognition by peptidases [28]. These lipophilic groups are subject to conversion and/or removal by specific enzymes. In designing a peptide targeting system for the CNS, the unique architecture of the BBB is exploited. Targeting is provided by a dihydropyridine-type functional group, a lipoidal 1,4-dihydrotrigonellyl, which can be converted by enzymatic oxidation to a water-soluble, lipid-insoluble quaternary pyridinium salt (This conversion occurs ubiquitously and is analogous to the oxidation of NAD(P)H, a coenzyme associated with many oxidoreductases and cellular respiration, and redox brain-targeting by this moiety alone has been possible for small molecules such as dopamine [29]). An additional lipophilic modification is the C-terminal ester [27] subject to removal by esterases and/or lipase, and the bulkiness of this group is also utilized to prevent or restrict access of degrading enzymes to the peptide sequence embedded in the molecule. Upon systemic administration, passive transport should partition the molecule into several body compartments, including the brain, due to its enhanced lipophilicity. Then, the 1,4-dihydrotrigonellyl moiety undergoes an enzymatically-mediated oxidation that converts the group to a hydrophilic, membrane impermeable trigonellyl ion which is trapped behind the lipoidal BBB and, in essence, remains 'locked' in the CNS. However, any of the oxidized salt formed in the periphery will be rapidly lost, as it is now polar and an excellent candidate for elimination by the kidney and bile, which reduces systemic, dose-related toxicities. Sequential removal of the attached functional groups is expected to allow the action of peptidyl glycine α-amidating monooxygenase (PAM) and glutaminyl cyclase to produce the centrally active peptide analogue from the CNS-targeted conjugate. The existence of these enzyme activities in the CNS has been well-documented [27, 30, 31]. However, attachment of the 1,4-dihydrotrigonellyl directly to the N-terminal residue may not permit the release of the target peptide because of the low amidase activity of the brain tissue. Therefore, a spacer function separating the progenitor peptide sequence from the trigonellyl with an additional amino acid residue or a pair of residues is applied in order to render the release of the desired peptide dependent on peptidases known to be active in the brain. In our preliminary work [26], an endopeptidase was superior to dipeptidyl peptidase for the possible release of the peptide when the effect of [Leu^2]TRH targeting on the amelioration of drug-induced cholinergic hypofunction was tested. A Pro residue was also found to be more beneficial than Ala in the spacer portion for similar chemical targeting systems for peptides [27]. Therefore, the spacer function applied here is optimized for prolyl oligopeptidase (POP) or post-proline cleaving enzyme (PPCE), EC 3.4.21.26.32 [32]. Besides, PPCE activity was found to be elevated in the brain of patients with AD [33]; thus, the present design may also 'take advantage' of this elevated enzyme activity to further improve the efficacy of the discussed brain-targeting approach for [Leu^2]TRH when possible treatment of AD is considered.

2.2. *Synthesis*

The chemical brain-targeting system for [Leu]^2-TRH (**10**) was synthesized by stepwise elongation of the peptide chain in the C-to-N direction using solution–phase synthesis (*figure 2*). First, *tert*-butoxycarbonyl-(Boc-)protected glycine was esterified with cholesterol using dicyclohexylcarbodiimide (DCC) as a coupling agent and dimethylaminopyridine (DMAP) as catalyst to obtain compound **2**. The Boc protecting group was removed with trifluoroacetic acid (TFA) in methylene chloride (50%, v/v) and the glycine cholesteryl ester trifluoroacetate was neutralized with N,N-diisopropylethylamine (DIEA). The protected dipeptidyl ester (**3**) was prepared by coupling glycine cholesteryl ester with Boc-proline using hydroxybenzotriazole (HOBt) activation and DCC as a coupling agent. Similar method of coupling was used to synthesize the peptide cholesteryl ester intermediates (**4**–**7**). The deprotected compound **7** was coupled with nicotinic acid to afford **8**, whose N-alkylation with dimethyl sulfate yielded the quaternary pyridinium salt (**9**). The chemical targeting system, a 1,4-dihydrotrigonellyl compound (**10**), was obtained by sodium dithionite reduction in aqueous/methanolic sodium bicarbonate solution.

After each condensation step, the intermediates (**2**–**8**) were purified by column chromatography. The quaternary salt (**9**) was stable, but hygroscopic, and co-crystallized with the alkylating agent [27]. The dihydropyridine derivative (**10**) was the 1,4-isomer, characterized by its typical UV absorption at around 348 nm.

3. Results and discussion

In animals bearing bilateral fimbrial lesions and treated with the vehicle, performance in the passive avoidance paradigm was poor (*table I*). Of the six rats tested, only one performed to the criteria of a 100 s delay before transferring to the dark side of the two-way shuttle box. The animals receiving the unmodified TRH-analogue (**1**) were similarly poor in their memory of the adversive

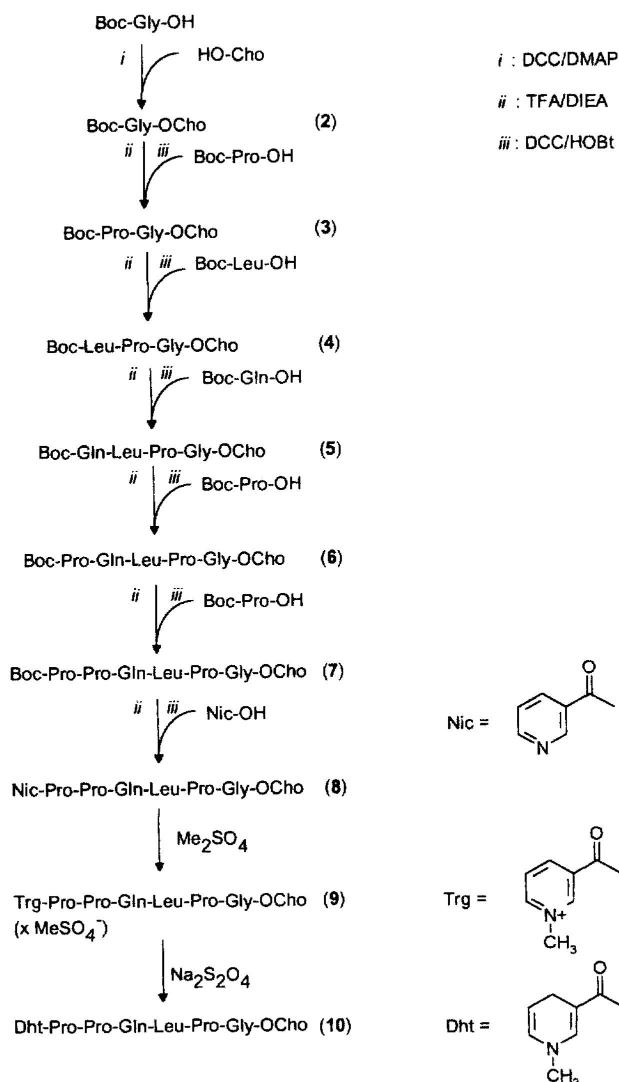

Figure 2. Synthetic pathway to obtain the chemical brain-targeting system **10**.

experience, having similar mean latencies to transfer, and only one of six rats exhibited learning in the task. By contrast, rats treated with **10** showed a fourfold increase in latency, and all animals tested reached criteria.

Daily administration of high doses of **1** or **10** had no effect on thyroid function, as no changes in thyroid weights, T3, T4 or TSH levels were observed (*table II*). These data are consistent with reports that [Leu^2]-TRH only weakly interacts with the high-affinity TRH receptor; therefore, it has little effect on TSH secretion. The present study indicates that chronic administration of **1** and **10** is, similarly, without effect on thyroid function.

Table I. Effects of [Leu^2]TRH (**1**) and its brain-targeting system (**10**) on performance in the passive avoidance test.

Treatment	Training transfer (s)	Test transfer (s)	Number of rats reaching 100-s criteria
Vehicle	25 ± 6	56 ± 12	1 of 6
1	22 ± 10	68 ± 48	1 of 6
10	54 ± 19	238 ± 36 [a]	4 of 4 [b]

Values are mean ± SEM. [a] $p < 0.05$ vs vehicle or **1** by Scheffe's post hoc analysis after ANOVA; [b] $p < 0.05$ vs vehicle or **1** by chi-squares analysis.

Table II. Effects of [Leu^2]TRH (**1**) and its brain-targeting system (**10**) on the thyroid function in adult male rats.

Treatment	Total T3 (ng/dL)	Total T4 (μg/dL)	TSH (ng/mL)	Thyroid weight (mg)
Vehicle	48 ± 8	2.1 ± 0.2	1.14 ± 0.23	23.8 ± 1.8
1	43 ± 5	2.9 ± 0.3	0.66 ± 0.15	22.5 ± 1.3
10	47 ± 5	2.7 ± 0.3	1.47 ± 0.24	22.3 ± 1.6

Values are mean ± SEM. No significant differences were observed among groups for any parameters.

These results have shown that treatment with **10** improves memory-related behavior in a passive avoidance paradigm without altering thyroid function. Evaluation of brain slices collected has shown no difference in the group treated with the chemical CNS-targeting system for [Leu^2]TRH from the control group in the average number of cholinergic neurons. Therefore, the observed effect of **10** on memory-related behavior is not a function of preservation of cholinergic neurons damaged by the lesion, but probably coming from the activation of those that remained intact due to incomplete lesion.

In conclusion, targeting a centrally active TRH analogue via the strategy that considers both physicochemical and enzymatic aspects of the blood–brain barrier may significantly improve the neuropharmaceutical efficacy of the peptide for potential treatment of various CNS maladies associated with cholinergic hypofunction such as Alzheimer's disease. We have, indeed, obtained significant enhancement of memory-related effects of a TRH analogue upon designing specific covalent modifications that allow for enhanced transport due to increased lipid-solubility, as well as retention and release of the biologically active peptide in the CNS via sequential metabolism. TRH analogues are the simplest models to optimize the variables of such targeting systems in a systematic

way, and the strategy may be directly transferable to more complex peptides.

4. Experimental protocols

4.1. Chemistry

All chemicals used were of reagent or peptide synthesis grade. All solvents were from Fisher Scientific. Boc-protected amino acids and benzhydrylamine (BHA) resin (0.7–1.2 meq/g substitution level) were purchased from Bachem (Torrance, CA). All amino acid derivatives were of L-configuration. Cholesterol was purchased from Eastman Kodak Co. (Rochester, NY). Melting points were determined on a Fisher–Johns melting point apparatus and were uncorrected. Thin layer chromatography (TLC) was carried out on silicagel-coated (Kieselgel 60 F254, 0.2 mm thickness) glass or aluminum plates (EM Science, Gibbstown, NJ). Column chromatography was performed using Davisil grade 643 silica gel. UV spectrophotometry was done on Cary 210 and Cary 3E UV-Visible Spectrophotometer (Varian, Walnut Creek, CA). Electrospray ionization mass spectra (ESI-MS) were recorded on a Vestec 200 ES quadrupole mass spectrometer (Vestec Co., Houston, TX) using methanol or methanol/chloroform (containing 10^{-5} M NaI, or 2% acetic acid, v/v, for **1** and **9**) as a solvent. Elemental analyses of compounds synthesized were performed by the Atlantic Microlab, Inc. (Norcross, GA), and by the Department of Chemistry, University of Florida (Gainesville, FL). Analyses indicated by the symbols of the elements or functions were within ±0.4% of theoretical values.

*4.1.1. [Leu[2]]TRH (pGlu-Leu-Pro-NH_2) **1***

This compound was prepared by solid-phase peptide synthesis (SPPS) using Boc-chemistry on a BHA resin with a Coupler 1000 (Du Pont-VEGA, Wilmington, DE) synthesizer. The peptide was cleaved with hydrogen fluoride using an apparatus purchased from Peninsula Laboratories (Belmont, CA). The crude peptide was purified by semi-preparative reversed-phase high-performance liquid chromatography (SpectraSeries P200 binary gradient pump, Rheodyne 7125 injector, Spectra 100 UV/VIS detector, ThermoSeparation, Fremont CA) using 0.1% (v/v) of TFA in water and 0.08% (v/v) of TFA in acetonitrile mobile phase (5.0 mL/min, linear gradient). The freeze-dried peptide was an off-white, hygroscopic solid; TLC: $CHCl_3$/MeOH (75:25), $R_f = 0.55$; m.p. 111–117 °C. MS (ESI): *m/z* 339 $[M + H]^+$, Anal. $C_{16}H_{25}N_4O_4 \times 1.5H_2O$ (C, H, N).

*4.1.2. Boc-Gly cholesteryl ester **2***

A solution of Boc-glycine (24.7 g, 14.1 mmol), DMAP (24.0 g, 196.5 mmol) and DCC (43.7 g, 21.1 mmol) in methylene chloride (165 mL) was cooled to 0 °C with an ice-bath. Cholesterol (28 g, 72.4 mmol) in methylene chloride (100 mL) was added and the mixture was stirred at 0 °C for 30 min, then continued for 24 h at room temperature. The precipitate (DCU) was filtered off, then the filtrate was successively washed with citric acid (5%, 5 × 100 mL), saturated sodium bicarbonate (5 × 100 mL), distilled water (5 × 100 mL). The organic layer was dried over sodium sulfate, and the solvent was removed in vacuo. The crude product was purified by column chromatography (silicagel) with CH_2Cl_2/MeOH (99:1, v/v) as an eluent. White solid (29.6 g, 75% yield) was obtained; TLC: $CHCl_3$/MeOH (100:1), $R_f = 0.61$; m.p. 115–116 °C. MS (ESI): $[M + Na]^+$ *m/z* 567; Anal. $C_{34}H_{57}NO_4 \times 0.25H_2O$ (C, H, N).

4.1.3. General procedure for the elongation of the peptide chain (3–7)

Boc-Gly or Boc-peptide cholesteryl ester, dissolved completely in methylene chloride, was deprotected with an aliquot of TFA for 60 min at room temperature, then the solvent was removed in vacuo. The residue was dissolved in methylene chloride, and neutralized with DIEA. A solution of Boc-protected amino acid (1.5 eq.; 1.5:1 molar ratio to the cholesteryl ester), HOBt (1.7 eq.) and DCC (2.8 eq.), dissolved in methylene chloride, was added and the mixture was stirred at room temperature overnight. The precipitate (DCU) was filtered off, the filtrate was diluted with methylene chloride, then washed successively with 5% citric acid solution (4–5 times), saturated aqueous sodium bicarbonate (4 times), and distilled water (4 times). The organic layer was dried over anhydrous sodium sulfate, and solvent was removed in vacuo. The crude Boc-peptide cholesteryl ester was purified by column chromatography. Purification procedure and analytical data of the compounds prepared are given below.

*4.1.4. Boc-Pro-Gly cholesteryl ester **3***

Column chromatography with CH_2Cl_2/MeOH (99:1, v/v), yield 63%. TLC ($CHCl_3$/MeOH 98:2, v/v): $R_f = 0.3$; m.p. 173–175 °C. MS (ESI): $[M + Na]^+$, *m/z* 664; Anal. $C_{39}H_{64}N_2O_5 \times 0.5H_2O$ (C, H, N).

*4.1.5. Boc-Leu-Pro-Gly cholesteryl ester **4***

Column chromatography with CH_2Cl_2/MeOH (98:2, v/v), yield 75%. TLC ($CHCl_3$/MeOH 90:10, v/v): $R_f = 0.65$; m.p. 59–61 °C. MS (ESI): $[M + Na]^+$, *m/z* 777; Anal. $C_{45}H_{75}N_3O_6 \times H_2O$ (C, H, N).

*4.1.6. Boc-Gln-Leu-Pro-Gly cholesteryl ester **5***

Column chromatography with CH_2Cl_2/MeOH (93:7, v/v), yield 77%. TLC ($CHCl_3$/MeOH 90:10, v/v): $R_f = 0.4$; m.p. 105–107 °C. MS (ESI): $[M + Na]^+$, *m/z* 905; Anal. $C_{50}H_{83}N_5O_8 \times H_2O$ (C, H, N).

*4.1.7. Boc-Pro-Gln-Leu-Pro-Gly cholesteryl ester **6***

Column chromatography with CH_2Cl_2/MeOH (92:8, v/v), yield 75%. TLC (CHC_{13}/MeOH 90:10, v/v): $R_f = 0.35$; m.p. 128–130 °C. MS (ESI): $[M + Na]^+$, *m/z* 1002; Anal. $C_{55}H_{90}N_6O_9 \times H_2O$ (C, H, N).

*4.1.8. Boc-Pro-Pro-Gln-Leu-Pro-Gly cholesteryl ester **7***

Column chromatography with CH_2Cl_2/MeOH (90:10, v/v), yield 82%. TLC ($CHCl_3$/MeOH 90:10, v/v): $R_f = 0.47$; m.p. 137–139 °C. MS (ESI): $[M + Na]^+$, *m/z* 1099; Anal. $C_{60}H_{97}N_7O_{10} \times H_2O$ (C, H, N).

*4.1.9. Nicotinoyl-Pro-Pro-Gln-Leu-Pro-Gly cholesteryl ester **8***

Compound **7** was dissolved completely in methylene chloride, then deprotected with an aliquot of TFA for 60 min at room temperature. The solvent was removed in vacuo. The residue was completely dissolved in methylene chloride, and neutralized with DIEA. A methylene chloride solution of nicotinic acid (1.5 eq.; 1.5:1 molar ratio to the cholesteryl ester), HOBt (1.7 eq.) and DCC

(2.8 eq.) was added, and the mixture was stirred at room temperature overnight. The precipitate (DCU) was filtered off, the filtrate was diluted with methylene chloride, then washed successively with 5% citric acid solution (4–5 times), saturated aqueous sodium bicarbonate (4 times), and distilled water (4 times). The organic layer was dried over anhydrous sodium sulfate, and solvent was removed in vacuo. Column chromatography with CH_2Cl_2/MeOH (86:14, v/v), yield 95%. TLC ($CHCl_3$/MeOH 88:12, v/v): $R_f = 0.37$; m.p. 150–152 °C. MS (ESI): $[M + Na]^+$, *m/z* 1104; Anal. $C_{61}H_{92}N_8O_9 \times H_2O$ (C, H, N).

4.1.10. Trigonellyl-Pro-Pro-Gln-Leu-Pro-Gly cholesteryl ester ***9***

The nicotinoyl peptide cholesteryl ester (**8**) was dissolved completely in methylene chloride, and dimethyl sulfate (15 eq.; 15:1 molar ratio to **8**) was added. The mixture was stirred at room temperature for 24 h. The solvent was removed in vacuo, and the solid residue was washed with diethyl ether. The crude product was purified by recrystallization from chloroform and diethyl ether. Yield 92%. m.p. 186–188 °C (dec.); MS (ESI): Quaternary ion (Q^+) at *m/z* 1096; UV_{max} (MeOH): 264 nm.

4.1.11. 1,4-Dihydrotrigonellyl-Pro-Pro-Gln-Leu-Pro-Gly cholesteryl ester ***10***

To the quaternary compound **9** dissolved in 50% (v/v) degassed aqueous methanol, the mixture of sodium bicarbonate (12 eq.; 12:1 molar ratio to **9**) and sodium dithionite (10 eq.) was added under ice-cooling, and the reaction mixture was allowed to stand at 0 °C for 15 min and then stirred at room temperature for 40 min under nitrogen atmosphere (the progress of the reduction was monitored by TLC and UV spectroscopy). Then the mixture was extracted with cold, degassed methylene chloride and the organic layer was washed several times with cold, degassed water. The organic layer was dried under nitrogen atmosphere and the solvent was carefully removed in vacuo resulting in a pale yellow solid with 24% yield. TLC ($CHCl_3$/MeOH 88:12, v/v): $R_f = 0.41$; m.p. 140–142 °C (dec.). UV_{max} (MeOH): 348 nm; MS (ESI): $[M + Na]^+$, *m/z* 1120, Anal. $C_{62}H_{96}O_9N_8 \times 3.5H_2O$ (C, H, N).

4.2. Pharmacology

4.2.1. Animals

Young adult male Sprague–Dawley rats (210–240 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA). Animals were housed two per cage in an environmentally controlled room on a 12-h light, 12-h dark cycle. They were maintained ad libitum on water and rodent chow. All procedures performed on animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Florida before initiation of the study.

4.2.2. Treatment

The rats were implanted with atrial cannulae for the daily administration of the vehicle (propylene glycol–dimethyl sulfoxide, 2:1, v/v), as well as **1** and **10** in equimolar doses (15 µg/kg body weight). The effects of chronic treatment on cholinergic neuronal function following injury by treating were assessed by daily treatment with vehicle, **1** and **10** for 2 days, respectively, then subjecting the rats to bilateral fimbrial lesions. Daily treatment with the compounds continued for 8 days thereafter. On the tenth day of treatment, the rats were trained in a passive avoidance paradigm, and the following day they were tested for memory of the training.

One day after completion of the behavioral testing, the animals were anesthetized with sodium pentobarbital, and blood samples were taken for the determination of serum total T3, total T4, and TSH concentrations. The thyroid gland was weighed, and the rats were perfused with phosphate-buffered formaldehyde for processing brain slices to count the choline-acetyltransferase-(ChAT-) positive cells in the medial septa.

4.2.3. Fimbria–fornix lesion

Rats were subjected to bilateral fimbrial lesion at 2 days following the onset of daily treatment with the TRH analogue (**1**) and its chemical targeting system (**10**), the vehicle solution of the compounds as a control (this focused study design without sham controls for each group was based on our earlier data that indicated only slight differences in passive avoidance behavior between the lesioned and non-lesioned groups). Animals were anesthetized with intraperitoneal injections of sodium pentobarbital (0.7 mL/rat; 50 mg/mL). Surgery was carried out on a Kopf stereotaxic apparatus. With the skull lying flat between bregma and lambda, a small hole was drilled at 1.1 mm posterior and 1.1 mm lateral to bregma. A concentric bipolar electrode was lowered 4.5 mm from the dura and a current of 750 mA was passed through the electrode for 30 s to make the fimbrial lesion. At the end of surgery, the wounds were sutured and all animals were given a subcutaneous injection of penicillin (0.1 mL).

4.2.4. Passive avoidance behavior

Passive avoidance behavior was conducted as previously described [34]. Briefly, rats were placed in the lighted compartment of a two-way shuttle box. The door connecting the lighted and the dark compartments was opened. When the animal entered the dark compartment, it received a mild footshock (0.8 mA) for 1 s. The latency for the animal to enter the dark compartment was recorded. 24 h later, the trial was repeated and the latency to enter the dark compartment was determined and recorded. A longer latency to enter the dark compartment was taken as an indication that the animal had retained memory of the electrical shock delivered upon entering the dark compartment during the training trial, 24 h earlier.

4.2.5. Assay of serum hormones

TSH was assayed using reagents provided by the Dr. A.F. Parlow and the National Hormone and Pituitary Program (NIDDK, Baltimore, MD). TSH concentrations are expressed as nanograms/mL serum in terms of the NIDDK reference preparation TSH-RP-2. T3 and T4 were assayed using kits provided by Diagnostic Products (Los Angeles, CA). For all three hormones, duplicate samples were assayed from each animal. The intra-assay coefficient of variation for the TSH, T3 and T4 assay used were less than 10%, 5% and 5%, respectively. All samples were determined in a single assay for each hormone, so no inter-assay coefficient of variation was determined.

4.3. Immunohistochemistry

4.3.1. Sample preparation

Animals were anesthetized with intraperitoneal injections of sodium pentabarbital (0.7 mL/rat; 50 mg/mL) and then perfused transcardially with 100 mL of 0.9% saline followed by 400 mL of

fixative containing 4% paraformaldehyde, 0.2% glutaraldehyde in phosphate buffered saline (PBS) at pH 7.4. The brains were left in situ for 2 h at 4 °C and then removed and placed in PBS containing 30% glucose at 4 °C for 72 h. For immunohistochemical analysis, the brains were frozen and coronally sectioned on a cryostat microtome into 50 micron sections and collected in PBS. Sections were then washed with 3% hydrogen peroxide for 10–15 min and then washed twice with PBS. Next, sections were incubated in PBS containing normal goat serum (3%) and the primary antibody (anti-ChAT, 1:250 dilution, Chemicon Inc., Temecula, CA) overnight at room temperature under gentle movement of the incubation medium. After washing with PBS twice, the sections were incubated overnight in biotinylated Mouse IgG (1:1000 dilution, Chemicon Inc.) in PBS and 3% normal goat serum. After two washes the sections were incubated overnight in extravidin peroxidase in PBS (1:1000 dilution, Sigma, St. Louise, MO). In all incubation steps, 0.1% Triton X-100 was added to the incubation medium. Sections were washed twice and incubated for 15 min in a solution of 0.5 mg/mL diaminobenzidine (DAB) with 3% hydrogen peroxide in Tris-buffered saline (pH 7.6). Sections were washed again with PBS and mounted on glass slides and coverslipped. Sections containing the area of lesion were collected separately, mounted on glass slides and later stained with cresyl violet. The extent of lesion was later determined by measuring its diameter and location.

4.3.2. Image analysis

Images were captured and computer-assisted cell counts were conducted using the 'Image Pro Plus' (Media Cybernetics, MD) software package. The medial septum, not including the vertical diagonal band of broca, was defined by manually drawing an area outline on each image. Neurons were identified visually and images were segmented by stain intensity. ChAT-positive neurons were identified as round objects containing a dark brown coloration. To aid the computer in identifying the immunoreactive cells the threshold intensity was adjusted interactively by selecting the minimum and maximum image cutoffs in every slice. The intensity was adjusted interactively for each area slice because, depending on the staining of the slice, the intensity at which a ChAT-positive neuron was identified varied between slices. Following adjustment of the intensity (range between 0 and 255), a threshold value was determined by investigator for the medial septum in very section. The threshold value was the value at which the image could be segmented into its constituent elements, thereby aiding the investigator in analyzing the individual shape and size when a neuronal shape was identified. This thresholding was optimized to maximize the number of separate neurons present while including as much of the cell body as possible and then the cells were counted automatically by the computer. From a total of approximately 18 sections of the medial septum in every animal, every third one was counted.

4.3.3. Statistics

Analysis of variance (ANOVA) was used to assess the treatment effects on medial septal ChAT-positive cell number, serum hormones and behavior response to drug treatment. Scheffe's post-hoc test was used to test for differences between individual treatment conditions. The significance of the difference in the number of animals reaching a behavioral criteria was assessed using chi-square analysis. $P < 0.05$ was considered significant.

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