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Neurohypophyseal hormones, analogs, and fragments: Their effect on puromycin-induced amnesia

(memory consolidation/peptides/structure-activity relationship/mice)

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ABSTRACT **Neurohypophyseal hormones and several of their analogs, as well as N-terminal and C-terminal fragments, have been studied for their ability to attenuate puromycin-induced amnesia in mice. [8-Lysine]vasopressin, [8-arginine]vasopressin, and the analogs des-9-glycinamide-[8-lysine]vasopressin, [1-β-mercaptopropionic acid, 8-lysine]vasopressin, [1,6-aminosuberic acid, 8-lysine]vasopressin, [4-leucine, 8-lysine]vasopressin, glycyl-glycyl-glycyl-[8-lysine]vasopressin, [1-β-mercaptopropionic acid, 8-D-arginine]vasopressin, and [1,6-aminosuberic acid, 8-arginine]vasopressin are active. [8-Arginine]oxytocin as well as oxytocin and all of its other analogs tested are inactive with the striking exception of glycyl-glycyl-glycyl-oxytocin. The structural aspects of the neurohypophyseal hormones which appear to be important for significant activity in memory consolidation include the combination of a cyclic moiety containing the Tyr and Phe residues along with a basic residue in position 8. Another series of active compounds comprises C-terminal neurohypophyseal peptides and analogs thereof, including the naturally occurring Pro-Leu-Gly-NH_2 and, most surprisingly, Leu-Gly-NH_2, as well as its derivatives D-Leu-Gly-NH_2 and the diketopiperazine, *cyclo*(-Leu-Gly-).**

Peptides possessing behavioral and electrophysiological activities are the focus of increasing attention (1–9). Among these peptides are the antidiuretic hormone of pig, [8-lysine]vasopressin (LVP) (for primary structures see Fig. 1), and des-9-glycinamide-LVP (DGLVP), which was isolated from hog pituitary (6). The peptides delay extinction of active avoidance behavior in intact rats (10–12) and restore the ability of hypophysectomized rats to acquire a conditioned avoidance response (3, 5). Furthermore, rats with hereditary diabetes insipidus, which are unable to synthesize vasopressin (13), fail to exhibit passive avoidance behavior (14) as do rats treated intracerebrally with antiserum to [8-arginine]vasopressin (AVP) (15). These results, in conjunction with the dependence of LVP effects on the time of hormone administration (16), have been interpreted to suggest that LVP and AVP affect consolidation of some aspect of the memory process (12, 15, 16). Furthermore, while memory of maze-learning in mice is blocked for long periods by intracerebral injections of puromycin one or more days after training (17), treatment with DGLVP markedly attenuates the puromycin-induced amnesia (18).

In the present work we report on the protective effects of LVP and AVP on the amnestic action of puromycin in mice and on similar effects of several neurohypophyseal hormone analogs. The protective effects of DGLVP observed earlier using DGLVP obtained after tryptic digestion of LVP (18) have been confirmed with a preparation of DGLVP secured by direct synthesis; this approach excludes the possible contamination of DGLVP by LVP. In addition, we find that Pro-Leu-Gly-NH_2 (PLG), which is formed by hypothalamic enzyme(s) from oxytocin (19, 20) and which was isolated from ox hypothalamus (21), attenuates the puromycin-induced blockade of memory. The structure–activity relationship study stimulated by these results indicates that certain derivatives of PLG such as Z-PLG and Leu-Gly-NH_2 are even more active than PLG itself. Tentative deductions have been made regarding the structural requirements of these various groups of peptides for their effects on memory.

MATERIALS AND METHODS

LVP, AVP, oxytocin, PLG, Z-Pro-Leu-Gly-NH_2, Leu-Gly-NH_2·AcOH, Z-Leu-Gly-NH_2, D-Leu-Gly-NH_2, and thyrotropin releasing hormone (<Glu-His-Pro-NH_2) were from the same batches used in earlier studies (19, 22), as were Pro-Lys-Gly-NH_2, <Glu-Leu-Gly-NH_2 (22), [Phe^3,Ala^8]oxytocin, and [Ala^8]oxytocin (23), arginine vasotocin, and [Ala^2]AVP (24), deamino-LVP (25), arginine vasopressinoic acid (26), human neurophysin I (27), [$Asu^{1,6}$]AVP and [$Asu^{1,6}$]LVP (28), and the diketopiperazine of Leu-Gly (29). [Tri-Gly]LVP (30) and [tri-Gly]oxytocin were kindly supplied by Drs. I. Vávra and J. Mulder of Ferring AB, Malmö, Sweden. Pressinoic acid was a gift of Dr. S. Sakakibara, Protein and Peptide Research Institute, Osaka, Japan; [Mpr^1,D-Arg^8]vasopressin (DDAVP) (31) was from Dr. M. Zaoral, Czechoslovak Academy of Sciences, Prague, Czechoslovakia; [Leu^4]LVP (32) was a gift of Dr. V. du Vigneaud, Cornell University, Ithaca, N.Y., and Cys(Me)-Phe-Ile-NH_2, Cys(Me)-Tyr-Phe-NH_2, and Met-Tyr-Phe-NH_2 (33) were from Dr. E. Breslow, Cornell University Medical School, New York, N.Y. Gly-His-Lys·AcOH was a gift of Dr. L. Pickart, University of California, San Francisco. Cys-Tyr-Phe-Gln-Asn and Pro-Ile-Gly-NH_2 were synthesized by Dr. S. Nakagawa and *N*-formyl-PLG by J. Darnell, in our laboratory. Pro-Leu-Sar·AcOH was purchased from Fox Chemical Co.

Nomenclature is in accord with the IUPAC-IUB Rules on Biochemical Nomenclature (1972) *Biochem. J.* **126**, 773–780; and (1967) *J. Biol. Chem.* **242**, 555–557. All optically active amino acids are of the L configuration unless otherwise noted. Abbreviations used: LVP, lysine vasopressin, [8-lysine]vasopressin; [tri-Gly]LVP, glycyl-glycyl-glycyl-[8-lysine]vasopressin; [tri-Gly]oxytocin, glycyl-glycyl-glycyl-oxytocin; deamino-LVP [1-β-mercaptopropionic acid, 8-lysine]vasopressin; AVP, arginine vasopressin, [8-arginine]vasopressin; DDAVP, [1-β-mercaptopropionic acid, 8-D-arginine]vasopressin; AVP acid, arginine vasopressinoic acid, [8-arginine]vasopressinoic acid; DGLVP and DGOXY, des-9-glycinamide-[8-lysine]vasopressin, des-9-glycinamide-oxytocin; DGDLOXY, des-9-glycinamide, des-8-leucine-oxytocin; AVT, arginine vasotocin, [8-arginine]oxytocin; PLG, prolyl-leucyl-glycinamide; Asu, aminosuberic acid; *cyclo* (-Leu-Gly-), the diketopiperazine of Leu-Gly; Mpr, β-mercaptopropionic acid; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; Bzl, benzyl; Sar, sarcosine.

[Des-Gly-NH_2[9]]Lysine vasopressin (DGLVP) was prepared from Z-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Pro-Lys(N^{ϵ}-Z)-polystyrene-2%-divinylbenzene obtained by the general method of solid-phase synthesis (34) as applied to the synthesis of AVP (35). Appropriate *N*-Boc protected amino acids were added to the peptide as their *p*-nitrophenyl or *N*-hydroxysuccinimide esters in the presence of 1,2,4-triazole. Cleavage of the peptide from the resin and removal of the *N*-Z and *O*-Bzl groups was achieved by treatment with HBr in trifluoroacetic acid to give the hydrobromide of the *S*-protected octapeptide. The *S*-Bzl group was removed with Na in boiling NH_3, and the linear peptide was cyclized to the disulfide by treatment with potassium ferricyanide. The peptide was purified by partition chromatography on Sephadex G-25 in the solvent system *n*-BuOH/EtOH/pyridine/1 N AcOH (4:1:1:7); R_F = 0.19; 23% yield; $[\alpha]_D^{22}$ 32.1° (*c* 0.5, 1 N AcOH); the material was homogeneous upon thin-layer chromatography on silica gel G (*n*-BuOH/AcOH/H_2O, 4:1:5; upper phase; R_F 0.4) and electrophoresis (Whatman 3 MM paper; pyridine–acetate buffer, pH 6.5; 1.5 hr; 3000 V). Amino-acid composition: Asp, 1.0; Glu, 1.0; Pro, 1.0; Cys, 1.9; Tyr, 0.8; Phe, 1.0; Lys, 1.1; NH_3, 2.1 (R. T. Havran and R. Walter *et al.*, unpublished). [Des-Gly-NH_2[9]]Oxytocin (DGOXY) and [des-Gly-NH_2[9], des-Leu[8]]oxytocin (DGDLOXY) were prepared in an analogous manner. DGOXY and DGDLOXY were purified by partition chromatography on Sephadex G-25 in the solvent system *n*-BuOH/PrOH/H_2O containing 0.125% pyridine and 0.625% AcOH (6:1:8); R_F = 0.29 and 0.15, respectively. Details of syntheses and characterization of these compounds will be published elsewhere (Walter *et al.*, in preparation).

Behavioral Training and Testing. The behavioral techniques have been fully described (36). Swiss-Webster mice (6–7 months old, 30–35 g) from our inbred colony were trained in a single session in a Y-maze to a criterion of nine out of 10 correct responses. Intermittent foot shock, manually applied (0.2–0.4 mA from a dc source; 2 sec on, 2 sec off) was given for failure to move from the stem of the Y within 5 sec and for errors of left-right discrimination. Shock was adjusted with individual mice to the minimal level consistent with the desired behavioral response. The same procedure was used in tests, given 1 week after treatment with puromycin, for retention of memory of the training experience. A final test of retention of relearning was given 1–2 weeks after the first retention test. Mice which scored poorly on this final retention test were discarded.

Total errors were the sum of latencies greater than 5 sec and of incorrect choices, i.e., all mistakes were added until, in 10 consecutive runs in the maze, the mouse had performed correctly in nine of them. Memory was evaluated in the retention tests in terms of the percentage savings of errors. These percentages were calculated by subtracting the number of errors to criterion in the retention tests from the number to criterion in training, dividing by the number in training and multiplying by 100. Savings of 100% indicate perfect memory; zero savings, complete loss of memory. The Mann-Whitney U test, one-tailed, was used for determining statistical significance.

Injection of Puromycin and Peptides. Intracerebral injections of puromycin were bitemporal and were given 24 hr after training as described (36). Each injection site received 90 μg of puromycin·2 HCl (neutralized with NaOH) in 12 μl of water. As judged by their inhibition of protein synthesis, these bitemporal injections primarily affect the hippocampi and entorhinal cortices (37). Peptides used in this study were dissolved in 15% gelatin. In all instances 0.1 mg in 0.2 ml of solution was injected subcutaneously immediately after training. Two sets of controls were used: in the first, animals were trained, injected intracerebrally with 12 μl of water 24 hr later, and tested 7 days later. In the second, animals were trained and injected bitemporally with puromycin, but did not receive injections of peptides. Mice receiving bitemporal injections of water (n = 8) showed a mean of 89% savings of errors; those receiving puromycin (n = 10) showed 0% savings. Results with the peptides are compared to the latter group.

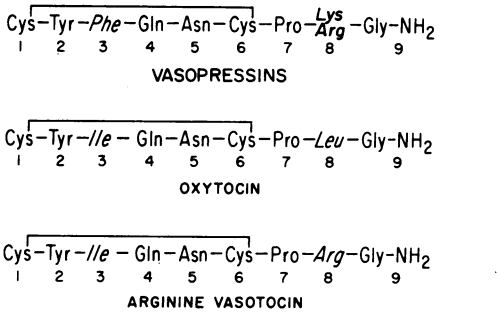

FIG. 1. Amino-acid sequence of neurohypophyseal hormones.

RESULTS AND DISCUSSION

LVP and five analogs of LVP (Fig. 2: DGLVP, [tri-Gly]LVP, deamino-LVP, [Leu[4]]LVP, and [Asu[1,6]]LVP) were assayed for their effects on memory. All of these compounds significantly decreased puromycin-induced amnesia; the most active were LVP and [tri-Gly]LVP, with the rest of the analogs having somewhat reduced, but highly significant effects.

As may have been expected on the basis of structural likenesses (Fig. 1), AVP is similar to LVP in its effects on memory retention (Fig. 2). DDAVP is also very active and [Asu[1,6]]AVP has a lesser, but significant effect. Pressinoic acid, which possesses the same covalent 20-membered ring as LVP and AVP but lacks the C-terminal tripeptide of the hormones, is inactive against puromycin-induced amnesia, as are [Ala[2]]AVP and AVP acid.

Oxytocin, which previously de Wied found inactive in inhibiting extinction of an avoidance response in rats (12), and all of its analogs tested in this study (DGOXY, DGDLOXY, [Phe[3],Ala[8]]oxytocin, and [Ala[8]]oxytocin), have undetectable activity in protecting against puromycin-induced amnesia. The striking exception is [tri-Gly]oxytocin which gave 82% savings (n = 4; P = 0.001). Another naturally occurring neurohypophyseal hormone, arginine vasotocin (AVT), which has the C-terminal tripeptide of AVP and the covalent ring of oxytocin, also shows no significant activity.

Some conclusions regarding structural characteristics of the neurohypophyseal hormones necessary for effectiveness in memory retention may be deduced from our results. Both LVP and AVP, with identical 20-membered ring structures, are active, but not oxytocin and AVT, which have ring structures differing from the vasopressins by replacement of the amino-acid residue with an aromatic sidechain in position 3 by one with an aliphatic sidechain. The aminosuberic acid analogs of the vasopressins, which have a 20-membered ring containing an ethylene moiety in place of a disulfide bridge (28), and which have been suggested to have a less compact ring structure than the natural hormones (28, 38), have reduced, but significant activity. While alterations of the 20-

Table 1. Comparison of endocrine* and extraendocrine† activities of lysine vasopressin and [des-Gly-NH_2^9]lysine vasopressin

	Rat uterotonic	Avian vasodepressor	Rat pressor	Rat antidiuretic	Memory retention†
LVP	4.8‡	48‡	300§	203‡	76
DGLVP	0.22 ± 0.04	Inhibitor	0.05 ± 0.003	2.36 ± 0.21	61

* Activities are given in international U/mg ± standard errors. A four-point design was used with the USP posterior pituitary reference standard. *Uterotonic Activity:* Holton (60) and Munsick (61); *Avian Vasodepressor Activity:* Munsick *et al.* (62) and Coon (63); *Rat Pressor Activity:* The Pharmacopeia of the United States (64); *Antidiuretic Activity:* Jeffers *et al.* (65) and Sawyer (66). Inhibitory properties were determined by injecting the analog into the wing vein of the chicken immediately prior to injecting oxytocin, and comparing the result to that caused by oxytocin alone.

† Values represent per cent savings of errors in memory retention tests of mice. For details of experimental method and controls, see *text*. For LVP, $n = 4$ and $P = 0.001$; for DGLVP, $n = 8$ and $P < 0.001$.

‡ Kimbrough *et al.* (67).

§ Meienhofer and Sano (68).

membered ring such as removal of the N-terminal amino group of LVP, or replacement of the Gln in position 4 by Leu, do not greatly influence the memory retention effects of LVP, replacement of the aromatic Tyr in position 2 of AVP by an aliphatic residue ([Ala^2]AVP) virtually eliminates activity. It is noteworthy that pressinoic acid is inactive in memory retention. This may reflect a difference of the preferred conformation of pressinoic acid as compared with vasopressins at the receptor sites. In fact, as early as 1971 (40) it was suggested that the Pro residue plays a role in stabilizing the β-turn of the preferred solution conformation proposed for the ring moiety of neurohypophyseal hormones (24, 39–42). Thus, it was no surprise that for tocinamide a conformation different from that of oxytocin was reported (43). By analogy, the structure of pressinoic acid is thought to differ from that of the ring moiety of vasopressin.

Contributions of residues in the C-terminal tripeptide are also evident: [Phe^3,Ala^8]oxytocin, containing the vasopressin ring, but a neutral alanine in position 8 in place of a basic residue, has no measurable activity. However, an analog with a basic residue in position 8 but without the Phe^3 in the ring, also has no detectable activity, as demonstrated with arginine vasotocin.

These results suggest that the features which distinguish vasopressins from oxytocin, i.e., the presence of the aromatic residue in position 3 of the 20-membered covalent ring in combination with a basic residue in position 8, and the influence of these features on the overall hormone conformation, are important for detectable attenuation of puromycin-induced amnesia in mice. The lack of activity of [Ala^2]AVP and AVP acid indicates that other structural modifications can, nevertheless, strongly influence these basic requirements. For the most part these tentative conclusions are in line with those proposed by de Wied and coworkers (44) using a different behavioral test system.

A striking dissociation for DGLVP of endocrine effects and activity in memory retention is found upon comparison of biological activities of synthetic LVP and DGLVP (Table 1). DGLVP is potent in protecting mice against amnestic effects of puromycin, while it has drastically reduced endocrine activities characteristic of neurohypophyseal hormones. This confirms the results of de Wied *et al.*, who found similarly reduced endocrine activities for DGLVP in association with high activity in inducing resistance to extinction of active avoidance behavior (10). The dissociation of behavioral and endocrine effects is also supported by results with [Leu^4]LVP, which is effective in memory retention (Fig. 2) while exhibiting low agonistic or inhibitory endocrine activities (32). An even more active LVP derivative in terms of memory retention is [tri-Gly]LVP (Fig. 2), an analog with low specific endocrine activities but significantly prolonged duration of action (45, 46).

DDAVP and [tri-Gly]oxytocin also possess high activity in memory retention. Apparently, the structural modification, i.e., elongation of the N-terminus with a tri-glycyl moiety, which has a tendency to enhance the inherent activity of LVP, also allows expression of activity undetectable in oxytocin *per se*. The protracted endocrinological actions of both DDAVP and [tri-Gly]LVP (45–47) may possibly result from slow enzymatic modification or from a change in hydrophobicity with a concomitant alteration in distribution patterns. Since radioactively labeled neurohypophyseal hormones are taken up both *in vitro* and *in vivo* by the neurohypophysis

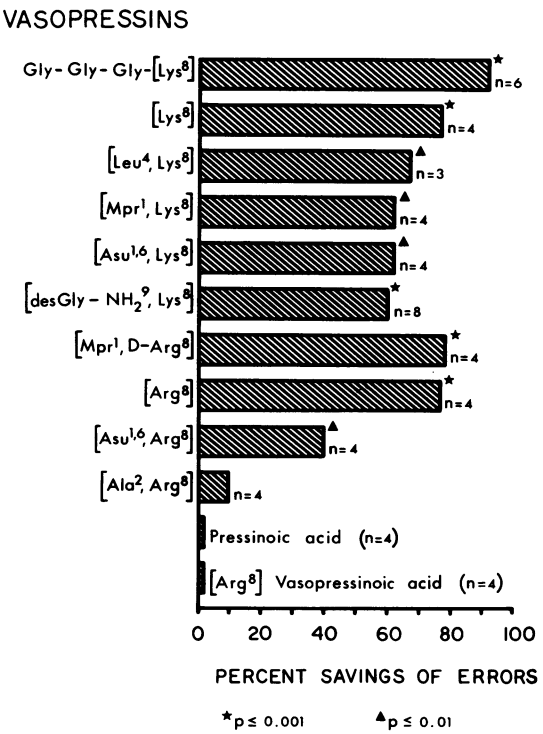

FIG. 2. Effectiveness of LVP, AVP, and their analogs in attenuating puromycin-induced amnesia in mice. For details of method, see *text*. One hundred percent savings of errors indicates total attenuation of the puromycin effect; 0% indicates no effect of the compound on the amnestic action of puromycin. n = number of animals tested.

and are rapidly enzymatically degraded (48–51), structural characteristics influencing distribution or breakdown of hormone analogs might be relevant to their effects on memory.

In fact, in addition to DGLVP, which is probably derived enzymatically from LVP, other neurohypophyseal hormone fragments have activity in attenuating puromycin-induced amnesia, as was found during screening of a series of peptides. While the N-terminal pentapeptide of vasopressin, Cys-Tyr-Phe-Gln-Asn, and a number of related peptides [i.e., Cys(Me)-Phe-Ile-NH_2, Cys(Me)-Tyr-Phe-NH_2, Met-Tyr-Phe-NH_2] as well as model peptides (<Glu-His-Pro-NH_2 [thyrotropin releasing hormone], Gly-His-Lys·AcOH) and human neurophysin I are all inactive, the results in Fig. 3 demonstrate the protective effects of C-terminal fragments of the neurohypophyseal hormones and analogs of these fragments. The C-terminal tripeptide of oxytocin, Pro-Leu-Gly-NH_2, originally proposed to be the natural factor inhibiting the release of melanotropin (MSH) from the pituitary (19), has more recently been found to possess extrapituitary central nervous system effects (e.g., 52–55). As shown in Fig. 3, it is also active in attenuating puromycin-induced amnesia. Structure-activity studies on the MSH-release-inhibiting activity of PLG revealed that there are specific structural requirements for the N- and C-terminal residues of the tripeptide and that modifications of the side chain of the center residue are permitted (provided the L configuration is maintained) (22). Similarly for memory-retention activity, Pro-Lys-Gly-NH_2 is even more potent than PLG. Pro-Ile-Gly-NH_2 has greatly reduced and statistically nonsignificant, but still detectable effects. Varying the N- and C-terminal residues (i.e., <Glu-Leu-Gly-NH_2 and Pro-Leu-Sar·AcOH) eliminates significant activity. On the other hand, blocking of the free N-terminal imino group of PLG by a carbobenzoxy group greatly enhances activity, while blocking with a formyl group significantly reduces activity. It is of interest to note that small peptides such as PLG (56) and thyrotropin releasing hormone (57, 58) seem to be taken up as intact molecules, after intravenous injection, by pituitary gland, and PLG also by pineal gland.

The most surprising finding to us was that the C-terminal dipeptide of oxytocin, Leu-Gly-NH_2, its D isomer as well as its cyclic analog, are very effective in memory retention (Fig. 3). It is not clear at present whether the biological effect of the C-terminal peptides is elicited by the same mechanisms as that of the neurohypophyseal hormones. Notably, larger molar doses of the C-terminal peptides than of the hormones have been used in this study.

In earlier studies using DGLVP (18), the possibility that this peptide might alter the cellular interactions of puromycin rather than processes concerned with memory was shown to be untenable. We tentatively assume that the same holds true for the compounds found here to attenuate puromycin-induced amnesia. It will be of value to determine both the dose range over which the hormones, analogs, and fragments are active, as well as to study the effect of variations in time of injection relative to time of training, particularly with the "long-acting" hormone analogs. Moreover, it is important to note that the lack of information regarding permeability of the blood-brain barrier to peptides, and the metabolic fate of these peptides in brain, suggests caution in interpretation of the present results. In this context, it should be mentioned that [^{3}H]PLG has been reported to penetrate readily the blood-brain barrier after intracarotid injection into rats, while [^{14}C]AVP does not (59). At this time the results presented here appear to be best explained by supposing that the active compounds modify consolidation of memory in such a way that expression or retrieval of memory becomes insensitive to puromycin, and that there are distinct structural requirements if the peptides are to be active in this way.

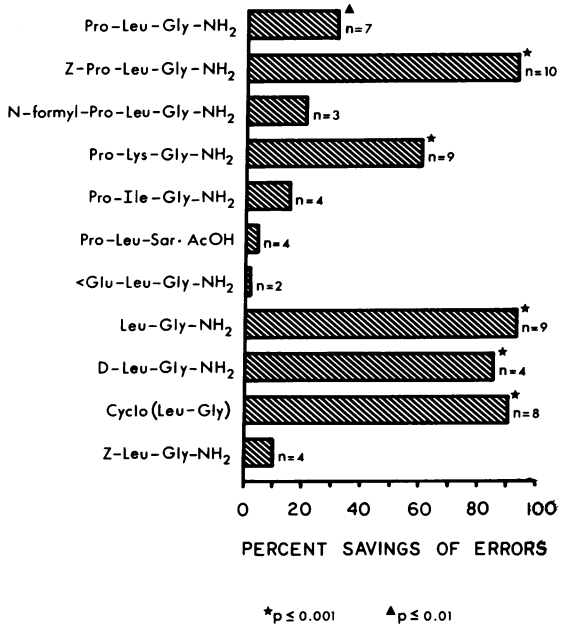

FIG. 3. Effectiveness of C-terminal fragments of neurohypophyseal hormones and analogs of the fragments in attenuating puromycin-induced amnesia in mice. See legend to Fig. 2 for details.

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