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Synthesis and biological evaluations of brain-targeted chemical delivery systems of [Nva^2]-TRH

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Abstract

Various chemical delivery systems for [Nva^2]-TRH were synthesized and their CNS activity was investigated and compared with that of a similar chemical delivery system of [Leu^2]-TRH, previously studied. Sequential metabolism of the chemical delivery system delivered to the brain, starting with the conversion of the dihydrotrigonellyl (DHT) to the trigonellyl (T^+) moiety, will provide the lock-in to the brain of the T^+-chemical delivery system, which will undergo hydrolysis of the cholesteryl ester, formation of the Pr-amide and cleavage of the spacer-T^+ part, allowing ultimately the sustained release of the active [Nva^2]-TRH. The CNS activity was assessed by measuring the extent of antagonizing barbiturate-induced sleeping time in mice. The fully packaged DHT-Pro-Pro-Gln-Nva-Pro-Gly-OCh produced robust antagonism, reducing sleeping time from 89 min to 48 min, similar to the Leu^2-analogue (49 min). However, the partially substituted [Nva^2]-TRH analogues showed little or no CNS activity. The results indicate that the fully packaged delivery system is necessary to produce the successful brain targeting of the precursor construct and effective release of the Gln-Nva-ProNH_2.

Introduction

Thyrotropin-releasing hormone (TRH, PyroGlu-His-Pro-NH_2) is primarily responsible for the neuroendocrine regulation of thyrotropin (TSH) secretion from the pituitary (Boler et al 1969; Brownstein et al 1974). TRH, however, induces numerous behavioural effects (Webster et al 1982; Andrews & Sahgal 1983), the most interesting and best-known being the reduction of barbiturate narcosis or haloperidol-induced catalepsy (Schmidt 1977; Horita et al 1989). More importantly, TRH and its analogues can accelerate acetylcholine turnover and improve memory and learning (Santori & Schmidt 1980; Itoh et al 1994). Thus, these peptides can be beneficial for treating motor neuron disease (Yarbrough 1983), spinal cord trauma (Faden et al 1989) and Alzheimer's disease (Sunderlana et al 1989). The therapeutic use of TRH in the treatment of age-related brain dysfunction is limited by its short half-life (Basirri & Utiger 1973) and its inability to penetrate the blood–brain barrier (Metcalf 1982). Since TRH is also rapidly metabolized in the general circulatory system, by prolylendopeptidase and pyroglutamyl aminopeptidase (Hussain & Tait 1983), its brain-targeting strategies must include protection of the peptide before and during brain delivery, as well as enhancing its penetration through the blood–brain barrier. As indicated, TRH is not the best choice for the various targeting manipulations, but several of its analogues have been synthesized to separate the endocrine effect (TSH-releasing) from the CNS effects (Szirtes et al 1984; Yamamoto & Shimizu 1987). Thus, analogues where [His^2] is replaced by an aliphatic amino-acid residue, such as leucine, isoleucine or norvaline (Szirtes et al 1984), show significant enhancement in their CNS-vs-endocrine activity ratio. But these analogues have the same problem as TRH (Zlokovic et al 1985) in terms of penetrating the blood–brain barrier.

We have previously developed a general method (Bodor et al 1992) for brain targeting of peptides, using a chemical targeting system based on retrometabolic design. Accordingly, a peptide, like a Leu-enkephalin analogue, is coupled at the amino terminus via a spacer (one or two strategically chosen amino acids) to the redox-targeting 1-4-dihydrotrigonellyl moiety. A large lipophilic function, like cholesteryl, is

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placed on the C-terminus. This lipophilic construct is not an easy substrate for peptidases and penetrates the blood–brain barrier by passive transport. Sequential metabolism, starting with the enzymatic oxidation of the dihydropyridine moiety in the brain, yields retention (lock-in) of the conjugate due to the formation of a quaternary pyridinium salt. Removal of the cholesteryl function, followed by endopeptidase cleavage of the trigonellyl-spacer part affords the active neuropeptide in a sustained manner (Bodor 1994; Prokai-Tatrai et al 1996). A modification of this was successful in brain targeting of kyotorphin analogues (Chen et al 1998), as well as with further modifications to brain targeting of some TRH analogues, like [Leu^2]-TRH (Prokai et al 1994, 1999) and [Leu^2, Pip^3]-TRH (Yoon et al 2000).

In our continuing efforts to explore brain targeting of all possible TRH analogues for their potential use in Alzheimer's disease, we are reporting the synthesis and pharmacological studies of a chemical delivery system for [Nva^2]-TRH analogue, the dihydrotrigonellyl (DHT) derivative DHT-Pro-Pro-Gln-Nva-Pro-Gly-OCh (where Ch is cholesteryl). The targeted compounds were compared, in the pentobarbital-induced sleeping time assay in mice, with TRH, [Nva^2]-TRH, the various partially derivatized [Nva^2]-TRH analogues and the previously evaluated [Leu^2]-TRH chemical delivery system (Prokai et al 1999).

Materials and Methods

Instrument and chemicals

All chemicals used were of reagent or peptide-synthesis grade. TRH, Fmoc-amino acids, and other chemicals were purchased from Chem-Index Inc., USA. Solvents were purchased from Fisher Scientific Inc. Thin-layer chromatography was performed on either silica-gel-coated (Merck Kiesel 60 F254, 0.2 mm thickness) plates or neutral-alumina-coated (Merck Kiesel 60 F254, 0.2 mm thickness) plates. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Elemental analyses were supplied by Atlantic Micro labs. Inc. (Norcross, GA). Mass spectra were recorded on a Kratos MS80RFA instrument (Kratos Analyticals, Manchester, UK). UV spectra were recorded in methanol on a Lambda 11 UV/vis Perkin Elmer (Perkin Elmer Anal. Inst., Norwalk, CT). The following abbreviations are used: AA, amino acid; Ch, cholesteryl; DCC, 1,3-dicyclohexylcarbodiimide; DCU, 1,3-dicyclohexylurea; DIC, 1,3-diisopropylcarbodiimide; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; HOBt, 1-hydroxybenzotriazole; DCM, dichloromethane; DHT, dihydrotrigonellyl; DMA, *N*,*N*-dimethylacetamide; DMF, *N*,*N*-dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; EtOAc, ethyl acetate; IPA, isopropyl alcohol; MeOH, methanol; TFA, trifluoroacetic acid; DIEA, *N*,*N*-didisopropylethylaime, TEA, triethylamine; Nic, nicotinoyl; Su, *N*-hydroxysuccinyl; T^+, trigonellyl.

General procedure for solid-phase peptide synthesis

Fmoc-AA-resin was sealed into a nylon bag (called tea bag), and was placed in DMF (2×2 min). It was washed twice with DMF and the Fmoc group was deprotected by 50% piperidine–DMF solution (3 min and then 7 min).

Fmoc-AA-OBt (activated amino ester) was prepared by mixing Fmoc-AA-OH, HOBt and DIC in DMA in same equivalent amount. The mixture was stirred for 30 min before being used for coupling reaction.

The deprotected resin was washed successively with DMF (4×1 min), IPA (1×1 min), DMF (1×1 min) and DCM (2×1 min). After drying the bag, a solution of Fmoc-AA-OBt (3.0 equiv.) was added for coupling reaction. The mixture was shaken for 45 min at room temperature. The bag was washed with DCM (2×1 min), IPA (1×1 min), DMF (1×1 min), 5% DIEA–DCM (1×1 min), and DCM (2×1 min).

The coupling reaction was repeated for 45 min and the resin was washed with DCM (2×1 min), IPA (1×1 min), DMF (1×1 min), and DCM (2×1 min).

The coupling efficiency was monitored by the ninhydrin test.

*PyrNvaPro-NH$_2$ (**2**)*

Rink resin (Fmoc-NH-resin, 1.0 g, 0.40 mmol) was used in this synthesis. After coupling each residue to the resin step by step, the peptide was cleaved off by using TFA. After evaporation of solvent, the residue was washed with ether (3×30 mL) and dried. Purification with HPLC afforded the pure product (93.7 mg, 72% yield) as white crystals, mp 118–120°C, FAB MS. $[M+H]^+$ m/z 325.

*TFA salt of Nic-ProProGlnNvaProGly-OH (**3**)*

The flask containing a bag of Nic-ProProGlnNvaProGly-O-resin, which was prepared by following the general solid-phase peptide synthesis starting with Fmoc-Gly-resin (200 mg), was treated with 15 mL of TFA. The resulting mixture was shaken for 45 min. The solution was transferred into another flask and the bag was washed twice with TFA. After the TFA was removed in vacuum, the residue was diluted with ethyl ether (20 mL). The precipitate was washed once with ether (20 mL), and then it was re-precipitated from chloroform–ether. The precipitate was dried and recrystallized from CH_2Cl_2–hexane to give 140 mg of product (81% yield), mp 138–140°C. ESI MS $[M+Na]^+$; m/z 721. Elemental analysis for $C_{35}H_{47}N_8O_{11}F_3$; Calc., C 51.72, H 5.83, N 13.79. Found, C 51.44, H 5.99, N 13.88.

*Nic-ProProGlnNvaProGly-OCh (**4**)*

To a solution of the TFA salt of Nic-ProProGlnNva-ProGly-OH (81.2 mg, 0.10 mmol), cholesterol (85.1 mg, 0.22 mmol) and 4-pyrrolidinopyridine (6.0 mg, 0.04 mmol) in chloroform (2 mL) was added DCC (45.5 mg, 0.22 mmol). After the mixture was stirred for 40 h at room temperature under nitrogen atmosphere, the precipitated

DCU was filtered, and the filtrate was diluted with methylene chloride (20 mL). The combined organic layers were washed with 5% citric acid solution (2×25 mL), saturated sodium bicarbonate solution (2×25 mL) and water (2×25 mL) successively, and dried over anhydrous sodium sulfate. After the solvent was filtered and concentrated, the residue was purified by HPLC to give the pure compound (8.0 mg, 15%). R_f = 0.13 (chloroform–methanol, 10:1), ESI MS $[M+Na]^+$; m/z 1090.

*Trig-ProProGlnNvaProGly-OCh (**5**)*
To a solution of Nic-ProProGlnNvaProGly-OCh (1.63 g, 1.53 mmol) in 25 mL of chloroform (25 mL) was added dimethyl sulfate (2.89 g, 22.95 mmol). The mixture was stirred at room temperature for 14 h. After evaporation of the solvent, the residue was diluted with ether (50 mL). The resulting yellow precipitate was filtered and washed with ether (50 mL) again. Recrystallization from chloroform–ether (1:20) gave the pure product (1.83 g, quantitative yield) as white crystals. mp 190–192°C, ESI MS $[M+H]^+$; m/z 1082, UV (MeOH) 254 nm (max).

*1,4-Dihydrotrigonellyl-ProProGlnNvaProGly-OCh (**6**)*
To an ice-cold solution of Trig-ProProGlnNvaProGly-OCh (200 mg, 0.16 mmol) in de-aerated water (30 mL) were added sodium bicarbonate (290 mg, 3.5 mmol), sodium dithionate (1.05 g, 6.0 mmol) and ethyl acetate (5 mL) under nitrogen. The mixture was stirred for 5 min at 0°C and additional cold ethyl acetate (50 mL) was added. The mixture was stirred for another 30 min at 0°C, then for 50 min at room temperature. The ethyl acetate was separated and the aqueous layer was extracted with ethyl acetate (60 mL). The combined ethyl acetate extracts were washed with 5% sodium bicarbonate solution (40 mL), dried over sodium sulfate, filtered and then distilled on a rotary evaporator to give the product (70 mg, 38.5% yield) as a yellowish solid. De-aerated petroleum ether (30 mL) was added immediately to prevent the oxidation. R_f = 0.30 ($CHCl_3$–MeOH–Et_3N, 86:9:5), UV($\boldsymbol{k}_{max}$) 348 nm.

*Trig-ProProGlnNvaProGly-OH (**7**)*
To a solution of Trig-ProProGlnNvaProGly-OCh (400 mg, 0.335 mmol) in chloroform (3 mL) was added drop-wise methanesulfonic acid (10 mL) and anisole (1 mL) at 0°C. The reaction mixture was stirred for 10 min at 0°C and then stirred for 60 min at room temperature. After evaporation of chloroform, the residue was diluted with ether (200 mL) to give precipitate. The precipitate was washed with ether (2×200 mL) and then dissolved in 50% acetonitrile–water. The resulting solution was neutralized to pH 7 with 1 M NaOH to give precipitate, which was filtered and dried to give the product (244 mg, 90% yield), mp 180°C, FAB MS $[M+H]^+$; m/z 713.

*Dihydrotrigonellyl-ProProGlnNvaProGly-OH (**8**)*
To an ice-cold solution of Trig-ProProGlnNvaProGly-OH (30 mg, 0.036 mmol) and sodium bicarbonate (147 mg, 1.75 mmol) in de-aerated water (10 mL) was added sodium dithionate (261 mg, 1.5 mmol) and ethyl acetate (2 mL) under nitrogen atmosphere. After the mixture was stirred for 5 min at 0°C, additional cold ethyl acetate (15 mL) was added. The mixture was stirred for another 30 min at 0°C, and then for 50 min at room temperature. The following work-up procedure was carried out in a dry box filled with nitrogen: The ethyl acetate layer was separated and the aqueous layer was extracted with ethyl acetate (3×20 mL). The combined ethyl acetate extract was washed with 1% sodium bicarbonate solution (150 mL) containing 1% $Na_2S_2O_4$ (1.5 mL) and then dried over sodium sulfate. Evaporation of the solvent gave the product (8.85 mg, 38.5% yield) as a yellow solid. De-aerated petroleum ether was added immediately to prevent the oxidation. R_f = 0.48 ($CHCl_3$–MeOH, 9:1), UV($\boldsymbol{k}_{max}$) 348 nm.

*PyrNvaProGly-OH (**9**)*
Fmoc-Gly-O-Wang resin (1.40 g, 0.882 mmol) was used for the solid-phase peptide synthesis. After coupling each amino acid onto the resin according to the general procedure of solid-phase synthesis, the peptide was cleaved from the resin by treating with TFA. The evaporation of solvent gave a residue, which was washed with ethyl ether (3×50 mL) to give the product (336 mg, quantitative yield) as a white solid. mp 89–91°C, ESI MS $[M+Na]^+$; m/z 406.

*PyrNvaProGly-OCh (**10**)*
To a solution of PyrNvaProGly-OH (153 mg, 0.40 mmol) and cholesterol (1.74 g, 4.50 mmol) in chloroform (5 mL) was added TFA (45.6 mg, 0.40 mmol) followed by 4-pyrrolidinopyridine (26.7 mg, 0.40 mmol). After the mixture was cooled down to 0°C, DCC (111 mg, 0.54 mmol) was added and then stirred for 1 h at room temperature. Additional DCC (111 mg, 0.54 mmol) was added and the resulting mixture was stirred overnight at room temperature. The precipitate was filtered and the filtrate was diluted with methylene chloride (50 mL). The solution was washed with 5% citric acid solution (2×25 mL), water (2×25 mL), saturated sodium bicarbonate solution (2×25 mL) and water (2×25 mL) successively, and then dried over sodium sulfate. After evaporation of the solvent, the residue was dissolved in chloroform (10 mL) and then subjected to neutral alumina column chromatography with 0.5% methanol–chloroform (150 mL), 5% methanol–chloroform (150 mL), and 25% methanol–chloroform (150 mL) as an eluent. The product (90 mg, 30% yield) was obtained as a white solid. R_f = 0.54 ($CHCl_3$–MeOH, 9:1), mp 136–138°C, ESI MS $[M+Na]^+$; m/z 774.

Effect of [Nva²]-TRH chemical delivery system and analogues on the barbiturate-induced sleeping time in mice

Swiss Webster mice (body weight, 30±3 g) were used. Compounds were dissolved in propylene glycol–dimethyl sulfoxide (DMSO) (2:1). Vehicle only (1.5 mL kg^{-1}) or compounds at a dose of 20.7 $\boldsymbol{l}$mol kg^{-1} (equimolar to 7.5 mg kg^{-1} of TRH) was injected into the mice through the tail vein to compare the CNS activity. Ten minutes after intravenous injection of the compound, each mouse

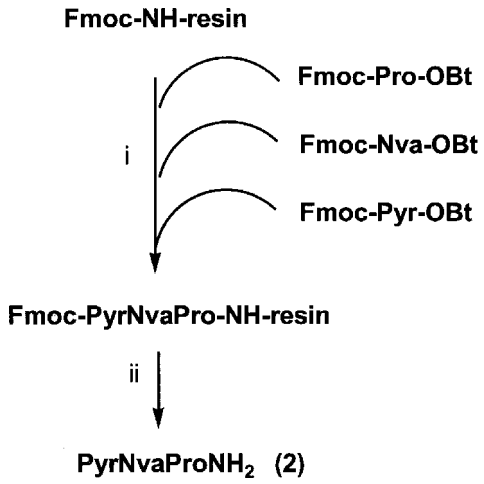

Figure 1 Synthesis of PyrNvaPro-NH_2. Reagents: (i) HOBt/DIC, (ii) TFA.

received an intraperitoneal injection of sodium pentobarbital (30 mg mL^{-1}) at a dose of 60 mg kg^{-1}. The sleeping time was recorded as the time elapsed from the onset of loss of the righting reflex until the reflex was regained. Groups of 6–7 mice were used for testing each compound. Statistical significance was evaluated using analysis of variance.

Results and Discussion

Synthesis

[Nva^2]-TRH (**2**) was prepared by step-wise elongation of the peptide chain from Fmoc-NH-resin with the corresponding activated ester of an amino acid using solid-phase peptide synthesis followed by cleavage of the resin with TFA (Figure 1).

The chemical delivery system of DHT-ProProGlnNva-ProGly-OCh (**6**) for brain-targeted delivery was prepared

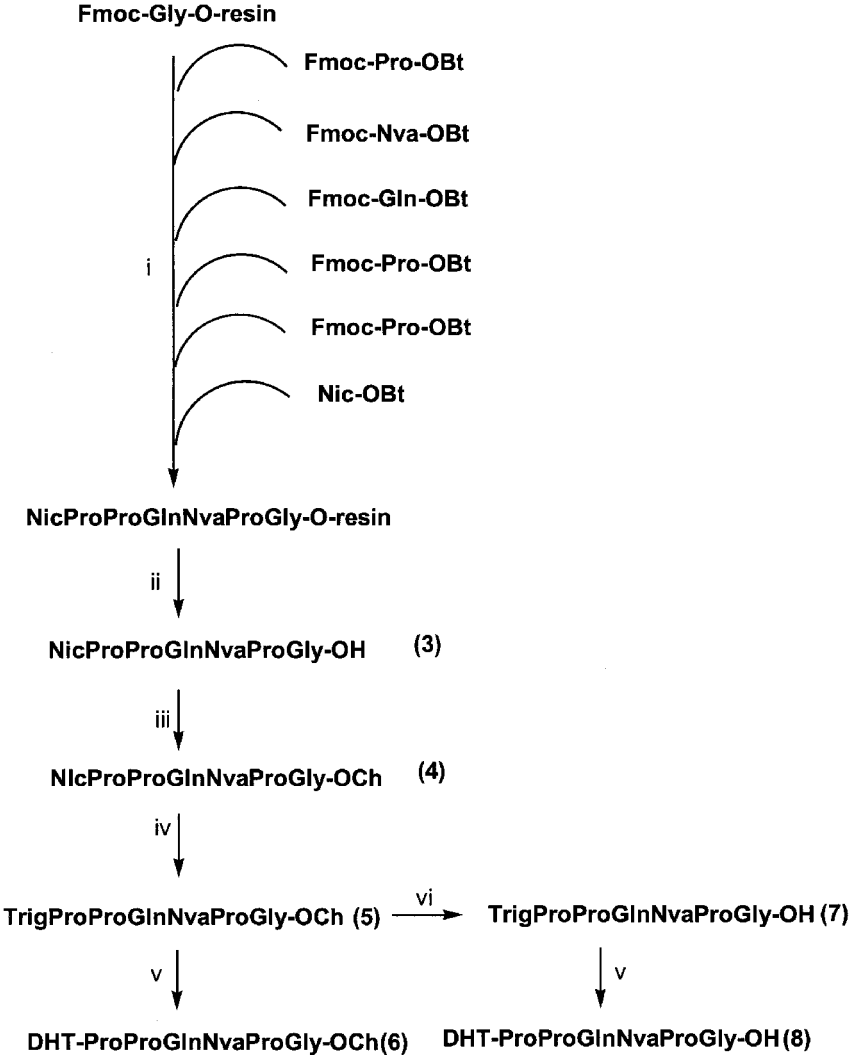

Figure 2 Syntheses of DHT-ProProGlnNvaProGly-OCh and DHT-ProProGlnNvaProGly-OH. Reagents: (i) HOBt/DIC, (ii) TFA, (iii) cholesterol–DCC–DMAP, (iv) $(CH_3)_2SO_4$, (v) $Na_2S_2O_4$, (vi) CH_3SO_3H, anisole.

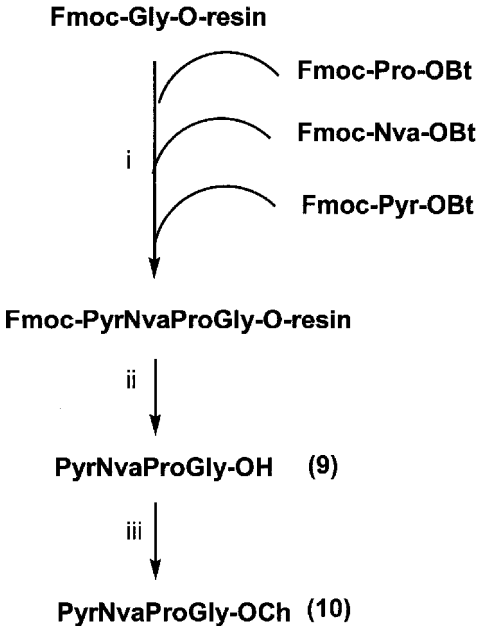

Figure 3 Synthesis of PyrNvaProGly-OCh. Reagents: (i) HOBt/DIC, (ii) TFA, (iii) cholesterol–DCC–DMAP.

by step-wise elongation of the peptide chain from Fmoc-Gly-O-resin with the corresponding activated ester of the amino acid using solid-phase peptide synthesis followed by solution-phase synthesis (Figure 2). Thus, NicProProGlnNvaProGly-O-resin was prepared by solid-phase peptide synthesis using an automated Fmoc chemistry protocol. The DIC-mediated coupling was accelerated with HOBt. After completion of step-wise elongation, the crude peptide of NicProProGlnNvaGly-OH (**3**) was cleaved off the resin with TFA, and identified by EI-MS. Since the purity of the crude peptide was about 99%, it was used in the next reaction without further purification. The coupling of NicProProGlnNvaGly-OH (**3**) with cholesterol by using DCC–HOBt system in DCM gave the crude product, **4**, which was purified by column chromatography on neutral alumina with 5% MeOH–DCM eluent. Methylation of the pyridine ring in **4** with dimethyl sulfate gave **5**. Finally, reduction of the trigonellyl group with sodium dithionate gave the desired 1,4-dihydro product **6**.

The partially packaged delivery systems of Trig-Pro-ProGlnNvaProGly-OH (**7**) and DHT-ProProGlnNvaProGly-OH (**8**) were prepared from Trig-ProProGlnNvaProGly-OCh (Figure 2). After the removal of the cholesteryl group of **5** in the acidic condition, the trigonellyl group of **7** was reduced with sodium dithionate to give **8**.

PyrNvaProGly-OCh (**10**) was prepared from Fmoc-Gly-O-resin (Figure 3). Thus, after step-wise elongation of the peptide chain with the corresponding activated ester of amino acid using solid-phase peptide synthesis, the resulting PyrNvaProGly-OH (**9**) was cleaved off the resin with TFA. The coupling of PyrNvaProGly-OH (**9**) with cholesterol by using DCC–DMAP system in chloroform gave the desired product **10**.

The chemical delivery system of [Leu²]-TRH for brain-targeted delivery was prepared by following our previously reported method (Prokai et al 1999), consisting of step-wise elongation of the peptide chain from Fmoc-Gly-O-resin with the corresponding activated ester of amino acids, cleavage of the peptide resin, coupling reaction of the NicProProGlnLeuProGly-OH with cholesterol, methylation with dimethyl sulfate and reduction with sodium dithionate.

Pharmacology

The antagonism of barbiturate-induced sleeping time in mice was used to assess the effect on mice cholinergic neurons of CNS-delivered [Nva²]-TRH. Ten minutes

Table 1 Comparison of pentobarbital-induced sleeping time in mice after administration of the test compounds.

Compound	Sleeping time (min)
Vehicle	88.89±9.89
Pyr-His-Pro-NH_2 (**1**)	83.89±13.85
Pyr-Nva-Pro-NH_2 (**2**)	73.31±9.60*
Nic-Pro-Pro-Gln-Nva-Pro-Gly-O-cholesterol (**4**)	84.64±18.62
Trig-Pro-Pro-Gln-Nva-Pro-Gly-O-cholesterol (**5**)	84.00±15.31
DHT-Pro-Pro-Gln-Nva-Pro-Gly-O-cholesterol (**6**)	48.09±3.84***
Trig-Pro-Pro-Gln-Nva-Pro-Gly-OH (**7**)	79.98±14.17
DHT-Pro-Pro-Gln-Nva-Pro-Gly-OH (**8**)	69.42±11.99**
Pyr-Nva-Pro-Gly-O-cholesterol (**10**)	71.71±15.19*
DHT-Pro-Pro-Gln-Leu-Pro-Gly-O-cholesterol (**11**)	49.19±15.71

Ten min after intravenous injection of a compound (20.7 μl mol kg^{-1}; TRH (**1**) 20.7 μl mol = 7.5 mg), pentobarbital, 60 mg kg^{-1}, was injected intraperitoneally. The sleeping time was recorded as the time elapsed from the onset of the loss of the righting reflex until it was regained. A mixture of propylene glycol and DMSO (2:1) was used as vehicle. Seven Swiss Webster mice (30±3 g) were used in each group. Table entries are mean±s.d. *$P < 0.05$, **$P < 0.01$, when compared with vehicle control using analysis of variance; ***$P < 0.01$ when compared with **2**, **8**, **10** using analysis of variance.

after intravenous administration of vehicle (propylene glycol–DMSO, 2:1, 1.5 mL kg^{-1}) or drug solution (20.7 *I* mol kg^{-1}) in the tail vein of mice, 60 mg kg^{-1} of pentobarbital was injected intraperitoneally. The sleeping time was recorded as the time elapsed from the onset of the loss of righting reflex until the reflex was regained. In this study, the CNS activity of TRH (**1**), [Nva^2]-TRH (**2**), various lipidized intermediates of the fully packaged chemical delivery system of [Nva^2]-TRH (**4**–**8**, **10**), and the previously reported (Prokai et al 1994, 1999) chemical delivery system of [Leu^2]-TRH (**11**) have been evaluated. As shown in Table 1, the sleeping time after administration of vehicle and [Leu^2]-TRH-CDS (**11**) was 88.89±9.89 min and 49.19±15.71 min, respectively. The new [Nva^2]-TRH chemical delivery system (**6**), where the Leu residue was replaced by Nva, also showed a significant decrease in sleeping time (48.09±3.84 min), similar to the effect of [Leu^2]-TRH chemical delivery system (**11**). The sleeping times observed after administration of PyrNvaProNH$_2$ (**2**), DHT-ProProGlnNvaProGly-OH (**8**) and Pyr-NvaProGly-O-cholesterol (**10**) were 73.31±9.60 min, 69.42±11.99 min and 71.71±15.19 min, respectively. These results were significantly ($P < 0.05$) shorter than the control group, indicating that **2**, **8** and **10** have induced some CNS activity. However, these structural changes did not improve the CNS delivery of **2**, as there was no difference in CNS activity within this group. The sleeping times observed after administration of NicProProGlnNvaProGly-O-cholesterol (**4**), Trig-ProProGlnNvaProGly-O-cholesterol (**5**) and Trig-ProProGlnNvaProGly-OH (**7**) showed no significant CNS activity compared with vehicle control. It can be concluded that a fully packaged delivery system is necessary to deliver the active peptide to the brain, and replacement of Leu residue with Nva in the TRH analogue did not significantly affect its CNS activity.

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