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Antiamnesic Effects of D-Pipecolic Acid and Analogues of Pro-Leu-Gly-NH_2 in Rats

GÁBOR L. KOVÁCS,*[1] GYULA SZABÓ,* GYULA TELEGDY,* LAJOS BALÁSPIRI,† ÉVA PÁLOS AND LÁSZLÓ SZPORNYI‡

*Institute of Pathophysiology, University Medical School, Szeged, Hungary
†Institute of Medical Chemistry, University Medical School, Szeged
‡Gedeon Richter Pharmaceutical Works, Budapest, Hungary

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KOVÁCS, G. L., G. SZABÓ, G. TELEGDY, L. BALÁSPIRI, É. PÁLOS AND L. SZPORNYI. *Antiamnesic effects of D-pipecolic acid and analogues of Pro-Leu-Gly-NH_2 in rats.* PHARMACOL BIOCHEM BEHAV 31(4) 833–837, 1988.— The antiamnesic effects of prolyl-leucyl-glycinamide (PLG) and analogues of this tripeptide were investigated in rats. Retrograde amnesia was induced by electroconvulsive shock treatment and the degree of amnesia was characterized by the attentuation of one-trial learning passive avoidance response. PLG resulted in dose-dependent attenuation of retrograde amnesia. Structural modifications included N-terminal protection, substitution of the C-terminal NH_2 group, replacement of the N-terminal amino acid, and replacement of the second amino acid of the tripeptide. Some tripeptides, all of them containing D-pipecolic acid instead of the N-terminal proline, were more effective than PLG. Therefore, D-pipecolic acid, D-pipecolamide and their N-terminally protected analogues were also investigated, and were found to have powerful antiamnesic effects.

Retrograde amnesia Prolyl-leucyl-glycinamide D-Pipecolic acid

PREVIOUS studies have shown that the postulated hypothalamic peptide hormone, melantropin release inhibiting factor (prolyl-leucyl-glycinamide, PLG), exerts wide-ranging central nervous system effects, including changes in higher nervous activity (13, 15, 26), motor coordination (9), narcotic tolerance (16,25), etc. The tripeptide facilitated passive avoidance behaviour in rats (8) and its effect was more pronounced in the retrieval of stored information than on the consolidation of memory (8,17). PLG also normalized retrograde amnesia induced by protein synthesis inhibition (10), CO_2-intoxication (21) or electroconvulsive shock (ECS) treatment (14, 15, 18).

However, the effectiveness of PLG is modest insofar as relatively high peptide doses have to be administered in order to achieve antiamnesic effects. Experiments were therefore carried out to test some structural analogues of the tripeptide. Structural modification included N-terminal protection of the tripeptide with carbobenzoxy (Z), 9-fluorenylmethyloxycarbonyl (FMOC) or tertiarybutyloxycarbonyl (BOC) groups; through replacement of the C-terminal NH_2 group by OMe, CH_3 or NHC_2H_5 groups; through replacement of the N-terminal amino acid (proline) by homoproline, D-proline, D-pipecolic acid, L-pipecolic acid or pyroglutamine, and through replacement of leucine by D-leucine or valine. The antiamnesic effects of pipecolic acid derivatives (D-pipecolic acid, D-pipecolamide, Z-D-pipecolic acid and Z-D-pipecolic amide) were also investigated. Seven analogues, all of them containing D-pipecolic acid (or its amide), appeared to have more powerful antiamnesic effects than that of PLG.

METHOD

Subjects

Experimentally naive, sexually mature (90–100 days old) male rats of an inbred Sprague-Dawley CFY strain were used. The animals were kept on a standard illumination schedule, with lights on between 7 a.m. and 7 p.m. Five animals were kept in each experimental cage. The animals received standard laboratory diet and drinking water ad lib. The body weight of the animals was 160–180 g.

Behavioural Procedures

Retrograde amnesia was induced as described in detail earlier (16). Briefly, animals were first trained in a one-trial learning passive avoidance apparatus (1). The animals were placed on an illuminated platform and were allowed to enter a dark compartment. Since rats prefer dark to light, the

[1]Requests for reprints should be addressed to Gábor L. Kovács M.D., Director, Central Laboratory, Markusovszky Teaching Hospital, Hámán Kató u. 28, Szombathely 9701, Hungary.

animals entered the dark compartment within 10 seconds. After three trials had been given on two consecutive days, the rats received unavoidable electric footshocks (0.75 mA for 2 sec) in the dark compartment (learning trial). Immediately thereafter, the animals were removed from the conditioning apparatus and ECS treatment (220 V, AC, sinusoid wave 0.5 sec, Minicoma, Hungary) was given via ear-clip electrodes. Only those animals were evaluated in which tonic-clonic convulsions developed in response to ECS treatment. The retention of passive avoidance was measured 24 hr after the learning trial, by placing the rats on the platform and measuring the latency to reenter the dark compartment. A cut-off time of 300 sec was used. All treatments were given subcutaneously (SC) in a volume of 0.5 ml. Rats were divided into four experimental groups.

Normal control (no-ECS) group. These rats were trained in the passive avoidance apparatus, but no ECS treatment was given after the learning trial. Thus, the passive avoidance latency was high in this control group.

ECS control group. These rats received ECS treatment, as described in the Method section. They were not injected with peptides but only with vehicle.

PLG control group. These control animals received ECS treatment, and PLG (100 μg/animal, ca. 0.6 mg/kg SC) was injected as reference substance. The peptide was dissolved in concentrated (98%) ethanol and then diluted with saline to give a solution containing 6 vol. % ethanol. This procedure was necessary, since some peptides were poorly soluble in water. The peptide was given 1 hr prior to the retention test of passive avoidance, i.e., 23 hr after ECS treatment.

Experimental group. Various peptide analogues (Tables 1 and 2) were injected in doses of 100 μg/rat. Peptides were given 23 hr after ECS treatment. The peptides were synthesized by one of the authors (L.B.). Detailed information on the peptide syntheses will be published elsewhere. The chemical purity of the peptides was in all cases 98% by HPLC.

Calculation and Statistics

The degree of retrograde amnesia was expressed as follows:

$$\text{Retrograde Amnesia (\%)} = \frac{\text{Latency of ECS control group}}{\text{Latency of no-ECS control group}} \times 100$$

The antiamnesic effect of a compound was calculated according to the formula:

$$\text{Antiamnesic Effect (\%)} = \frac{\text{Latency of experimental group} - \text{Latency of ECS control group}}{\text{Latency of no-ECS control group} - \text{Latency of ECS control group}} \times 100$$

For those compounds which appeared to have an antiamnesic effect stronger than that of the reference compound, the relative activity was calculated according to the following formula:

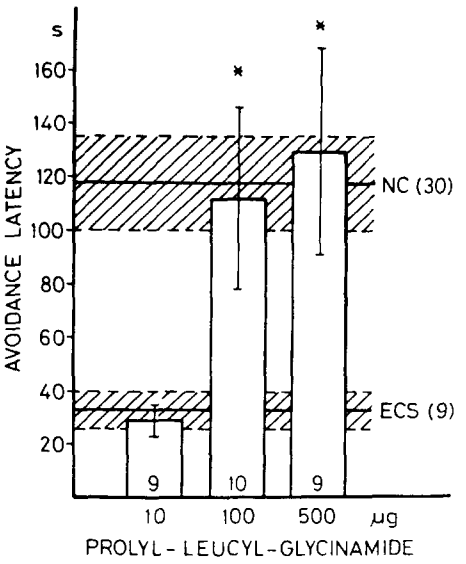

FIG 1. Dose-related effect of prolyl-leucyl-glycinamide on ECS-induced retrograde amnesia in rat. (The numbers in bars and brackets indicate the numbers of experimental animals.) *$p<0.01$ vs. ECS group.

$$\text{Relative Activity (\%)} = \frac{\text{Latency of experimental group}}{\text{Latency of PLG control}} \times 100 - 100$$

Statistical analysis of the data was performed with analysis of variance, followed by the Scheffe test for multiple comparison. A probability level of 0.05 was accepted as indicating a significant difference.

RESULTS

Dose-Related Antiamnesic Effect of PLG

The dose-response effect of PLG on ECS-induced retrograde amnesia is illustrated in Fig. 1. Passive avoidance behaviour developed in each group of rats as a result of electric footshocks in the dark compartment. Thus, rats entered the dark compartment significantly later than before electric footshocks (not documented). However, there were marked differences in the level of passive avoidance between the various groups, $F(4,481)=38.68$, $p<0.001$. Those rats which received ECS treatment after the learning trial, entered the dark compartment significantly ($p<0.001$) faster than those which did not receive ECS treatment. Thus, ECS resulted in retrograde amnesia. PLG injection dose-dependently alleviated the symptoms of retrograde amnesia. Accordingly, rats treated with 100 or 500 μg PLG entered the dark compartment significantly later ($p<0.01$ and $p<0.001$, respectively) than ECS-treated controls. The PLG dose of 100 μg was selected for further studies, since this amount of the tripeptide caused a significant, but submaximal antiamnesic effect.

The Antiamnesic Effects of Tripeptide Analogues

Data on the antiamnesic effects of tripeptide analogues are given in Table 1. Of the 41 tripeptide analogues tested, 16

TABLE 1

EFFECTS OF PLG AND SOME ANALOGUES ON RETROGRADE AMNESIA

Group	No. of Animals	Passive Avoidance Latency	Anti-amnesic Effect	Significance
		A		
Normal Control	184	154 ± 9	—	—
ECS Control	190	44 ± 3	− 70%	0.001
H-Pro-Leu-Gly-NH_2	93	90 ± 10	+ 52%	0.001
Z-Pro-Leu-Gly-NH_2	18	100 ± 28	+ 63%	0.001
Z-Pro-Leu-Gly-OMe	15	113 ± 27	+ 77%	0.001
Z-Pro-Leu-Gly-CH_3	14	29 ± 4	− 15%	NS
Z-Pro-Leu-Gly-NHC_2H_5	10	92 ± 27	+ 54%	0.01
BOC-Pro-Leu-Gly-OMe	15	77 ± 24	+ 30%	0.05
D-Pip-Leu-Gly-NH_2	20	151 ± 26	+119%	0.001*
Z-D-Pip-Leu-Gly-NH_2	11	106 ± 37	+ 69%	0.001
Z-D-Pip-Leu-Gly-OMe	20	163 ± 27	+132%	0.001†
BOC-D-Pip-Leu-Gly-OMe	9	21 ± 6	− 24%	NS
FMOC-D-Pip-Leu-Gly-OMe	10	78 ± 22	+ 38%	0.02
Z-D-Pip-Leu-Gly-CH_3	11	50 ± 10	+ 8%	NS
		B		
L-Pip-Leu-Gly-NH_2	5	47 ± 4	+ 4%	NS
Z-L-Pip-Leu-Gly-NH_2	13	37 ± 8	− 7%	NS
Z-L-Pip-Leu-Gly-OMe	19	115 ± 27	+ 79%	0.001
BOC-L-Pip-Leu-Gly-OMe	9	38 ± 12	− 9%	NS
Z-L-Pip-Leu-Gly-CH_3	8	34 ± 5	− 10%	NS
Hpro-Leu-Gly-NH_2	15	128 ± 33	+ 93%	0.001
Z-Hpro-Leu-Gly-NH_2	13	97 ± 31	+ 59%	0.001
BOC-Hpro-Leu-Gly-NH_2	10	33 ± 10	− 10%	NS
BOC-Hpro-Leu-Gly-OMe	13	117 ± 35	+ 81%	0.001
Z-D-Pro-Leu-Gly-OMe	15	103 ± 29	+ 54%	0.001
Z-D-Pro-Leu-Gly-CH_3	12	59 ± 16	+ 14%	NS
BOC-D-Pro-Leu-Gly-OMe	8	16 ± 4	− 30%	NS
Z-Pro-D-Leu-Gly-NH_2	10	70 ± 27	+ 24%	NS
Z-Pro-D-Leu-Gly-OMe	15	46 ± 19	+ 2%	NS
Z-Pro-D-Leu-Gly-CH_3	9	61 ± 25	+ 20%	NS
BOC-Pro-D-Leu-Gly-NH_2	15	69 ± 24	+ 23%	NS
BOC-Pro-D-Leu-Gly-OMe	10	36 ± 10	− 7%	NS
		C		
Z-Pro-Val-Gly-NH_2	14	73 ± 27	+ 26%	NS
Z-Pro-Val-Gly-OMe	10	59 ± 13	+ 14%	NS
BOC-Pro-Val-Gly-NH_2	9	43 ± 13	− 1%	NS
BOC-Pro-Val-Gly-OMe	14	40 ± 19	− 4%	NS
Z-D-Pro-D-Leu-Gly-CH_3	15	110 ± 28	+ 60%	0.001
D-Pip-DPip-DPip-NH_2	9	49 ± 14	+ 5%	NS
Z-D-Pip-Val-Gly-NH_2	10	43 ± 8	− 1%	NS
Z-D-Pip-Val-Gly-OMe	20	143 ± 28	+ 90%	0.001*
FMOC-D-Pip-Val-Gly-OMe	10	90 ± 36	+ 42%	0.01
Z-Pglu-Leu-Gly-CH_3	5	33 ± 9	− 11%	NS
Pglu-Leu-Pro-CH_3	13	59 ± 15	+ 14%	NS
Z-Pglu-Leu-Pro-CH_3	13	75 ± 19	+ 28%	NS

*Significantly ($p<0.05$) different from the PLG-treated group.
†Significantly ($p<0.01$) different from the PLG-treated group.

TABLE 2

ANTIAMESIC EFFECTS OF D-PIPECOLIC ACID, SOME OF ITS DERIVATIVES AND DIPEPTIDES

Group	No. of Animals	Passive Avoidance Latency	Anti-amnesic Effect	Significance
Normal Control	184	154 ± 9	—	—
ECS Control	190	44 ± 3	− 70%	0.001
Z-DPip-DPip-NH_2	19	108 ± 24	+ 58%	0.001
Z-DPip-NH_2	10	156 ± 33	+102%	0.001*
DPip-NH_2	10	163 ± 40	+108%	0.001*
Z-DPip-OH	14	165 ± 30	+110%	0.001*
DPip-OH	24	177 ± 24	+121%	0.001†
Z-Val-Gly-OMe	10	89 ± 23	+ 41%	0.01
Z-D-Val-Gly-OMe	20	111 ± 29	+ 61%	0.001
Z-D-Leu-Gly-OMe	7	40 ± 9	− 3%	NS

*Significantly ($p<0.05$) different from the PLG-treated group.
†Significantly ($p<0.01$) different from the PLG-treated group.

appeared to exert significant antiamnesic effects in a dose of 100 μg/animal, $F(41,735)=3.51$, $p<0.001$. Another 25 tripeptides were ineffective on retrograde amnesia. The active compounds could be divided into two subgroups: 13 tripeptides were as potent as the reference substance (PLG), while 3 tripeptides appeared to be significantly more potent than PLG. All three contained D-Pip in the N-terminal position (D-Pip-Leu-Gly-NH_2, Z-D-Pip-Leu-Gly-NH_2 and Z-D-Pip-Val-Gly-OMe).

Neither N-terminal protection of the tripeptide with the Z group, nor replacement of the C-terminal NH_2 group by the OMe or NHC_2H_5 group, led to a better antiamnesic potency than that of PLG. On the other hand, replacement of the C-terminal NH_2 group by a CH_3 group resulted in a complete loss of the antiamnesic action.

Replacement of proline by L-Pip resulted in an attenuation or the loss of the antiamnesic effects. Replacement of proline by homoproline did not improve the antiamnesic effects, while replacement of proline by D-proline, leucine or valine greatly reduced the antiamnesic action.

The antiamnesic effects of pipecolic acid and some dipeptides: D-Pip, a protected analogue and the amide of this amino acid (Z-D-Pip-OH, Z-D-Pip-NH_2 and D-Pip-NH_2) were more effective than PLG against ECS-induced retrograde amnesia. Z-D-Pip-D-Pip-NH_2, Z-Val-Gly-OMe and Z-D-Val-Gly-OMe exerted antiamnesic effects which were in the range of that of PLG. Z-D-Leu-Gly-OMe, on the other hand, did not normalize retrograde amnesia [Table 2, ANOVA: $F(9,382)=15.27$, $p<0.001$].

DISCUSSION

The present results confirm the previous findings from our own (14, 15, 18) and other laboratories (10,21) that PLG can normalize, partially at least, retrograde amnesia in rats. The added finding was made that some structural analogues of the tripeptide were significantly more potent against retrograde amnesia than PLG itself. All these tripeptides contained D-Pip in place of the N-terminal proline. It should be noted, however, that the replacement of proline by D-Pip is itself not sufficient to improve the antiamnesic action. Some analogues containing D-Pip were just as active as PLG, but some of them were ineffective. In contrast to D-Pip, the tripeptides in which proline was replaced by L-Pip were much less active (4 out of five were ineffective).

These data suggest the possibility that the increased antiamnesic activity of the tripeptide analogues containing D-Pip was due to the D-Pip itself. D-Pip, its amide and their protected analogues were very active against ECS-induced retrograde amnesia in rats. Thus, the possibility exists that the antiamnesic effects of the tripeptides containing D-Pip were directly related to the biological action of this synthetic D-amino acid. D-Pip also affects ethanol tolerance in mice (22,23). Further detailed studies, involving dose-response curves and ED_{50} calculations, are required ro resolve the question of whether D-Pip and its derivatives are quantitatively more potent than PLG. It has to be borne in mind that there is about a 1:3 difference in the molecular weights of this amino acid and the tripeptides. In molar terms, therefore, a 100 μg dose of D-Pip is approximately three times larger than a 100 μg dose of PLG.

As far as the biological significance of the present findings is concerned, there is great theoretical and clinical interest in compounds which could eliminate (even partially) the retrograde amnesia induced by various treatments. PLG, a postulated hypothalamic hormone (3) also present in the C-terminal portion of oxytocin, is one of peptides exerting such an antiamnesic effect.

As concerns the significance of the structure, it should be stressed that L-Pip is a naturally-occurring compound in the brain. L-Pip is the next higher homologue of proline and is a major metabolite of lysine in the mammalian brain (4). Recent biochemical and pharmacological evidence suggests the involvement of L-Pip in the regulation of synaptic mechanisms (11, 19, 20, 24). L-Pip also affects behavioral reactivity (5). The present findings show that replacement of L-Pip by D-Pip may result in opposite CNS effects, e.g., D-Pip and some tripeptides substituted with this amino acid normalized retrograde amnesia. It is known that peptides containing D-amino acids may exert central nervous effects opposite to those induced by the L-enantiomers of the same amino acids (2, 6, 7, 12).

In conclusion, the present results suggest that D-Pip or some oligopeptides in which proline is replaced by D-Pip

might have a beneficial therapeutic effects in the treatment of retrograde amnesia. Further experiments are required to elucidate the pharmacological, behavioural and molecular mechanisms of this antiamnesic effect.

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