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Structure-activity relationship of memory enhancing peptide ZNC(C)PR analogs

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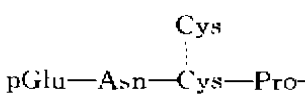
ABSTRACT ZNC(C)PR has been found to be a new neuropeptide in rat brain as a significant enhancer of learning and memory. In this work, the structure-activity relationship of ZNC(C)PR was studied. First, the roles of every residue in ZNC(C)PR were investigated theoretically and analogs were designed according to the predicted conformational properties. Five analogs were synthesized following the design. Passive avoidance behavior tests in rats showed that NIPR, NVPR, and NAPR have positive effects to facilitate memory, while NSPR has no effect, and DLPR has somewhat inhibitory effect. As the experimental results are in good agreement with that of theoretical calculations, it is suggested that further research may help us to understand more details on the structure-activity relationship of ZNC(C)PR and provides a way for further design of potent agonist, antagonist and possible nonpeptide mimetics of ZNC(C)PR.

KEY WORDS learning; memory; neuropeptides; structure-activity relationship

ZNC(C)PR is a neuropeptide in rat brain[1,2] and has primary structure as follows:



ZNC(C)PR is no longer recognized by argipressin (AVP) receptors and totally void of pressor and antidiuretic activities, but surprisingly shows higher potency than AVP itself in behavior responses[3-5]. ZNC(C)PR and its analogs not only induce facilitation of passive avoidance behavior, but also remarkably facilitate the acquisition and subsequent maintenance of brightness discrimination in immature and mature rats by neonatal administration[6,7]. Therefore, ZNC(C)PR is thought to be a new neuropeptide.

To study the structure-activity relationship of ZNC(C)PR, a series of ZNC(C)PR analogs had been synthesized and tested[3-5]. From these experiments, NXXR was proposed to be the essential group for keeping a high behavioral potency[3]. In a 2D-NMR study[8], it was found that ZNC(C)PR is in a compact structure, while [*D*-Arg]ZNC(C)PR is not. Due to the potency on behavioral response of the latter is only 3 % of that of the former, it implied that the activities closely related to the conformation. Based on the 2D-NMR data, conformation of ZNC(C)PR has been built[9] and an algorithm to predict the conformations of peptide by systematic search has been proposed[10]. It was suggested[8-10] that the compact structure and moderate stability were essential for the high activity of ZNC(C)PR. This provided the basis for our designing new analogs and studying structure-activity relationship of ZNC(C)PR.

In this paper, the influences of each residue in ZNC(C)PR on conformation and activity were investigated, and five analogs with

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substitution at cystine position were synthesized and testified subsequently.

MATERIALS AND METHODS

All the calculations were performed on a Graphics 4D/70GT workstation (Silicon Graphics Inc, USA), implemented with QUANTA program (Polygen Inc) for molecular modeling. And a program of CHARMM[11] was employed in conformational calculations.

Conformational prediction of peptide The algorithm[10] was employed to predict the conformations of the peptide. First, one conformation with extended main chain was generated, then energy minimization was performed to eliminate atom collisions and contacts with slight constraints of the heavy atoms in the main chain. From *N*-terminal, 2 neighboring dihedral angles were rotated synchronously with an increment of 10°. Energies were evaluated and 36^2 energies in total were obtained. An energy contour map was plotted according to the 36^2 energies, and the energy minimum regions were selected. The 2 dihedral angles were rotated to let the energy minimum. This process was repeated until reached *C*-terminal The side chain dihedral angles were rotated in the same way as did the main chain dihedral angles. At last, many possible conformations were obtained. Energy minimization was then performed and only the conformations with the energy less than 3 kcal·mol^{-1} to the lowest were kept. The remaining conformations were divided into several classes. The criterion to classify the 2 similar conformations was taken as their coordinate RMS less than 0.10 nm.

Design strategy As the early experiments[5] and theoretical calculations[8] showed that the pyroglutamyl residue (pGlu) was not essential to the activity of ZNC(C)PR, only the tetrapeptide without pGlu, NC(C)PR, was considered in this design. Because it did not take too much time to calculate the conformations of such a short peptide by use of the previous algorithm, various analogs from residue substitution in NC(C)PR were considered and their conformations were calculated. Thus the influences of residue substitutions on the conformation were investigated theoretically and the roles played by every residue were deduced.

The analogs with ZNC(C)PR activity should have the similar conformational properties as ZNC(C)PR. ZNC(C)PR is characterized by sidechain contacts between Asn and Arg[14] and a moderate stability[9,10]. The conformational characteristics were chosen as parameters to classify the analogs, so as to find out the analogs with similar properties as ZNC(C)PR.

Peptide synthesis and purification All chemicals and solvents were analytical reagents. Five ZNC(C)PR analogs were synthesized by a stepwise solid-phase method in this laboratory[5,6]. The protected tetrapeptide was condensed on an *N*-tosyl-*L*-arginine (1 % DVB) polystyrene resin with *tert*-butyloxycarbonyl (Boc-) amino acids. The protected tetrapeptides were blocked and freed from the resin by hydrogen fluoride (HF) treatment for 1 h at 0 ℃. The synthetic products were fractionated by chromatography on a Sephadex G-15 column with elution by 0.25 % acetic acid. The main product thus generated was proved to be the tetrapeptide we expected by amino acid composition analysis.

Further purifications were performed on a set of Waters system reverse-phase HPLC (Waters model 510, Millipore Corp, USA) with DELTA PAK C_{18}-300A column (7.8 mm×300 mm, Nihon Waters Ltd) with a consecutive gradient of acetonitrile from 2 % to 30 % containing 0.1 % trifluoroacetic acid within 25 min. Absorbance at 214 nm were recorded. These peptides synthesized were purified to homogeneous material which showed a single peak before bioassay.

Bioassay and statistics The activities of peptides on memory processes were tested in a passive avoidance manner using a single learning trial in ♂ Wistar rats (135±15 g). The apparatus consisted of a small illuminated (60-W bulb) chamber connected with a large dark chamber via a door. The 2 chambers were equipped with a grid floor. On d 1, the rat was habituated to the dark chamber (3 min), the latency to enter the dark was recorded. Since rats prefer dark to light, they normally enter within 15 s. Trials were commenced on the following day. The rat was placed in the small chamber. As soon as the rat entered the dark chamber, the door was shut and an inescapable foot shock was given (80 V, 2 s) through the grid floor of the dark chamber. And peptide or saline were injected sc immediately. On d 3, 24 h after the acquisition of the trial, the rat was placed in an illuminated chamber, the latency that it entered the dark chamber was recorded. On d 4, a 48-h latency was

recorded. Passive avoidance respondence latencies were recorded up to a maximum of 300 s (cut-off time) on the retention tests. All tests were carried out in a double-blind way.

The results of the passive avoidance tests were expressed as a mean latency $\bar{x} \pm s$. Results of the treatments were compared with those of the saline control and the significance of the difference was analyzed with ANOVA.

RESLUTS

Design of ZNC(C)PR analogs Forty-five ZNC(C)PR analogs were constructed by considering various situations including the substitution of residues with hydrophobic or hydrophilic properties (Tab 1 and Fig 1).

The criterion to define the conformational stability of ZNC(C)PR analogs were as follows: unstable ($E > -50$ kcal·mol^{-1}), stable (-80 kcal·mol$^{-1} < E < -50$ kcal·mol^{-1}), and very stable ($E < -80$ kcal·mol^{-1}). The analogs in Tab 1 with closed side chains and moderate stability still had ZNC(C)PR activity, while the analogs with very low energies had inhibitory activity (Tab 2).

Synthesis of ZNC(C)PR analogs It is predicted that NIPR, NVPR, and NAPR are predicted to possess the ZNC(C)PR activity, while NSPR has no activity, and DLPR shows an inhibitory activity (Tab 2). These peptides were synthesized and purified to homogeneous material which showed a single peak in reverse-phase HPLC (Fig 2).

Effects of ZNC(C)PR analogs on passive avoidance behavior The effects of these synthetic peptides on the passive avoidance behavior of rats were measured following sc immediately after the learning trial. NIPR, NVPR, and NAPR induced facilitation of passive avoidance behavior, NSPR did not enhance memory, while DLPR showed a somewhat inhibitory effect ($P<0.05$) at 48 h (Tab 3).

Tab 1. ZNC(C)PR analogs and predicted conformational properties. A and B represent the side chains between the first and the last residue closed and open, respectively. Alternative conformations are in parentheses.

	Analog	Energy, kcal·mol^{-1}	Conf	Distance ($C^1_\alpha - C^5_\alpha$)
1	LLPR	−45.61	B	8.38
2	FLPR	−45.70	B	8.49
3	DLPR	−105.39	A	8.33
4	SLPR	−48.85	B	8.39
5	QLPR	−61.60	B(A)	8.32
6	HLPR	−53.66	A(B)	7.78
7	NAPR	−63.90	A	7.38
8	NLPQ	−61.63	A	6.63
9	NLPE	−63.72	A	6.73
10	NLPH	−51.97	B	7.47
11	NLPK	−68.42	A	7.42
12	NIPR	−68.50	A	7.44
13	NFPR	−68.99	B	6.81
14	NYPR	−75.66	A	8.08
15	NWPR	−77.64	A(B)	7.13
16	NVPR	−70.33	A(B)	7.42
17	NMPR	−72.92	A	7.42
18	NSPR	−79.24	B	7.09
19	NTPR	−82.62	B	8.08
20	NDPR	−91.98	B	6.63
21	NEPR	−94.33	B	6.67
22	NQPR	−83.58	A(B)	7.43
23	NCPR	−73.62	A(B)	7.31
24	NLLR	−107.96	B	4.67
25	NIFR	−103.62	B	4.95
26	NFFR	−104.89	B	4.78
27	NWIR	−89.86	B	5.15
28	NVWR	−109.51	B	4.94
29	NYIR	−103.90	B	4.83
30	NLGR	−115.19	B	4.94
31	NSTR	−96.07	B	7.86
32	NEHR	−125.74	B	4.74
33	NDKR	−127.65	B	4.92
34	NTQR	−111.49	B	8.19
35	SLPH	−35.17	B	6.49
36	SLPK	−44.89	B(A)	8.43
37	TLPK	−47.20	B	8.48
38	DLPK	−85.75	A	9.42
39	ELPK	−80.23	A	9.64
40	LLPN	−36.46	B	6.12
41	DLPF	−41.32	B	10.17
42	NLPL	−44.92	B	6.44
43	WLPL	−21.68	B	7.88
44	FLPI	−10.22	A	7.77
45	LLPV	−8.87	A	8.94

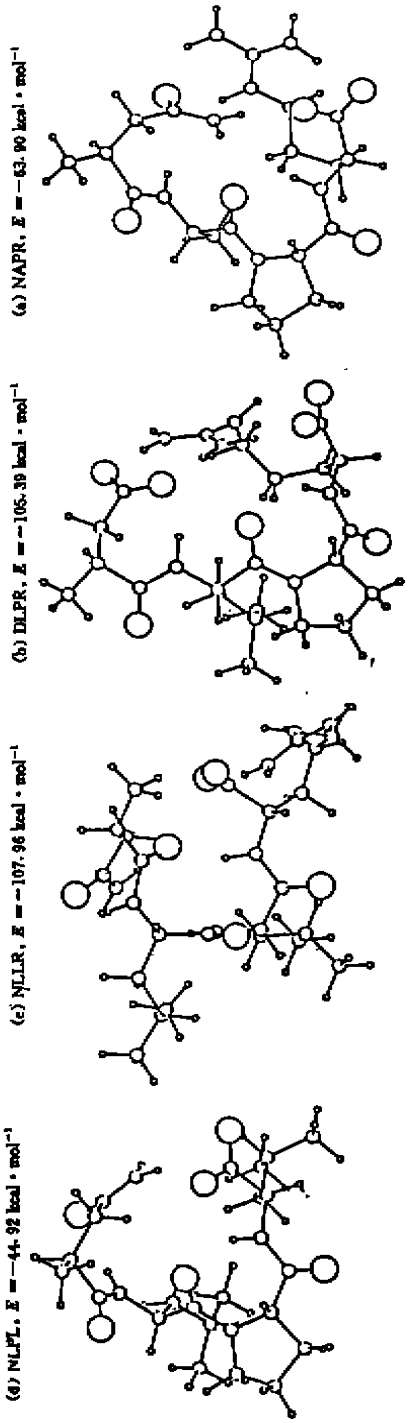

Fig 1. Predicted conformations of ZNC(C)PR analogs.

Tab 2. ZNC(C)PR analogs and predicted bioactivities on behavioral response. A and B are the same as in Tab 1.

Analogs	Energy, kcal·mol^{-1}	Distance (C^1_α—C^4_α)	Conf	Predicted effect on memory
NIPR	−68.50	7.44	A	enhanced
NVPR	−70.33	7.42	A	enhanced
NAPR	−63.90	7.38	A	enhanced
NSPR	−72.94	7.09	B	no effect
DLPR	−105.39	8.33	A	diminish

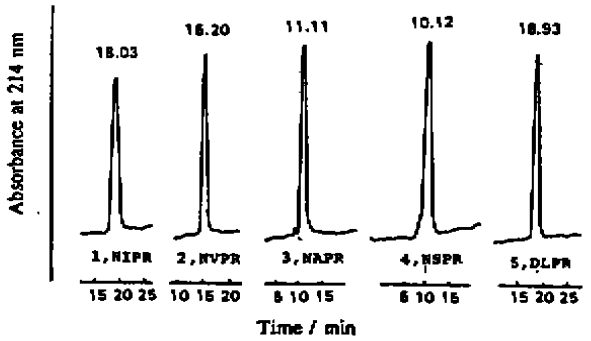

Fig 2. Reverse-phase HPLC of synthetic peptides. Column: DELTA PAK C18-300A (7.8 mm × 300 mm). Eluent: (A) 0.1 % trifluoroacetic acid; (B) 90 % acetonitrile and 0.1 % trifluoroacetic acid. Flow rate: 2 ml·min^{-1}. Detection: 214 nm. Linear gradient 2—30 % (B) in 25 min.

Tab 3. Effects of synthetic peptides on retention of passive avoidance behavior in rats. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* saline.

	Dose, ng/rat	n	Latency, s at 24 h	Latency, s at 48 h
Saline		10	78±28^a	111±28^a
NIPR	3	10	112±33^b	118±54^a
	100	10	80±22^a	107±27^a
NVPR	3	10	114±19^c	142±41^a
	100	10	102±44^a	138±40^a
NSPR	3	10	86±30^a	130±43^a
	100	10	88±36^a	104±45^c
Saline		20	97±39^a	107±43^a
NAPR	3	19	133±91^a	119±54^a
	30	19	147±65^b	125±65^a
	100	19	108±61^a	107±61^a
Saline		19	59±61^a	117±53^a
DLPR	100	18	40±53^a	73±43^b
	300	18	74±107^a	91±49^c

These results are in agreement with our predictions.

DISCUSSION

After a cycle of recognition from theory to experiment was completed, the following conclusions about the structure-activity relationship of ZNC(C)PR can be deduced. pGlu in *N* terminal of ZNC(C)PR is not essential to the activity, because the analogs without pGlu may still show ZNC(C)PR activity. While Arg and Asn are essential to the activity of ZNC(C)PR. Inhibitory effect will appear, when Asn was substituted by Asp. Cyt can be substituted by hydrophobic residues without losing the ZNC(C)PR activity, and the activity is related with the hydrophobicity. From the calculation, Pro might be essential to the maintenance of the conformation. In general, the experimental results are in agreement with our expectation; a relative compact structure is necessary for their behavioral activity.

According to the predicted conformational properties, many possible analogs can be eliminated prior to the experimental investigations. But the theoretical calculation algorithm to predict the conformation of peptide is not perfect, since the predicted conformations are sometimes different from the actual.

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REFERENCES

1 Burbach JPH, Leboulle JLM. Proteolytic conversion of arginine-vasopressin and oxytocin by brain synaptic membranes. J Biol Chem 1983; **258**: 1487—94.

2 Burbach JPH, Kovacs GL, de Wied D, Nispen JWV, Greven HM. A major metabolite of arginine vasopressin in the brain is a highly potent neuropeptide. Science 1983; **221**: 1310—12.

3 Lin C, Liu RY, Du YC. Cysteinyl methyl ester of AVP (4—8), a potent agonist on the maintenance of passive avoidance in rats. Peptides 1990; **11**: 673—9.

4 Du YC, Lin C, Xu LF, Lu ZX, Gu BX, Gou NN. Structure-dependent behavior function of AVP analogs. In: Rivier J E, Marshall G R, editors. Peptides, chemistry, structure and biology. Proceedings of the Eleventh American Peptide Symposium; 1989 Jul 9—14; La Jolla (CA), USA. ESCOM, 1990; 299—301.

5 Du YC, Gu BX, Tong T, Rong XW, Chen XF, Chen ZF. Studies on action mechanism of memory enhancing peptide — ZNC(C)PR. In: Du YC, *et al*, editors. Peptides, biology and chemistry — Proceedings of the Chinese Peptide Symposium; 1990 Aug 11—15; Shanghai, China. Beijing: Science Press, 1991; 71—4.

6 Chen XF, Chen ZF, Liu RY, Du YC. Neonatal administrations of a vasopressin analog (DDAVP) and hypertonic saline enhance learning behavior in rats. Peptides 1988; **9**: 717—21.

7 Liu RY, Lin C, Du YC. Facilitation of arginine vasopressin analogs on learning and memory in rats. Acta Pharmacol Sin 1990; **11**: 97—100.

8 Xu LF, Xu CJ, Du YC, Lin C, Lu ZX. Conformation studies on pentapeptide ZNC(C)PR and [D-Arg]ZNC(C)PR from vasopressin by 2D NMR. Acta Biochim Biophys 1990; **22**: 553—41.

9 Wang PL, Lai LH, Du YC, Jiang SP, Xu XJ. Conformation of ZNC(C)PR and molecular dynamics simulation. Chin Biochem J 1992; **8**: 619—23.

10 Wang PL, Du YC, Jiang SP, Lai LH, Xu XJ. Prediction of the conformation of ZNC(C)PR analogs. In: Du YC, *et al*, editors. Peptides, biology and chemistry. Proceedings of the Chinese Peptide Symposium; 1990 Aug 11—15; Shanghai China. Beijing: Science Press, 1991; 63—4.

11 Brooks BR, Bruccoleri RE, Olafson BD, States DJ, Swaminathan S, Karplus M. CHARMM: a program for macromolecular energy, minimization, and dynamics calculations. J Comput Chem 1983; 4: 187—217.

311-316 增强 记忆 神经肽 5

记忆增强肽 ZNC(C)PR 类似物的结构-活性关系

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A **摘要** ZNC(C)PR 是大鼠脑中具有促进学习记忆作用的神经肽。本文按前文2D-NMR 测

定结构和活性比较的结果以及分子动力学的关系式计算了45个 ZNC(C)PR 类似物的能量和可能的结构趋向，从理论上探讨了各残基对稳定结构的贡献，并据此设计和合成了5个新的类似物加以验证。结果与预测相符，即一定紧密度的分子结构是表现活性所必需的。

关键词 学习；记忆；神经肽；结构-活性关系

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Effects of low level lead exposure on behavior of young rats[1]

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ABSTRACT Dam rats were given Pb (0.58, 1.76, and 5.27 mmol·L^{-1}), containing water *ad lib* from d 16 of gestation to weaning of the offspring on d 21 postpartum. The pups continued drinking the same water till the postnatal d 30. The neurobehavioral function of pups was tested. The second-step-down latencies (SDL_2) were shortened and the number of step-downs in 5 min (NSD) were increased in step-down test. The prolongation of the lapse of time in passing through the whole course (LTPWC) and the increase in number of entries into the blind alley (NEBA) were measured in water maze test. The number of ambulations and rearings were increased in locomotor activity. The results indicated that Pb exerts adverse effects on the learning ability and memory function, and induces hyperactivity in young rats.

KEY WORDS lead poisoning; animal behavior; learning disorders; memory disorders; locomotion

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Pb-induced neurobehavioral effects were reported both in children and rats [1-3]. However, relatively few studies on behavioral status of young rats exposed to low level Pb had been reported. The present study described the findings of neurobehavioral effects in terms of cognition, spontaneous activity, and muscle-coordination due to low level Pb exposure.

MATERIALS AND METHODS

Rats Sprague-Dawley rats (♀, $n=21$) on d 16 of pregnancy (weighing 332 ± 28 g) were randomly divided into 3 Pb-poisoned groups and a control group. The poisoned groups were dosed with a solution containing lead acetate (from Shanghai Fourth Chemical Reagent Factory) 5.27, 1.76, and 0.58 mmol·L^{-1}, respectively, in drinking water and the control group was given distilled water from d 16 of gestation to weaning of the offspring on d 21 postpartum. The pups continued drinking the same water till postnatal d 30. On postnatal d 30, 6 ♂ and 6 ♀ pups per group were used for neurobehavioral tests.

Lead assay The rats were decapitated. Pb contents in blood and cortices were determined using a Hitachi-80 flameless atomic absorption spectrophotometer.

Neurobehavioral tests

1 Water maze A water maze of 100 cm × 50 cm × 35 cm was used. LTPWC and NEBA were