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Amnestic effects in mice of four synthetic peptides homologous to amyloid β protein from patients with Alzheimer disease

(amnesia/memory processing)

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ABSTRACT **Immediate post-training intracerebroventricular administration of a synthetic peptide homologous to β protein of brain amyloid, [Gln^{11}]β-(1–28), caused amnesia for footshock active avoidance training in mice in a dose-dependent fashion. This effect was specific to memory processing since the peptide did not cause amnesia when injected 24 hr after training nor did it disturb storage or retrieval of older memories. Shorter fragments of the amyloid β protein consisting of residues 12–28, 18–28, and 12–20 also were amnestic when given intracerebroventricularly, residues 12–20 being least effective. The hippocampus, a brain structure importantly involved in learning and memory, consistently shows severe pathological changes and deposition of amyloid in patients with Alzheimer disease. Immediate post-training bilateral intrahippocampal injection of [Gln^{11}]β-(1–28) produced amnesia at much lower doses than did [Gln^{11}]β-(1–28) injected intracerebroventricularly. Thus these experimental results suggest a possible direct role of amyloid β protein or fragments thereof in an aspect of the spectrum of cognitive deficit in Alzheimer disease.**

Much data suggest that in Alzheimer disease (AD) there may be genetically and/or environmentally induced defects in the enzymatic machinery involved in degradation of amyloid precursor protein (APP) (for reviews, see refs. 1 and 2). Alternative splicing of mRNAs gives rise to at least five forms of APP, two of which possess a Kunitz-type protease inhibitory domain. Normal lysosomal processing of APPs involves highly coordinated sequences of desulfation, dephosphorylation, deglycosylation, and proteolytic splitting. The APPs may belong to a family of polypeptide precursors or polyproteins that upon processing give rise to a number of different bioactive peptides that may act individually or in concert to regulate cellular activities (3–5). The processing of the parent molecules and/or the extracellular secretion of the resulting subunits may vary with species, tissue, age, hormonal status, extent of phosphorylation (6), etc. Although the APPs may be cell-surface receptors (7, 8), some of the peptidic fragments derived from them may be ligands (9) for specific membrane sites.

To some extent in normal aging and to a greater extent in AD and in adult Down syndrome, abnormal processing of APP gives rise to an insoluble self-aggregating 42-amino acid polypeptide [designated variously as amyloid β protein (AβP), A4, or βA4] that is found in amyloid (10–14). The extent of AβP deposition correlates with the degree of neuronal damage, cognitive impairment, and memory loss (15–18). Amyloid-like fibrils arise readily *in vitro* under physiological conditions even from the following smaller peptides homologous to AβP: β-(1–28), [Gln^{11}]β-(1–28), β-(12–28), and β-(18–28) (19–21). Extensive stacks of β-pleated sheets are formed from the latter peptide (21). Functional deficits arise in AD from damage to nerve circuitry *per se*, which is known to occur in late phases of the disease (22, 23). It also is possible that binding of AβP and related peptides to components of the extracellular matrix [e.g., proteoglycans (24)] or to receptors on endothelial, glial, or neuronal cells in particular brain regions could have disruptive effects on neuronal communications at earlier stages of the disease when the deposits of these substances are diffuse and typical cytopathological evidence of AD often is absent.

Should the latter be the case, we conjectured that AβP and, perhaps, smaller peptidic fragments thereof that are responsible for binding of AβP to cell membranes or components of the extracellular matrix might have amnestic effects upon appropriate administration to experimental animals. Soluble peptides or structurally mimetic nonpeptidic substances then might be devised that could antagonize the binding of the AβP and thus alleviate some of the symptoms of AD not caused by actual physical destruction of neural circuitry. Such substances also might be useful in attenuating progression of AD.

In the present experiments we tested the effects on memory in mice of a synthetic peptide homologous to AβP, [Gln^{11}]β-(1–28) or DAEFRHDSGYQVHHQKLVFFAEDVGSNK, first isolated from amyloid fibrils prepared from leptomeningeal vessels from AD brains (10, 11). Other sequences for AβP isolated directly from human brain or deduced from cDNA cloning are identical with this sequence with the exception that residue 11 was found to be glutamic acid and not glutamine (1). The reason for the discrepancy is not apparent. One of us (E.R.) recently was assured by G. G. Glenner (personal communication) that it is not attributable to technical error in the original sequence determinations (10, 11). Also tested were residues 12–28 (VHHQKLVFFAEDVGSNK), 18–28 (VFFAEDVGSNK), and 12–20 (VHHQKLVFF), in which residue 11 plays no role.

MATERIALS AND METHODS

Test Animals. After 1 week in the laboratory, CD-1 male mice obtained from Charles River Breeding Laboratories were caged individually 24–48 hr prior to training and remained singly housed until retention was tested 1 week later. Animal rooms were on a 12-hr light/dark cycle with lights going on at the hour of 0600. Median body weight was 35 g, with a range of 33–38 g. Mice were assigned randomly to

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Abbreviations: AD, Alzheimer disease; APP, amyloid precursor protein; AβP, amyloid β protein; FAAT, footshock active avoidance training; icv, intracerebroventricular; ih, intrahippocampal.

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groups of 15 and were trained and tested between the hours of 0700 and 1500.

Polypeptides Tested. The following substances were employed in the tests. Polypeptides used in this study were [Gln^{11}]β-(1–28), β-(18–28), β-(12–20), and β-(12–28), respectively, the numbering beginning with the N-terminal amino acid of AβP. The first three of these peptides were synthesized and analyzed to establish purity by standard methods by Bruce E. Kaplan of the Beckman Research Institute of the City of Hope (Duarte, CA). Samples of [Gln^{11}]β-(1–28) and β-(12–28) also were purchased from Peninsula Laboratories. The above peptides were dissolved appropriately for injection: [Gln^{11}]β-(1–28) and β-(18–28) were dissolved in dimethyl sulfoxide and diluted in physiological saline to a final concentration of 8% (vol/vol) dimethyl sulfoxide; β-(12–20) was dissolved in 0.01 M HCl followed by dilution and neutralization with 0.01 M NaOH; and β-(12–28) was dissolved directly in physiological saline. All solutions were prepared fresh daily and coded to eliminate experimenter bias.

Apparatus and Training and Testing Procedures. The T-maze used in footshock active avoidance training (FAAT) (25) consisted of a black plastic alley (46 cm long) with a start box at one end and two goal boxes (17.5 cm long) at the other. The start box was separated from the alley by a plastic guillotine door that prevented movement down the alley until training began. The alley was 12.5 cm deep and 9.8 cm wide. An electrifiable stainless steel rod floor ran throughout the maze.

Mice were not permitted to explore the maze before training. A block of training trials began when a mouse was placed in the start box. The guillotine door was raised and a muffled doorbell-type buzzer (intensity, 65 decibels) sounded simultaneously; footshock (0.35 mA; intertrial interval, 45 sec) was 5 sec later through a scrambled grid floor shocker (Colbourn Instruments, model E13-08). The goal box first entered during the first set of trials was designated as "incorrect" and footshock was continued until the mouse entered the other goal box, which in all subsequent trials was designated "correct" for the particular mouse. At the end of each group of trials, the mouse was removed to its home cage.

As training proceeded, a mouse made one of two types of responses. A response latency longer than 5 sec was classed as an escape from the footshock. A response latency less than or equal to 5 sec was considered an avoidance, since the mouse avoided receiving a footshock. Two exclusion criteria were applied to reduce learning variability among mice, as follows. On the first training trials, mice with escape latencies greater than 20 sec were discarded. Mice not having at least one errorless escape latency between 1.5 and 3.5 sec on training trials 3 or 4 were excluded. The total exclusions were fewer than 15%. Mice received five such training trials. One week after training and post-trial administration of vehicle alone or vehicle containing peptide, T-maze training was resumed until each mouse made five avoidance responses in six consecutive training trials (trials to criterion).

For lever-press training and testing (26), mice were placed in a fully automated apparatus in which the pressing of a lever on one wall caused a light to go on and a liquid dipper containing 100 μl of milk to appear on the opposite wall. On the first day, the milk reward could be obtained within 11 sec, but on subsequent days only 4 sec were allowed. Training took place for 30 min on each of 5 successive days and retention was tested during a 30-min session 13 days later, the total number of lever-press–milk-consumption performances being a measure of retention.

Surgical Procedure in Preparation for Intracerebroventricular (icv) and Intrahippocampal (ih) Administration of Substances. The following procedure was performed 24–48 hr prior to training (27). A single hole was drilled through the skull over the third ventricle (−0.5 mm relative to bregma, 0.5 mm right of central suture) while the mouse, appropriately anesthetized with methoxyflurane, was held in a stereotaxic instrument. The third ventricle was chosen as site of icv drug injection because only a single injection is required and the drug quickly reaches limbic system structures, believed to be associated with memorial processes. Immediately after training, mice were anesthetized with enflurane, a short acting anesthetic, and given an icv injection of 2 μl of vehicle alone or test substance in vehicle delivered over a 30-sec period through a 31-gauge needle attached to a 10-μl syringe; the injection was given within 3 min after the training. Accuracy of injection was determined to be greater than 95% by dye injection, monitored regularly.

In preparation for bilateral ih injections (28), mice anesthetized with methoxyflurane were placed in a stereotaxic instrument and, after deflecting the scalp, holes were drilled through the skull over the injection sites. Coordinates for bilateral injections into the rostral hippocampus were 1.6 mm with respect to bregma, 1.6 mm right and left of the central suture, and 1.6 mm deep. The coordinates were confirmed using a stereotaxic atlas (29). Mice were trained 24–48 hr after surgery. Immediately after training mice were again placed in the stereotaxic apparatus under enflurane anesthesia. Within 3 min after training, 0.5 μl of solution was injected bilaterally into the target structure during 60 sec through a 31-gauge needle fixed to a 10-μl syringe with PE-10 tubing and driven by a Sage syringe pump (model 341A). Reliability of the injections was established by injecting dye into the sites and determining the location of dye in frozen sections.

Statistical Treatment of Data. Results are expressed in terms of the mean and SEM. The overall significance of the peptide treatment was determined by a one-way or two-way analysis of variance or Student's *t* test. Further comparisons of differences between means used Dunnett's *t* test to make multiple comparisons between each group treated with peptide and the vehicle control group or Duncan's multiple range test for comparisons among peptide-treated groups (30–32).

RESULTS

Mice were given either icv or ih injections of [Gln^{11}]β-(1–28) immediately after FAAT and were tested 1 week after training. At all doses tested, the mean number of trials to criterion for the peptide-injected mice was greater than for mice receiving vehicle alone (Fig. 1). One-way analysis of variance showed there to be overall significant amnestic effects of icv [$F(5, 84) = 13.09$; $P < 0.001$] and ih [$F(5,84) = 6.34$; $P < 0.001$] treatments. The mean number of trials to criterion was higher relative to controls for individual groups receiving icv 1.535 ($P < 0.05$), 3.07 ($P < 0.01$), and 6.14 ($P < 0.01$) nmol of peptide per mouse and receiving ih 0.1535 ($P < 0.05$), 0.307 ($P < 0.05$), and 0.614 ($P < 0.01$) nmol of peptide per mouse. The maximal number of trials (mean) to which the curves in Fig. 1 could be extrapolated was lower than the mean number of trials for mice trained for the first time, when the others were tested for retention (designated "naive" on Fig. 1); i.e., the highest doses of [Gln^{11}]β-(1–28) by the respective routes produced highly significant but incomplete amnesia of the training.

To assure ourselves of the quantitative reproducibility of the results as well as of the effects of [Gln^{11}]β-(1–28) from more than one source, icv results of 15 mice per dose with [Gln^{11}]β-(1–28) synthesized at the Beckman Research Institute (BRI) were compared with results of groups of 8 mice obtained with [Gln^{11}]β-(1–28) purchased from Peninsula Laboratories (P). The trials to criterion (mean ± SEM) for 1.535-, 3.07-, and 6.14-nmol doses, respectively, were 8.07 ± 0.48 (BRI) vs. 8.00 ± 0.70 (P); 9.20 ± 0.39 (BRI) vs. 9.00 ± 0.57 (P); and 9.53 ± 0.49 (BRI) vs. 9.25 ± 0.53 (P). Differences

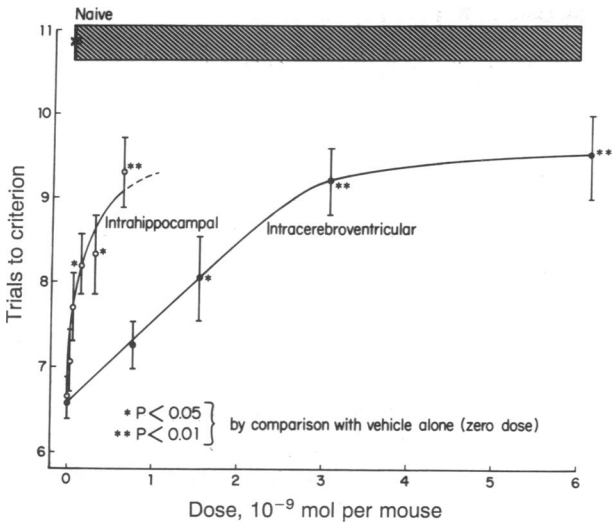

FIG. 1. Dose–response curves showing amnestic effects of [Gln[11]]β-(1–28) on retention of T-maze FAAT. Data for trials to criterion are expressed as mean ± SEM.

between results with peptides from the two sources were not significant ($t < 1$).

Groups of 15 mice were injected icv 24 hr after FAAT with 6.14 nmol of [Gln[11]]β-(1–28) or with vehicle alone. Upon testing for retention, trials to criterion (mean ± SEM) for the peptide-treated groups (7.00 ± 0.26) were not significantly different from the controls (6.58 ± 0.27; $t = 1.13$, $P > 0.5$). Thus, the peptide produced amnesia by impairing memory processing that occurred shortly after training but did not interfere with recall at the time of retention testing.

Mice trained to press a lever for milk reinforcement were divided into three 15-mouse groups, with the mean numbers of lever presses on the last day of training not differing significantly among them ($t < 1$ for all comparisons; Tukey's t test): A, 159 ± 9.8; B, 146 ± 8.4; C, 165 ± 9.5. Three days later groups A and B received FAAT, followed immediately by icv administration of 6.14 nmol of [Gln[11]]β-(1–28) to group A and vehicle alone to group B. Group C remained undisturbed in home cages. In a test of retention of FAAT 1 week after training, the peptide-injected mice required a greater number of trials to criterion than did those receiving vehicle alone: A, 10.08 ± 0.35 vs. B, 6.85 ± 0.26 ($t = 7.5$, $P < 0.001$). Tests of lever pressing in all groups of mice 3 days later showed there to be no significant differences among them ($t < 1$): A, 177 ± 8.5; B, 168 ± 12.6; C, 176 ± 16.7. T tests conducted on the number of lever presses for each group at the beginning and end of the experiment also showed there to be no significant changes. From the results with group C, we concluded that there was no time-related attenuation of memory retention of lever-press performance and from results with group B we concluded that intervening FAAT had no effect. The results with group A showed that the strong amnestic effect of [Gln[11]]β-(1–28) on intervening FAAT had no effect on retention of the lever-press task learned before. Thus, the peptide did not cause general impairment of ability to recall stored information.

The whole amino acid sequence of [Gln[11]]β-(1–28) is not required for its amnestic effect; peptides β-(12–28), β-(18–28), and β-(12–20) were significantly amnestic in the FAAT retention test by comparison with vehicle controls (Table 1). Peptide β-(12–28) is at least as potent as [Gln[11]]β-(1–28), whereas β-(12–20) is significantly less potent than β-(12–28) but not significantly different from β-(18–28).

DISCUSSION

Peptides β-(12–20) and β-(18–28), both amnestic, have only amino acid residues VFF at positions 18–20 in common, suggesting that the latter triad may be essential for there to be an amnestic effect. The greater efficacy of β-(12–28) indicates that amino acids flanking VFF may have modifying effects. Single or multiple VFF sequences exist in 469 of 15,409 (3%) of the protein sequences in the Swiss-Prot (version 18) data base. Among the VFF-containing proteins are receptors for serotonin, acetylcholine, norepinephrine, γ-aminobutyric acid, glucocorticoids, androgens, progesterone, natriuretic peptide, insulin, and transferrin. Only the AβPs of human, mouse, and rat were found to contain the β-(16–22) sequence KLVFFAE; and no protein was recorded in the above data base in which there are conservative substitutions for lysine, leucine, alanine, and glutamic acid in the latter peptide.

Peptides β-(1–28) and β-(18–28) are anionic, β-(12–28) is neutral, and β-(12–20) is cationic. Therefore, in these peptides net charge does not appear to be important for amnestic activity.

Depending on conditions, AβP has been found to exert both toxic and trophic effects on cells in culture (33–35). In the present experiments, four peptides homologous to AβP were found to be amnestic in mice, a demonstration of effects on quantitatively measurable variables in an intact behaving organism. However, effects of acute post-training administration of substances in tests with normal young mice cannot necessarily be equated to effects of endogenous deposition of related substances in brains of normal aging individuals or in those with AD. The main cause for poor recall in aging and in AD is the fragility of the patients' "working" memory [also known as primary or "short-term" memory (36–39)]. In the

Table 1. Amnestic effects of 6.14 nmol of icv-administered AβP-related peptides on retention of T-maze FAAT

Residues in peptide	AβP sequence	Trials to criterion, no.	P value for comparison with vehicle
	1 5 10 15 20 25		
1–28	DAEFRHDSGYQVHHQKLVFFAEDVGSNK	9.53 ± 0.49	<0.01
12–28	VHHQKLVFFAEDVGSNK	9.90 ± 0.48	<0.01
18–28	VFFAEDVGSNK	8.90 ± 0.33	<0.01
12–20*	VHHQKLVFF	8.33 ± 0.50	<0.01
None†	—	6.75 ± 0.29	—

Data for trials to criterion are expressed as mean ± SEM.

*β-(12–20) is significantly less amnestic than β-(12–28) ($P < 0.01$) but not significantly different from β-(18–28) by Duncan's multiple range test. β-(12–28) and β-(18–28) are not significantly different from each other.

†The vehicle is saline containing 8% dimethyl sulfoxide. In separate experiments it was shown that the mean of groups treated icv with saline alone or with dimethyl sulfoxide in saline did not differ significantly ($t < 1$).

present experiments the substances tested were shown to decrease retention, a measure of secondary or "long-term" memory. Whether or not these substances also are deleterious to acquisition of learning, a reflection of "working" memory, is more difficult to determine definitively.

Inhibition of synaptic information transfer (e.g., γ-aminobutyric acid, scopolamine, and *N*-methyl-D-aspartate antagonists), of genetic information transcription (e.g., actinomycin D) or translation (e.g., puromycin and anisomycin), and of macromolecular synthesis (e.g., amino acid and purine and pyrimidine antimetabolites) and generally unfavorable circumstances for cell growth and development, such as aminoacidopathies (hereditary and acquired), nutritional deficiencies, and circulatory insufficiency (blood vessel disease and arteriosclerosis), decrease the rate and extent of plastic changes that are required for memory formation. There is much literature in support of the latter (see ref. 40). Peptides homologous to AβP from patients with AD now join the ranks of the above. Determination of the nature of their effects awaits identification of the entities in particular cellular sites with which the peptides associate.

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