Pharmacology Biochemistry and Behavior, Vol. 46, pp. 799–805, 1993
Printed in the U.S.A.

0091-3057/93 $6.00 + .00

The Potent Free Radical Scavenger α-Lipoic Acid Improves Memory in Aged Mice: Putative Relationship to NMDA Receptor Deficits

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Received 22 May 1992

STOLL, S., H. HARTMANN, S. A. COHEN AND W. E. MÜLLER. *The potent free radical scavenger α-lipoic acid improves memory in aged mice: Putative relationship to NMDA receptor deficits.* PHARMACOL BIOCHEM BEHAV 46(4) 799–805, 1993.—α-Lipoic acid (α-LA) improved longer-term memory of aged female NMRI mice in the habituation in the open field test at a dose of 100 mg/kg body weight for 15 days. In a separate experiment, no such effect could be found for young mice. α-LA alleviated age-related NMDA receptor deficits (B_{max}) without changing muscarinic, benzodiazepine, and α_2-adrenergic receptor deficits in aged mice. The carbachol-stimulated accumulation of inositol monophosphates was not changed by the treatment with α-LA. These results give tentative support to the hypothesis that α-LA improves memory in aged mice, probably by a partial compensation of NMDA receptor deficits. Possible modes of action of α-LA based on its free radical scavenger properties are discussed in relation to the membrane hypothesis of aging.

Aging α-Lipoic acid Cognition Habituation in the open field Learning Memory
Mouse Thioctic acid

FREE radical damage is discussed as an important mechanism of brain aging and it may also be involved in the pathogenesis of neurodegenerative disorders including Alzheimer's disease (13) and Parkinson's disease (16). According to the membrane hypothesis of aging (39,41), chronic free radical damage may lead to changes in the composition and integrity of cell membranes. As a result, membrane fluidity decreases and intracellular density increases with age (40). This may finally lead to dramatic alterations of many membrane-bound mechanisms relevant for brain function (32). A specific example for such a mechanism are age-related deficits of the NMDA receptor system observed in many mammalian species, including man (17), monkeys (37), rats (12,33,37), and mice (4). These deficits have been suggested to contribute to age-related cognitive deficits, particularly to the pronounced cognitive impairments in Alzheimer's disease (5,25). An explanation for the age-related decline of NMDA receptor density may be changes of membrane fluidity as proposed by Müller (19). Accordingly, age-related NMDA receptor deficits might be one of the possible mechanisms linking the enhanced exposure of the aging brain to the damaging properties of free radicals (1) with the well-known decline of cognitive functions in many, but not all, aged individuals. According to this hypothesis, any pharmacological intervention protecting against enhanced free radical damage should improve biochemical and functional deficits of the aging brain (i.e., a treatment with appropriate radical scavengers may improve impaired cognitive functions in aging subjects). While this hypotheses is not new, only few supporting experimental data are available. There are even fewer data about the neurochemical mechanisms linking such a treatment with improvements in learning and memory. An improvement of radial maze performance was found in aged gerbils treated with the spin-trapping component *N-tert*-butyl-alpha-phenylnitrone (3). Ginsenoside Rg1 and Rb1 antagonize lipid peroxidation, act as oxygen radical scavengers, and improve one-trial avoidance learning in rats and mice (38). An improvement of reversal learning in a T-maze in middle-aged mice treated with centrophenoxine for 3 months was described (21,22). Lipofuscin accumulation in the frontal cortex and hippocampus of these mice was significantly reduced. The assumption that centrophenoxine acts mainly through its radical scavenger properties and not as a cholinergic precursor is

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supported by its effects on lipofuscin accumulation (11,21,22) and by the effects of the structurally similar substance BCE-001 on membrane properties (39).

α-Lipoic acid (α-LA) or thioctic acid (7) and its metabolite dihydrolipoate (26) are potent free radical scavengers with antioxidant properties. Our hypothesis that a treatment with an appropriate free radical scavenger improves age-related memory deficits, probably by restoring age-associated NMDA receptor deficits, was tested using a subchronic treatment of aged mice with α-LA. The aged NMRI mouse has been demonstrated to exhibit specific age-related deficits of cognitive functions (30,31) as well as deficits of NMDA receptor density (4). The possible effects of α-LA on several other neurochemical deficits of the aged mouse brain possibly relevant for cognitive functions were tested as well (14,19).

METHOD

Animals

All animals were purchased from Interfauna, Tuttlingen, Germany. Young female NMRI mice (3 months) had been transferred to our animal facilities 2 weeks prior to the beginning of the tests. Old female NMRI mice were received at the age of 12 months as retired breeders and were kept in our animal facilities from then on until they were 20 to 23 months old. All animals were screened for obvious pathologic changes like tumors, paresis, and cachexia before and after tests. Subjects with pathologic changes were not included in the statistical analysis.

Treatment

Animals received 100 mg/kg body weight α-LA dissolved in Methocel (1%) administered orally once per day for 15 days. Controls received Methocel alone. The treatment had started 14 days before the beginning of behavioral testing. During the first of 2 days of behavioral testing animals were treated after testing. On the second test day treatment was discontinued. A group of aged mice used for biochemical analysis was treated for 15 days as well.

Habituation in the Open Field

Habituation in the open field was carried out in a wooden box of 50 × 50 × 20 cm with white walls and white floor. The floor was divided in squares of 12.5-cm length. Horizontal movements were measured as the number of squares entered with the forepaws. Exploration time in the box was 3 min. Exposure to the box was repeated after 15 min and after 24 h. The same observer carried out all experiments. Untreated young and aged mice were tested on the same days for age comparisons. Treatment vs. control effects had to be assessed on different days for young and aged mice: it is not possible to test more than about 30 to 40 animals on the same day in the habituation design described without substantially increasing the variance of horizontal activities confounding analyses.

Binding Assay

Mice were killed by cervical dislocation, treated ones 24 h after the last treatment. Brains were removed immediately and frozen at a temperature of −20°C. Treated and control mice brains were prepared at the same time. In the following, experimental conditions for the NMDA receptor are described in detail. Experimental conditions for muscarinic, benzodiazepine, and α_2-adrenergic receptors are shown in Table 1. The frozen brains were dissected with a razor blade. Forebrains (including hippocampus) were weighed and homogenized in 15 ml ice-cold Tris-HCl buffer (pH 7.4 at 4°C, 5 mmol). After filling up the homogenate with Tris-HCl buffer to a volume of 40 ml, it was centrifuged for 25 min at 48,000 × *g*. The supernatant was discarded; the pellet was resuspended in the same buffer and centrifuged again for 20 min at 48,000 × *g*. This washing procedure was repeated once. After discarding the supernatant, the pellet was resuspended in the same buffer to give a final tissue concentration of about 4 mg original wet weight per ml. An aliquot of the homogenate was used for protein determination. The tissue homogenate (400 μl) was incubated at 20°C with 50 μl [^{3}H]MK-801 (final concentrations, see below) and 50 μl Tris-buffer (pH 7.4 at 4°C, 5 mmol) or Tris-buffer containing the blank (see below) for 90 min in the presence of 300 μmol/l glycine and 2 mmol/l glutamate. The incubation was terminated by rapid filtration through Whatman GF/C glass fiber filters under slight vacuum. Filters were washed three times with 3 ml ice cold Tris-buffer. The radioactivity on the filters was extracted with 4 ml scintillation fluid (Quickszint 402, Zinsser Frankfurt, Germany) and counted in a Beckman liquid scintillation counter (model LS 6800) at a counting efficiency of 40%.

TABLE 1

EXPERIMENTAL CONDITIONS FOR ASSAYING DENSITIES (B_{max}) OF MUSCARINIC, BENZODIAZEPINE, AND α_2-ADRENERGIC RECEPTORS IN DIFFERENT AREAS OF THE MOUSE BRAIN

					Incubation	
Receptor	Ligand	Range (nmol/l)	Blank	Area	Time (min)	Temperature (°C)
Muscarinic	[^{3}H]-*N*-methyl-scopolamine	0.06–2.6	atropine (10 μmol/l)	frontal cortex	45	25
Benzodiazepine	[^{3}H]-flunitrazepame	0.02–2.0	diazepam (10 μmol/l)	dorsal cortex	45	4
α_2 (total)	[^{3}H]-yohimbine	0.2 –5.0	phentolamine (10 μmol/l)	whole brain	30	25
α_2 (agonist)	[^{3}H]-UK-14304	0.1 –2.6	phentolamine (10 μmol/l)	whole brain	70	25

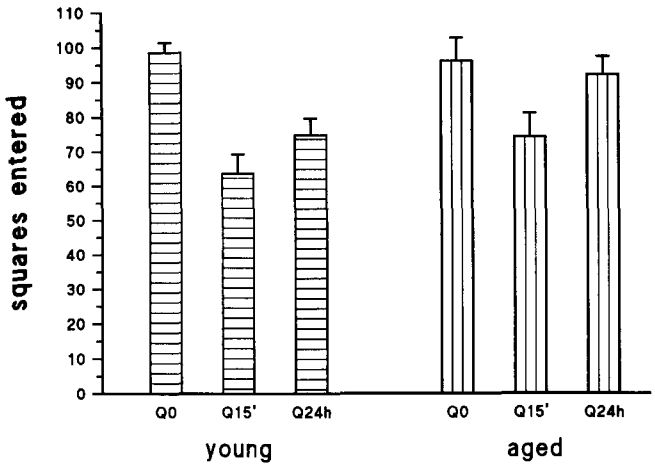

FIG. 1. Effects of age on the open field behavior of young and aged female NMRI mice. Q0 is the initial exploration activity, Q15′ the activity after 15 min, Q24h the activity after 24 h. All values are given with SEMs. The numbers of animals in each group are given in Table 2.

Calculations for the Binding Assay

Nonspecific binding was determined by parallel experiments in the presence of the blank (unlabeled (+)-MK-801 at a final concentration of 100 μmol/l for the NMDA receptor). It accounted for about 15–20% of total [^{3}H]MK-801 binding. Saturation experiments were carried out using eight concentrations of [^{3}H]MK-801 ranging from 0.3 nM to 15 nm. Dissociation constant (K_d) and maximal number of sites (B_{max}) were obtained from linear Scatchard plots by regression analysis. Protein concentration was determined according to Lowry (18) using bovine serum albumin as standard.

Preparation of Dissociated Brain Cells

Dissociated neurons were prepared according to Stoll and Müller (28). Animals were sacrificed 24 h after the last treatment. Brains were dissected immediately on ice. After removal of the cerebellum, brains were minced and suspended in ice-cold medium I (NaCl 138, KCl 5.4, Na_2HPO_4 0.17, KH_2PO_4 0.22, glucose 5.5, and sucrose 58.4, all mmol/l, pH 7.35). Tissue was dissociated by trituration through a nylon mesh (210 μm pore diameter) with a pasteur pipette and filtered through a tighter nylon mesh (102 μm pore diameter) by gravity. The resulting suspension (20 ml) was washed twice (400 × *g*, 3 min, 4°C) in 20 ml medium II [NaCl 110, KCl 5.3, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 25, sucrose 70, and *N*-2 hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) 20, all in mmol/l, pH 7.4].

PLC Stimulation Assay

The resulting pellet was resuspended in 6 ml medium III (like medium II, sucrose 40 mmol/l) and medium IV (like medium III, except $CaCl_2$ replaced by NaCl), respectively, for direct stimulation of PLC by F^- (15) and incubated for 20 min in a shaking water bath (37°C).

To quantify inositol phosphate metabolism, partly modified methods of Berridge et al. (2) were used. After washing, dissociated neurons were resuspended in 6 ml medium III (IV) and incubated with 25 μCi *myo*-[2-^{3}H(*N*)]-inositol (0.74 TBq/mmol) for 1 h. The incubation buffer was replaced by fresh, inositol-free medium and incubated for a further 15 min (37°C, shaking water bath) with LiCl (final concentration 10 mmol/l). Aliquots (460 μl) were placed in plastic tubes containing 20 μl atropine solution (final concentration 10^{-5}/ M) or 20 μl NaCl 0.9%, respectively, and were incubated again for 10 min. This step was omitted when NaF was used as stimulating agend. Stimulation was started by adding 20 μl of the agonist solution (carbachol 0.1 mmol/l, NaF 20 mmol/l). Stimulation was terminated after 60 min by adding 200 μl $HClO_4$, 20%, followed by sonification for 10 min. All experiments were performed in triplicate.

Samples were centrifuged for 5 min at 10,000 × *g* to separate precipitated protein and water-soluble metabolites. The residue was assayed for lipid extraction (see below), the aqueous phase was neutralized by precipitation of $KClO_4$ with KOH (10 N, titrated against phenol red), and freeze dried after centrifugation. The residue was dissolved in 100 μl distilled water and applied on columns containing Dowex-1 chloride ion exchange resin, 1 × 8–200. The columns were washed with 5 ml water and 16 ml 5 mM sodium tetraborate/60 mM sodium formate to elute inositol and glycerophosphoinositol. Inositol monophosphate ($InsP_1$) was eluted with 10 ml of 0.2 M ammonium formate/0.1 M formic acid. After a stimulation time of 60 min, no significant changes relative to baseline of bi- or polyphosphate concentrations were present (data not shown). Consequently, remaining polyphosphates were coeluted with 12 ml of 1.8 ammonium formate/0.1 M formic acid. The $InsP_1$ fraction was distributed in two vials, 13 ml Aquasafe 300 (Zinnser, Frankfurt, Germany) was added, and

TABLE 2

POST HOC COMPARISONS OF WITHIN-SUBJECTS OPEN FIELD ACTIVITIES IN UNTREATED YOUNG AND AGED MICE USING THE PAIRED *t*-TEST

	Comparison Between					
	Young			Old		
	p	*t*	*n*	*p*	*t*	*n*
Q0–Q15m	<0.0001	7.86	14	0.0011	4.01	16
Q0–Q24h	<0.0001	7.97		NS		
Q15m–Q24h	NS	2.22		0.0021	3.70	

Due to alpha-correction, the probability level for rejecting the null hypothesis was set to 0.017. Q0 is the initial exploration activity, Q15m the one in the trial after 15 min, Q24h the one after 24 h, *n* the number of animals per group.

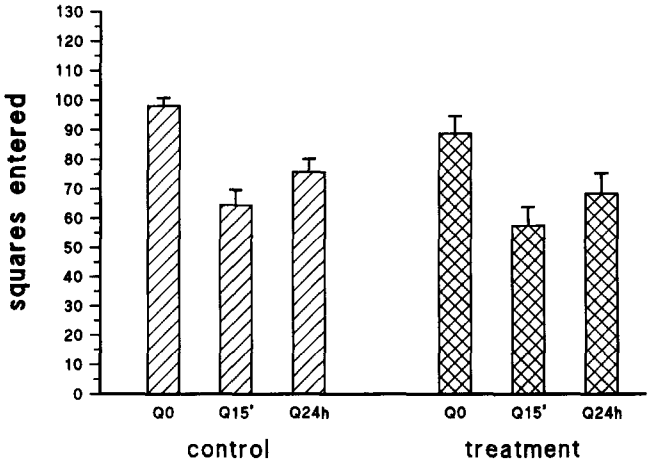

FIG. 2. Effects of treatment with α-lipoic acid on the open field behavior of young female NMRI mice. For an explanation of abbreviations see Fig. 1. The numbers of animals in each group are given in Table 3. All values are given with SEMs.

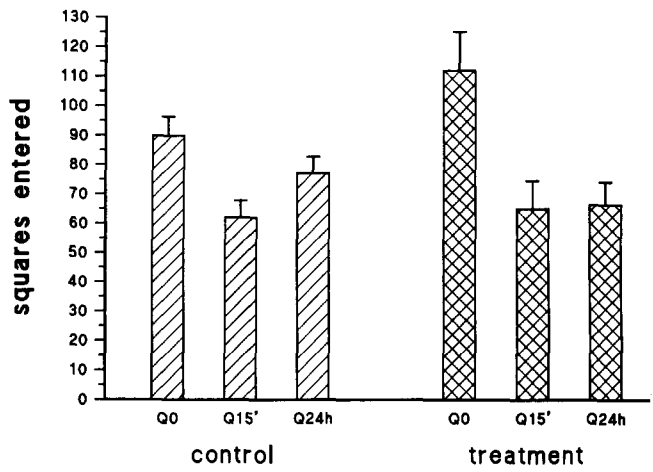

FIG. 3. Effects of treatment with α-lipoic acid on the open field behavior of aged female NMRI mice. For an explanation of abbreviations see Fig. 1. The numbers of animals in each group are given in Table 3. All values are given with SEMs.

radioactivity was determined on a Beckman liquid scintillation counter. To account for different counting efficiencies, cpm were transformed to dpm by quench correction.

To extract the radioactivity of the lipid fraction, the pellet was sonificated in 900 μl chloroform/methanol (1/2), acidified with 250 μl HCl 2.5 N, and further processed according to Fisher and Agranoff (8). After evaporation of chloroform, the remaining radioactivity was extracted in 4 ml Aquasafe 300 and determined as described above.

Stimulation was calculated as the ratio of $InsP_1$ accumulation to total incorporation ($[^3H]InsP_1$ + $[^3H]$ lipids). Basal accumulation (atropine and NaCl values) was subtracted.

Statistics

Statistical analyses were performed with SAS. The probability level for rejecting the null hypothesis was chosen to be 0.05. Since young and old animals were tested on different times (for reasons outlined in the Method section), no direct age comparisons were performed in the treatment groups.

RESULTS

Habituation in the Open Field: Age Effects

A highly significant repetition of trials effect could be found in ANOVA ($F = 33.86, 2\ df, p < 0.0001$) as well as a significant interaction between repetition of trials and age ($F = 4.27, 2\ df, p < 0.0187$). Data are shown in Fig. 1. Post hoc comparisons were performed with paired *t*-tests (Table 2) since the same subjects were tested three times. The horizontal activity of both young and aged animals declined significantly from the first trial to the second one after 15 min. In the third trial (24 h after the first one), activities of young mice remained close to 15-min activities. In aged animals, however, horizontal movements rose significantly above 15-min activities.

Habituation in the Open Field: Treatment Effects

A highly significant repetition of trials effect could be found in ANOVA for young (Fig. 2) and old (Fig. 3) mice (*F*

TABLE 3

POST HOC COMPARISONS OF WITHIN-SUBJECTS OPEN FIELD ACTIVITIES IN TREATED YOUNG AND AGED MICE USING THE PAIRED *t*-TEST

		Comparison Between					
		Young			Old		
Group		*p*	*t*	*n*	*p*	*t*	*n*
Control	Q0–Q15m	<0.0001	7.78	15	0.0001	7.40	19
	Q0–Q24h	<0.0001	7.21		0.0011	3.86	
	Q15m–Q24h	NS	2.41		0.003	3.43	
Treated	Q0–Q15m	<0.0001	6.87	10	0.0001	10.85	10
	Q0–Q24h	<0.016	2.97		0.0004	5.53	
	Q15m–Q24h	NS			NS		

Due to alpha-correction, the probability level for rejecting the null hypothesis was set to 0.017. Q0 is the initial exploration activity, Q15m the one in the trial after 15 min, Q24h the one after 24 h, *n* the number of animals per group.

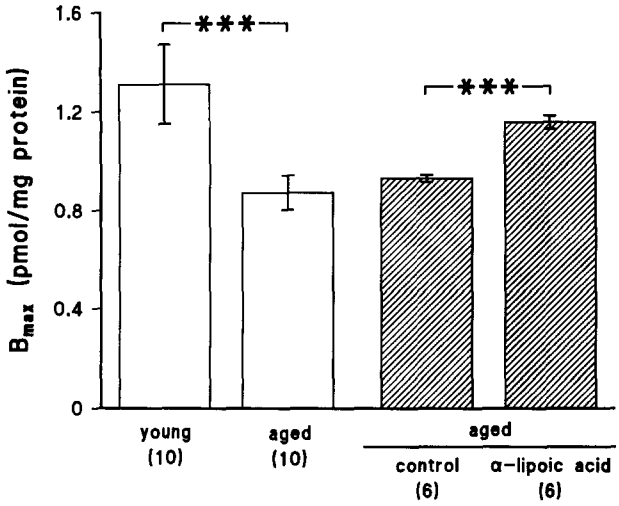

FIG. 4. Effects of age and treatment of aged animals with α-lipoic acid on the B_{max} of NMDA receptors in the frontal cortex of female NMRI mice. The data are mean ± SD of (n) determinations each representing an individual animal. ***Indicates a significant difference with $p < 0.0001$. For degrees of freedom and F-values see text.

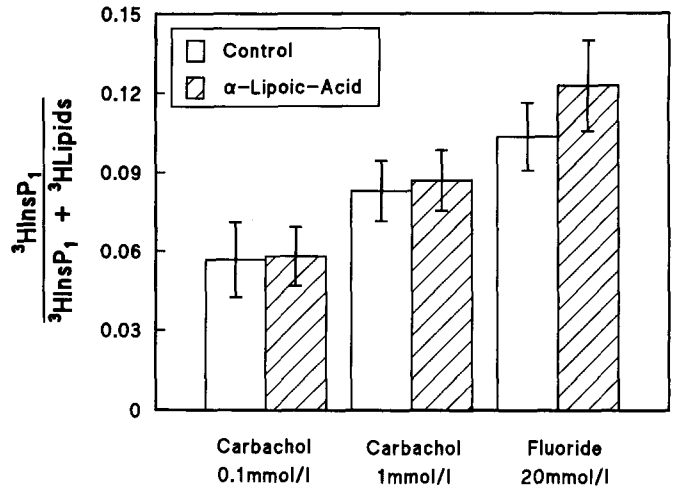

FIG. 5. Effects of α-lipoic acid on carbachol- and fluoride-stimulated accumulation of inositol monophosphates in dissociated brain cell aggregates (n = 8). There was no significant effect of treatment. Data are mean ± SD.

= 46.76, 2 df, $p < 0.0001$ for young and F = 63.33, 2 df, p 0.0001 for old subjects). Post hoc comparisons of activities with the paired t-test are shown in Table 3. Treated and control animals of both ages showed a pronounced decline of activity in the repetition after 15 min. Twenty-four hours after the first session, the activity of young treated and young control mice remained close to the 15-min activity (Fig. 2), whereas in old controls it was closer to the initial activity (Fig. 3). Yet in treated old animals activities after 24 h still were very close to the activities after 15 min (Fig. 3). Therefore, a highly significant interaction between repetition of trials and treatment could be found in aged animals (F = 11.20, 2 df, $p < 0.0001$) but not in young ones.

Neurobiochemistry

NMDA receptor densities of untreated young and aged mice as well as untreated and treated aged mice were compared with the t-test. They decline significantly with age, $t(18)$ = 7.568, $p < 0.0001$ (Fig. 4). The treatment of aged mice with α-LA led to a significant increase in the B_{max} $t(10)$ = 7.50, $p < 0.0001$, of the NMDA receptor without affecting K_d (Fig 4). No changes could be found in the K_d and B_{max} of muscarinic, benzodiazepine, and α_2-adrenergic receptors of aged animals after the same treatment (Table 4). Carbachol- and fluoride-stimulated accumulation of inositol monophosphates in dissociated brain cell aggregates of aged mice was not changed by treatment with α-LA (Fig. 5).

DISCUSSION

Learning is defined as a change in behavior caused by experience. This behavioral change leads to a repetition of trials effect in the ANOVA analysis of activities in the habituation in the open field procedure. Therefore, influences on learning and memory are expressed as interactions with this effect. In the present study, two factors influencing learning and memory have been under scrutiny: age and treatment with α-LA.

The effect of age alone was tested in the direct age comparison. Its results confirm our previous findings that long-term memory (but not shorter-term memory or learning) is impaired in aged female NMRI mice (30,31). Age effects on learning or memory are indicated by the interaction of age

TABLE 4

K_d AND B_{max} OF MUSCARINIC, BENZODIAZEPINE, AND α_2-ADRENERGIC RECEPTORS OF AGED ANIMALS AFTER TREATMENT WITH α-LIPOIC ACID

	Control		Treated	
Receptor Type	K_d (nmol/l)	B_{max} (pmol/mg prot.)	K_d (nmol/l)	B_{max} (pmol/mg prot.)
Muscarinic	0.99 ± 0.023	1.19 ± 0.13	0.093 ± 0.022	1.14 ± 0.07
Benzodiazepine	1.129 ± 0.0062	0.924 ± 0.194	1.060 ± 0.0068	0.991 ± 0.114
α_2-Adrenergic (total)	6.72 ± 3.02	0.074 ± 0.020	7.81 ± 2.44	0.093 ± 0.033
α_2-Adrenergic (agonist)	1.24 ± 0.31	0.078 ± 0.019	1.37 ± 0.15	0.100 ± 0.028

There was no significant effect of treatment on K_d and B_{max} values. Data are mean ± SD of 5–7 determinations each representing an individual animal.

with repetition of trials. No age effect could be found for learning and shorter-term memory: if learning or shorter-term memory were affected by age, horizontal activities in the second trial (after 15 min) should not differ from initial ones in aged animals. On the contrary, 15-min activities differed significantly from initial values in both young and aged mice. Impairments of long-term memory should result in an increase of 24-h activities compared to 15-min ones. This pattern could be found in aged, untreated mice, while young mice exhibited no deficit in long-term memory. Contrary to young animals, aged ones also showed no significant difference between initial and 24-h activities. The latter lack of difference, however, is not necessary to assume a deficit in habituation: mice have the opportunity to explore the open field in each activity measurement. Therefore, they have the experience of the open field in every measurement leading to a repetition of the initial learning experience in consecutive trials (i.e., 24-h activities should be influenced by the experience of the 15-min trial that repeated the learning experience of the initial trial). Not retaining or not retrieving the repeated experience leads to an increase of 24-h activities over 15-min activities. The lack of difference between initial and 24-h activities in aged, untreated mice only tells that the deficit in retention or retrieval was very pronounced by age.

An effect of treatment with α-LA on memory could be demonstrated in aged animals, but not in young ones. As outlined above, treated young and aged animals have not been treated simultaneously for procedural reasons. Because the exploration activity of female NMRI mice varies to some extent between experiments (31), the habituation data of treated young and old mice cannot be compared directly in the present experiment (see above). However, an indirect comparison of the course of exploration activities in the different groups of mice can be drawn: the lack of a significant difference between the 15-min and 24-h activities of treated old mice parallels the performance of young, untreated animals as outlined above (Tables 2 and 3). Aged controls (Table 3), on the other hand, show a long-term memory deficit similar to the old animals of the age comparison (Table 2) (i.e., a significant difference between 15-min and 24-h activities). The significant difference of initial and 24-h acitivities in aged treated and control mice indicates that long-term memory deficits in the treatment experiment were not as pronounced as in the age comparison. It is possible that the oral treatment over 15 days may have had an unspecific effect comparable to environmental enrichment (6). The lack of any treatment effect in young, treated mice suggests that α-lipoic acid compensates age-related, long-term memory deficits rather than improving memory in general. However, the lack of a direct comparison of young and aged treatment groups due to procedural reasons leaves these results as tentative.

The biochemical basis for the memory-enhancing effect of the free radical scavenger α-LA is not known as yet. Since this effect could only be observed in old animals, a specific mechanism associated with age-related cognitive deficits as the primary target may be suggested. According to this hypothesis, we have examined to which extent the treatment with α-LA restores biochemical deficits in the aged mouse brain possibly relevant for impaired cognitive performance. We have already described reductions of muscarinic cholinergic (9,24), NMDA (4), benzodiazepine (20), and α_2-adrenergic receptors (10). On the second messenger level we found an age-related decline in the carbachol-induced stimulation of phosphoinositide hydrolysis (29). The possible relevance of these biochemical deficits of the aging brain for the age-related decline of cognitive functions and for the mechanism of action of cognition-enhancing drugs has been discussed in detail elsewhere (14,19). In these studies, we have usually observed ameliorating effects of the cognition enhancers investigated on receptor properties in aged but not in young animals. Accordingly, we restricted our preliminary experiments about the possible effects of α-LA on receptor properties to aged animals. The deficit in NMDA receptor density found in aged mice was partially compensated. While this association does not prove that α-LA improves memory by enhancing NMDA receptor density, the coincidence is striking nevertheless, preliminary due to a lack of data on the effects of α-LA in young mice. In this context, it is of interest that the only common effect of three different cognition enhancers tested in the same receptor models of the aged mouse brain was an elevation of NMDA receptor density (4,14,19), supporting the tentative coincidence. It may be supported further by a close correlation between the degree of cognitive impairment and the reduction of NMDA receptor density that has been reported in the rat (23). Thus, our findings are consistent with the assumption of a causal relationship between the beneficial effects of α-LA on memory and NMDA receptor density in aged mice.

In summary, our results give tentative support to the hypotheses i) that there may be a causal link between α-LA's positive effects on memory and its improvement of NMDA receptor density, and ii) that these effects may be mediated by its free radical scavenging activity. Further treatment experiments using more robust or less time-consuming behavioral tests combined with neurobiochemical measurements for several age groups all done simultaneously over all age groups are necessary to support or to disprove these hypotheses.

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