Identification of a novel endogenous memory facilitating cyclic dipeptide cyclo-prolylglycine in rat brain

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Abstract Using high-performance liquid chromatography, gas-chromatography and chromato-mass spectrometry methods a novel endogenous cyclic dipeptide cyclo-prolylglycine was identified in rat brain. Its content according to gas chromatography is 2.8 ± 0.3 nmol/g wet brain. Synthetic cyclo-prolylglycine has demonstrated antiamnesic activity in the passive avoidance test in rats at a dose of 0.1 mg/kg i.p. Cyclic dipeptide cyclo-prolylglycine seems to be a memory facilitating substance and its presence in rat brain suggests the existence of a new mechanism of memory regulation.

Key words: Cyclo-prolylglycine; Endogenous cyclic dipeptide; Memory facilitating substance

1. Introduction

In spite of the fact that many neuropeptides influence training and memory, only some of them, it is presumed, directly control these processes [1,2]. The best known is the major arginine-vasopressin metabolite, [pGly^4,Cyt^6]AVP(4–9), which facilitates the consolidation stage of memory formation [3,4]. At the same time there are exogenous medicines, so-called nootropics, with piracetam as the main representative, which selectively facilitate processes of training and memory [5,6]. In 1985 we [7] put forward the hypothesis that the classical nootropic piracetam acts as a peptidomimetic. On the basis of this hypothesis active dipeptide analogues of piracetam were received that possessed proline or pyroglutamic acid as their N-terminal pyrrolidine-containing amino acid. Among the designed pyroglutamyl-containing dipeptides pyroglutamylasparagine amide turned out to be the most active [8]. It displayed nootropic activity in the passive avoidance test in rats at a dose of 0.01 mg/kg i.p. This effect depended on the stereochemistry of both amino acid residues [9]. The structure of pyroglutamylasparagine amide coincides with an N-terminal fragment of a major vasopressin metabolite, [pGlu^4,Cyt^6]AVP(4–9), responsible for the central effects of the latter [3]. These facts allowed us to assume that piracetam displays activity due to interaction with one vasopressin receptor subtype. We have found out, however, that unlike piracetam, which facilitates the initial processing of memory engram as well as consolidation, the dipeptide pGlu-Asn-NH_2 improved only the phase of information input and retrieval, but did not influence consolidation [10]. On the other hand, proline-containing dipeptide analogues of piracetam facilitated consolidation [11]. The cyclic dipeptide cyclo-(Pro-Gly) turned out to be the most active of them [12]. This cyclic dipeptide can be assumed to be one of the endogenous ligands of hypothetic 'nootropic receptors', which selectively regulates the processes of training and memory. This work is devoted to the identification of endogenous cyclo-(Pro-Gly) in rat brain.

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Abbreviations: AA, antiamnesic activity; amu, atomic mass unit; AVP, arginine vasopressin; FAB, fast atom bombarding mass spectrometry; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; MES, maximal electroconvulsive shock; MOPS, 3-morpholinopropanesulfonic acid; PDAH, pyrrolidino-[1,2-*a*]-2,6-diazacycloheptanedione-1,5; RIC, relative ion current; SIM, selected ion monitoring; UV, ultraviolet

2. Materials and methods

2.1. Chemicals

Diketopiperazine cyclo-prolylglycine (m.p. 213–214°C, $[\alpha]_D^{20}$ 214° in H_2O) was synthesized according to [13]. Pyrrolidino[1,2-*a*]-2,6-diazacycloheptanedione-1,5 (m.p. 169–170°C) was synthesized according to [14]. Acetonitrile (HPLC grade) and 3-morpholinopropanesulfonic acid (MOPS) were obtained from Merck.

2.2. Animals

Male outbred albino rats (180–240 g) were used in the experiments. The animals were housed under standard conditions and were allowed free access to food and water.

2.3. Preparation of extracts

Five animals were killed by decapitation, the brain was removed, washed thoroughly with ice-cold 0.9% saline, then crushed and immediately homogenized in 3 volumes of H_2O at 0°C. The homogenate was shaken for 10 min with 5 volumes of acetonitrile, then centrifuged at 9000 rpm for 10 min at 2°C and dried under a stream of N_2 at room temperature. The dry residue was redissolved in 2 ml H_2O and applied on an HPLC column.

2.4. High-pressure liquid chromatography

A Beckman 110B chromatograph equipped with the Tracor-970A absorbence detector UV (detection at 220 nm) was used. Chromatography was conducted on an Eiconosil C_{18} column, 250×4.6 mm, 10 μm, at a flow rate of 1.0 ml/min. The probe volume was 20 μl. The elution was carried out with acetonitrile containing 0.005 M MOPS (2:98 v/v). Fractions with a retention time corresponding to that of synthetic cyclo-(Pro-Gly) were combined, evaporated on a rotary evaporator to a small volume, then freed of MOPS by rechromatography on an Ultrasphere ODS column 250×4.6 mm, particle size of 5 μm, in system acetonitrile:water (2:98 v/v). The combined fractions left after rechromatography of the total material were evaporated to dryness on a rotary evaporator, the residue was dissolved in 100 μl of acetonitrile and used for GC and mass spectrometry.

2.5. Gas chromatography

A model 3700 gas chromatograph (Russia) equipped with a flame ionization detector was used. GC was performed using a glass 2 m×4 mm I.D. column packed with 3% OV-17 on Chromosorb G 125–150

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mesh washed 0.1 N NaOH. Nitrogen was used as the carrier gas. Gas flow rates were 40 ml/min (nitrogen), 30 ml/min (hydrogen), and 300 ml/min (air). A 3 μl aliquot was injected at 220°C. The oven temperature was 205°C, the detector temperature was 250°C and the bead power was set at 16 pA.

2.6. Mass spectrometry

Fast atom bombarding mass spectrometry (FAB) was performed using a FAB 50 device (Kratos, USA). Xenon was used as a reagent gas (8 keV). Glycerol was used as a standard matrix.

Chromato-mass spectrometric analysis was carried out with a Finnigan MAT SSQ-710 mass spectrometer. A Varian 3400 gas chromatograph equipped with a DB-5 glass capillary column (30 m×0.248 mm I.D., 0.25 μm film thickness, J. and W., Folsom, CA, USA) was used for sample separation with helium as a carrier gas at 30 ml/min flow rate. A 0.3 μl aliquot was injected at 250°C. The oven temperature was programmed from 40°C (held 0 min) to 250°C at 10°C/min, the latter temperature was then maintained until the end of the run. The total run time was 37 min. The GC-MS interface temperature was 250°C. The GC-MS system was operated in the electron impact mode with full scan at 1.34 u/s. The mass range was set for 50–650 amu. The source parameters were selected as ion energy 70 eV, ionization current 400 mA and ion source temperature 150°C.

Direct probe mass spectrometry was performed on the same device (Finnigan MAT SSQ-710) operated in the electronic impact mode with multiple selected ion monitoring (SIM) turned on m/z 154, 126, 111, 98, 83. The ionization energy was 70 eV. Ion source temperature was programmed from 25°C to 350°C at 162.5°C/min, then the temperature was held at 350°C for 4 min.

2.7. Recovery

Pyrrolidino-[1,2-*a*]-2,6-diazacycloheptanedione-1,5 (PDAH), a homologue of cyclo-(Pro-Gly), was used for recovery determination of the latter. The solution of 1.68 μg PDAH in 20 μl of H_2O was added to crushed brain ('sample') or 1.68 μg PDAH was dissolved in 100 μl of acetonitrile ('standard'). The sample was extracted as described above. The dry residue was redissolved in 100 μl of acetonitrile and used for GC. These analyses were performed in triplicate and average peak area of the sample was compared with that of the standard. The recovery of PDAH in the above extraction procedure was 95%.

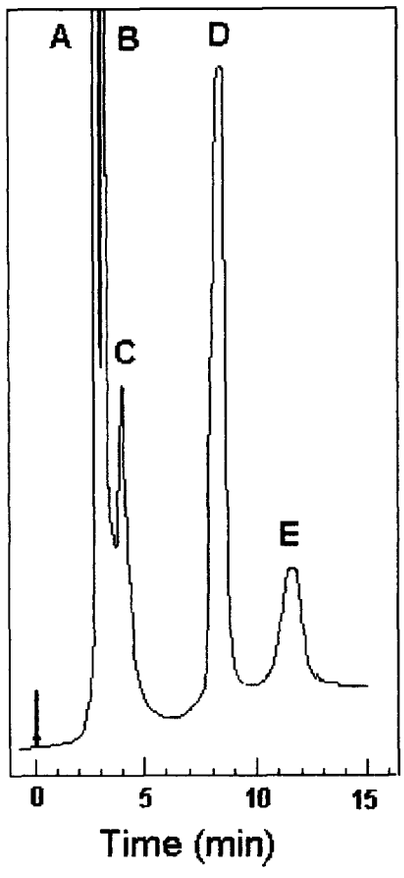

Fig. 1. HPLC separation of rat brain acetonitrile extract on an Eiconosil C_{18} column (250×4.6 mm, 10 μm particle size), UV detection at 220 nm. Mobile phase 98% 0.005 M MOPS/2% acetonitrile (v/v), flow rate 1.0 ml/min. Peak E coincided with synthetic cyclo-(Pro-Gly).

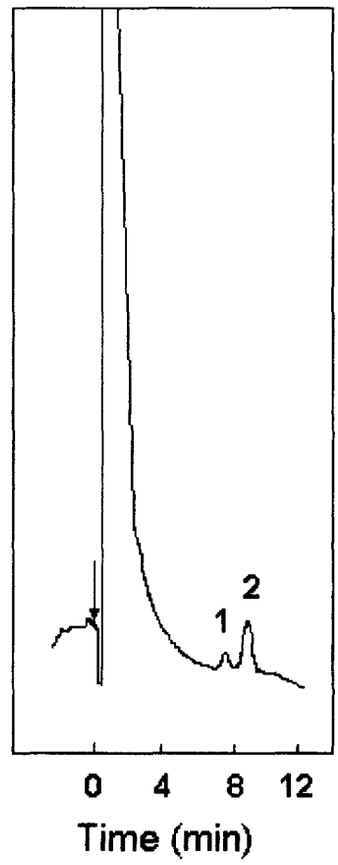

Fig. 2. GC analysis of fraction E on a glass 2 m×4 mm I.D. column, packed with 3% OV-17 on Chromosorb G 125–150 mesh washed 0.1 M NaOH. Gases flow rates were 40 ml/min (N_2), 30 ml/min (H_2) and 300 ml/min (air). The temperature was 220°C (injector), 205°C (oven), 250°C (detector). Peak 2 coincided with synthetic cyclo-(Pro-Gly).

2.8. Calibration graph

Standard at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 10.0 μM of synthetic cyclo-(Pro-Gly) in acetonitrile was prepared. A 3 μl volume of these solutions was injected onto the GC column. The calibration graph was calculated based upon peak areas.

2.9. Antiamnesic activity

Synthetic cyclo-(Pro-Gly) was evaluated for its ability to prevent the memory decline provoked by maximal electroshock (MES) in the passive avoidance step-through paradigm in rats [15].

The experiments were carried out on adult male outbred rats (Krjukovo, Moscow region) weighing 180–240 g. The step-through passive avoidance test, performed in a Lafayette Instrument Co. (USA) apparatus, was used for estimating memory retention according to Ader et al. [16]. The device consists of a lighted platform (25×7 cm) and a dark compartment (40×40×40 cm). A rat was placed on the lit start platform facing away from the dark compartment, which had an electrified grid floor. When the animal entered the dark compartment through a square guillotine door (6.5×6.5 cm) it received 8 unavoidable painful footshocks (0.45 mA). Then the rat was removed from the chamber. In the retention test performed 24 h after training the

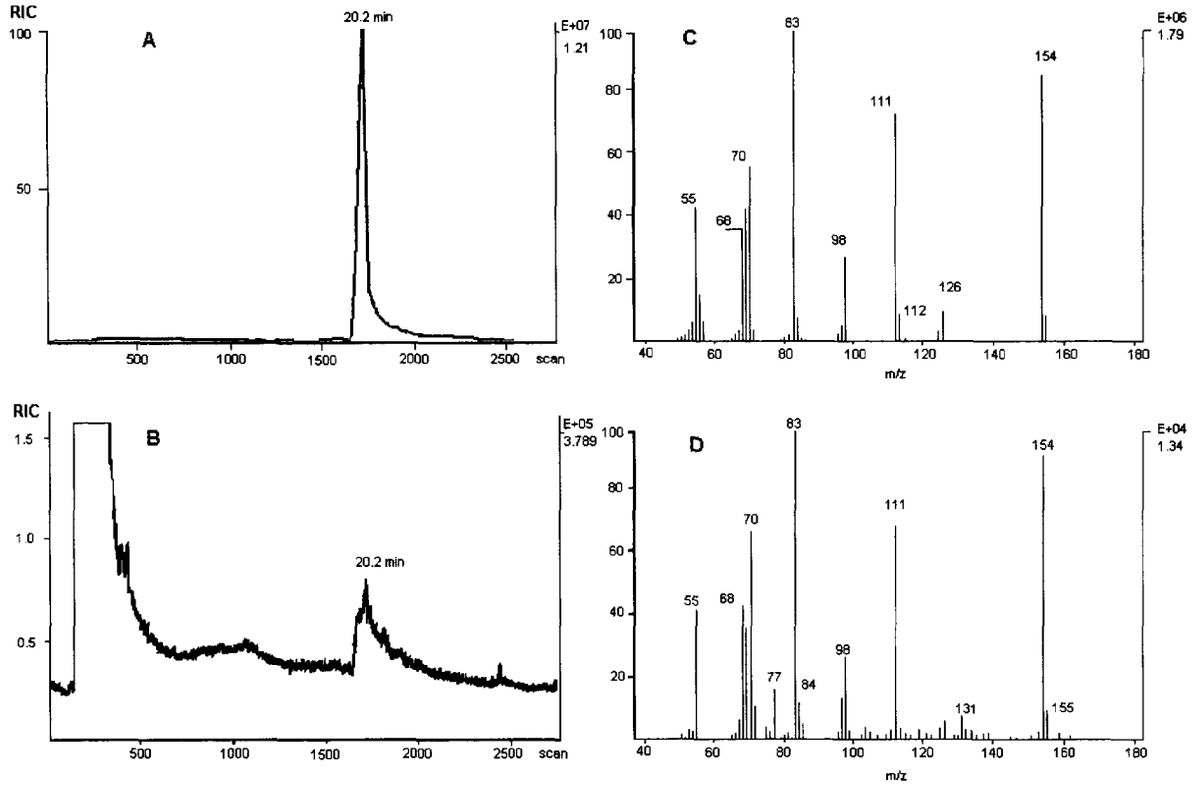

Fig. 3. Representative total ion GC-MS traces of synthetic cyclo-(Pro-Gly) (A) and fraction E (B). Positive electron impact spectra of synthetic (C) and endogenous (D) cyclo-(Pro-Gly) obtained by GC-MS.

animal was placed on the lit platform again. The latency to enter the dark compartment was registered. MES (70 V, 300 ms) was used immediately after training. Synthetic cyclo-(Pro-Gly) dissolved in saline was administered intraperitoneally in a dose range of 0.05–1.0 mg/kg, 15 min before the acquisition. Control animals were treated with saline. Antiamnesic activity (AA) was calculated according to the formula [17]:

$$\%AA = \frac{L(MES + compound) - L(MES)}{L(control) - L(MES)} x100\%$$

where L(MES) is the average latent time to enter the dark compartment for animals exposed to MES 24 h before; L(MES+compound) is the average latent time to enter the dark compartment for animals that had received the compounds and been exposed to MES; L(control) is the average time to enter the dark compartment for saline-treated animals with sham MES.

Statistical analysis was carried out using the Mann-Whitney *U*-test.

3. Results and discussion

HPLC separation of the acetonitrile extract of native rat brain demonstrated a symmetric peak (peak E, Fig. 1), absorbing at 220 nm, which retention time coincided with that of synthetic cyclo-(Pro-Gly). The symmetry of this peak was not infringed at cochromatography of the isolated sample (fraction E) with synthetic cyclo-(Pro-Gly) on the Ultrasphere ODS and Eiconosil C_{18} columns, when various elution rates and mobile phase compositions were used. These findings allow us to suggest that fraction E contains endogenous cyclo-(Pro-Gly). In the course of GC the above mentioned HPLC fraction E was separated into two peaks, one of which (peak 2, Fig. 2) according to its retention time coincided with synthetic cyclo-(Pro-Gly). GC cochromatography of HPLC fraction E and of synthetic cyclopeptide sample performed at various carrier gas rates and column temperatures showed that peak 2 and synthetic cyclo-(Pro-Gly) invariable coeluted as one peak. So, GC data do not conflict with the assumption that cyclo-(Pro-Gly) is present in rat brain.

To identify endogenous cyclo-(Pro-Gly) fraction E from the five rat brain extracts was isolated, collected and concentrated. It was analyzed using mass spectrometric methods.

Fraction E was examined by FAB mass spectrometry. The

Table 1
Antiamnesic activity of synthetic cyclo-(Pro-Gly)

Dose (mg/kg, i.p.)	Animals (*n*)	Latency (s) (mean ± SEM)			Antiamnesic activity (%)
		Control	Amnesia	Amnesia+substance	
0.05	18	126.7 ± 18.6	33.7 ± 13.0	49.2 ± 15.0	16.7
0.1	16	83.1 ± 34.3	18.2 ± 7.7	67.5 ± 25.8	75.9*
1.0	10	161.1 ± 18.9	77.5 ± 26.0	131.7 ± 21.3	64.8*

*$P < 0.05$ in comparison with MES treated animals (U-test).

presence of a quasimolecular ion with m/z 155 corresponding to the mass of protonized cyclo-(Pro-Gly) was revealed.

Then fraction E was analyzed by direct probe electron impact mass spectrometry. By temperature fractionation of fraction E in direct probe conditions with registration of total ion current a mixture of substances with evaporation temperatures from 130°C to 350°C was found. Selected ion monitoring showed the presence in this mixture of a substance that gave all characteristic molecule fragments of cyclo-(Pro-Gly), which are m/z 154, 126, 111, 98, 83 according to [18].

In GC-MS conditions total ion current monitoring of fraction E and synthetic cyclo-(Pro-Gly) demonstrated peaks with identical retention time values (Fig. 3A,B). The mass spectra of these peaks were superposed in details (Fig. 3C,D). Thus MS data strictly prove that cyclo-prolylglycine is present in rat brain.

The extraction efficiency (95%) of PDAH, of the cyclo-(Pro-Gly) homologue, was used for the determination of the concentration of cyclo-(Pro-Gly) in rat brain. PDAH in GC conditions does not interfere with cyclo-(Pro-Gly) or unknown peaks. The cyclo-(Pro-Gly) concentration in rat brain was determined by GC, using synthetic cyclo-(Pro-Gly) for preparation of the calibration curve. The calculated mean concentration of cyclo-(Pro-Gly) in rat brain was 2.8 ± 0.3 nmol/g wet brain.

To evaluate the effect of cyclo-(Pro-Gly) on memory, the passive avoidance test was used. MES applied immediately after the acquisition procedure significantly diminished the latency of entering the dark compartment. Cyclo-prolylglycine administered before the training was shown to be able to reduce this amnesic effect of MES. Doses of 0.1 and 1.0 mg/kg were found to be effective (Table 1). In our previous experiments cyclo-(-Pro-Gly) improved memory also under conditions of undertraining [13]. It is important to stress that cyclo-(Pro-Gly) exerted neither stimulating nor depressant action on general psychomotor activity. The cyclopeptide cyclo-prolylglycine seems to be a memory facilitating substance and its presence in rat brains suggests the existence of a new mechanism of memory regulation. Identification of cyclo-(Pro-Gly) in the brain is of special importance, taking into consideration the recent report of Prasad [19], that only one cyclic dipeptide, cyclo-(His-Pro), has been conclusively shown to be endogenous in mammals.

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