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N-Methyl-D-Aspartate Receptor Density and Membrane Fluidity as Possible Determinants of the Decline of Passive Avoidance Performance in Aging

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SCHEUER, K., S. STOLL, U. PASCHKE, R. WEIGEL AND W. E. MÜLLER. N-*Methyl-D-aspartate receptor density and membrane fluidity as possible determinants of the decline of passive avoidance performance in aging.* PHARMACOL BIOCHEM BEHAV **50**(1) 65–70, 1995.—The effect of aging on three different parameters possibly relevant for cognition was investigated in female Naval Medical Research Institute mice: a) *N*-methyl-D-aspartate (NMDA) receptor density, as determined by the specific binding of [^{3}H]MK-801 to forebrain membranes, decreased by 22% in aged (23 mo) and by 19% in middle-aged (12 mo) animals compared with young (3 mo) animals. b) In a passive avoidance acquisition task, the 24-h latency decreased significantly with age; the middle-aged mice also tended to show impairment in this task. c) The fluidity of the forebrain membranes also decreased significantly with age. Again, there was a significant reduction in the middle-aged group. A comparison of these parameters revealed significant correlations between NMDA receptor density and 24-h latency ($r = 0.52$, $p < 0.003$) over all three age groups, as well as significant correlations between membrane fluidity and either NMDA receptor density or 24-h latency. These findings do not prove a causal relationship, but are compatible with the hypothesis that changes of membrane fluidity, by decreasing the number of NMDA receptors, affect passive avoidance performance.

N-Methyl-D-aspartate receptor [^{3}H]MK-801 binding Aging Passive avoidance learning Membrane fluidity

N-METHYL-D-ASPARTATE (NMDA) receptor-mediated glutamatergic neurotransmission appears to be extremely important for learning, memory, and other cognitive functions in animals and humans (1,6,10,13,23). Accordingly, the blockade of NMDA receptor activation by competitive and noncompetitive antagonists profoundly impairs learning and memory in a variety of animal models (3,23,24,35). In many ways, cognitive deficits induced in young animals by the administration of NMDA receptor antagonists mirror age-associated cognitive deficits in the same animal species (1,16, 28,33). Thus, deficits of NMDA receptor-mediated glutamatergic neurotransmission have been postulated to be pathomechanisms of age-associated deficits of cognitive functions in animals (5,12,16,28) and humans (8,20,27,30).

A fairly consistent finding of a decrease of cortical and hippocampal NMDA receptors by about 20–30% in aging has been described for many animal species and humans (28–30), although the extent of the receptor loss varies among species and even between two mouse strains (29). In one of these studies, a significant relationship between NMDA receptor density and learning ability was found (28), suggesting reduced cognitive performance of the aged animals (rats) because of reduced NMDA receptor number (28). Although this positive relationship between individual learning ability and NMDA receptor density seems to be supported by findings in rats with high or low learning performance (19), conflicting data have also been reported (16).

Previous work from this laboratory has demonstrated an

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age-related decline of NMDA receptor density in the forebrain of female Naval Medical Research Institute (NMRI) mice (5). The present study reports to what extent this receptor loss is paralleled by deficits of passive avoidance learning using mice of three different age groups. Because changes of the fluidity of neuronal membranes with aging have been implicated in the age-related loss of central muscarinic cholinergic receptors (18,25), we also investigated the possible relationship between NMDA receptor density, passive avoidance performance, and membrane fluidity.

METHOD

Materials

Young (3 mo), middle-aged (12 mo), and aged (23 mo) female NMRI mice were obtained from Interfauna (Tuttlingen, Germany). [^{3}H]MK-801 (tritiated [+]-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclopotenten-5,10-imine hydrogen maleat) was obtained from New England Nuclear (Dreieich, FRG) (specific activity 25.7 Ci/mmol). Unlabeled (+)-MK-801 was purchased from Research Biochemicals (Natick, MA). All other chemicals were obtained from commercial suppliers.

Behavioral Testing

The Plexiglas passive avoidance (PA) box was divided into two compartments (dark and bright) by a sliding guillotine door. Both compartments had a metal grid floor. The mice were placed on the bright side of the PA box and the door was opened after 30 s (habituation). The latency to enter was measured (L-naive). In a training trial on the next day the procedure was repeated, again measuring the latency (L-before). Right after the mice entered the dark chamber, an electrical stimulus of 0.8 mA was delivered through the grid floor for 1 s. The animals remained in the dark compartment for approximately 30 s until they returned to the illuminated compartment. After 30 s, the door was opened again and the latency to enter was recorded as a measure of short-term memory acquisition (L-60 s). The retention test of the acquired memory was performed 24 h after the training trial, and step-through latency (maximum 300 s) was measured. (The higher the entry latency the better the retention performance.)

Binding Assay

The mice were decapitated. The brains were removed and immediately frozen at −20°C for at least 24 h. After thawing, the forebrain (including striatum, hippocampus, and overlying cortex) was homogenized in 15 ml of 5 mmol/l Tris-HCl buffer, pH 7.4, at 4°C and centrifugated for 20 min at 48,000 × *g*. The supernatant was discarded and the pellet was washed twice under the same conditions and frozen for at least 24 h. For the final incubation, the pellet was resuspended in the same buffer to give a tissue concentration of about 4.6 mg wet wt./ml. Saturating levels of glutamate and glycin (100 μmol/l each) were added to all binding assays to eliminate possible binding variability attributable to changes in the concentration of these amino acids that are known to affect the accessibility of NMDA-associated channel blockers such as MK-801 to their binding site (17,31). Triplicate aliquots of 350 μl tissue-suspension, 50 μl radioligand, 50 μl buffer or displacer, and 50 μl glutamate/glycin solution (each 1 mmol/l) were incubated at 21°C for 90 min, after which time binding was in equilibrium. Binding was terminated by rapid filtration through Whatman GF-C filters (Herolab, Wiesloch, Germany) under slight vacuum. Nonspecific binding was defined by 100 μmol/l (final concentration) of unlabeled (+)-MK-801 and accounted for 15--20% of total binding at low ligand concentrations.

The saturation experiments were always carried out using eight different [^{3}H]MK-801 concentrations ranging from 1–25 nmol/l. Data for dissociation constant (K_d and maximal number of binding sites (B_{max}) were calculated from Scatchard plots by linear regression analysis using the EBDA-LIGAND program (26).

Protein concentration was determined according to the Lowry method (22), using bovine serum albumin as the standard.

Membrane Fluidity Measurements

The individual forebrain homogenates, already prepared for the [^{3}H]MK-801 binding assays, were used for determination of membrane fluidity by fluorescence spectroscopy using the hydrophobic 1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescence probe (15). The protein content of each sample was determined by the method of Lowry et al. (22). The samples were diluted with 5 mmol/l Tris-HCl buffer, pH 7.4, to give 30 μg protein in 100 μl.

Samples (450 μl) were incubated with 4050 μl 5 mmol/l Tris-HCl buffer, pH 7.4 , 4500 μl of a 1 : 150 DPH-solution and 1000 μl Tris-HCl buffer for 60 min at 37°C, after which time the anisotropy was stable. The DPH solution was prepared from a stock solution of 5 mmol/l DPH in tetrahydrofuran by 1 : 150 dilution with 5 mmol/l Tris-HCl buffer, pH 7.4. The anisotropy was directly measured in an SLM 4800C Aminco spectrofluorometer (Urbana, IL) using excitation and emission wavelengths of 360 and 450 nm, respectively. Anisotropy measurements were determined in triplicates of each sample, with each measurement representing the average of 100 readings. The steady-state fluorescence polarization (P_s) was also expressed as the anisotropy (r_s) of the probe, using the following equation (21):

$$r_s = 2P_s/3 - P_s.$$

Statistics

For statistical analysis (two-tailed *t*-test, ANOVA, correlation analysis), we used the SAS-package (Cary, NC). Behav-

TABLE 1

EFFECT OF AGING ON DENSITY OF NMDA RECEPTORS IN THE FOREBRAIN OF FEMALE NMRI MICE

Age (mo [n])	B_{max} (pmol/mg protein)	K_D (nmol/l)	n_H
3 (12)	1.69 ± 0.08	2.89 ± 0.5	1.04 ± 0.08
12 (8)	1.38 ± 0.09*	2.82 ± 0.6	1.07 ± 0.07
23 (10)	1.31 ± 0.05*	3.73 ± 1.2†	1.03 ± 0.04

Data for dissociation constants (K_D), maximal numbers of sites (B_{max}), and Hill coefficients (n_H) were obtained from saturation experiments using [^{3}H]MK-801 as a radioligand, as described in METHODS. All experiments were performed in the presence of L-glutamate and glycin (each 100 μmol/l). Data are means ± SD of (*n*) experiments, each representing an individual animal.

*$p < 0.001$ vs. 3 mo.

†$p < 0.05$ vs. 3 of 12 mo.

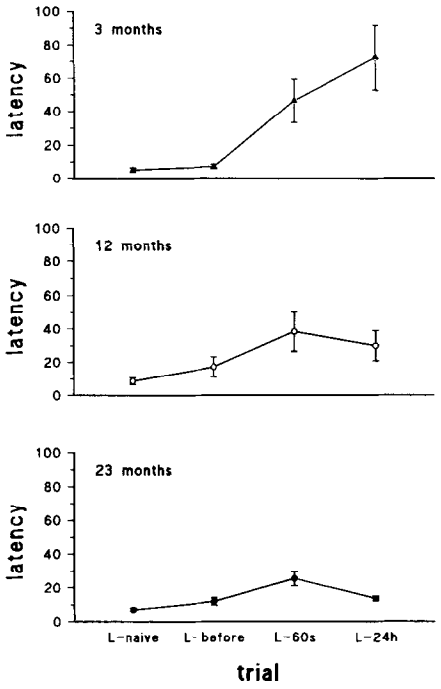

FIG. 1. Effect of aging on the behavioral acquisition response of female NMRI mice in a passive avoidance task. Measurements were recorded as described in METHODS. For statistical analysis, see text. For the number of animals in each age group, see Table 1.

ioral testing, binding assays, and fluidity measurement were done by different experimentors, who were blinded to the other experimental data.

RESULTS

In accordance with previous findings (5), we observed a highly significant age-related decrease of [^{3}H]MK-801 binding to membrane homogenates of the forebrain of the NMRI-mouse (Table 1). Hill coefficients close to unity indicate one single population of [^{3}H]MK-801 binding sites. The K_d were 2.89 ± 0.5 and 2.82 ± 0.6 nmol/l at 3 and 12 mo, respectively, and increased to 3.73 ± 1.2 nmol/l at 23 mo.

We confirmed a decline of B_{max} with age. The B_{max} of the middle-aged animals had declined by 19% of the maximal binding found in young animals and by 22% in the aged animals.

The behavioral assessment in this study was included to evaluate a possible relationship between the acquisition of passive avoidance learning (Fig. 1) and the density of NMDA receptors in aged, middle-aged, and young mice. As is seen in Fig. 1, the 60-s and 24-h latencies after electrical stimulus are increased compared with the L_{before} values, indicating that the animals might remember the stimulus as a negative experience that should be avoided. Multivariate analyses of this behavioral task over all three age groups revealed a highly significant effect of repeated measures ($p < 0.0001$, $F = 11.57$, $df = 3$), an almost significant effect of age ($p < 0.052$, $F = 3.22$, $df = 2$), and a highly significant interaction of repeated measures × age ($p < 0.0017$, $F = 3.90$, $df = 6$) (Fig. 1). Posthoc analyses indicated that the decrease in 24-h latency, normally used as an indicator for long-term memory capacity,

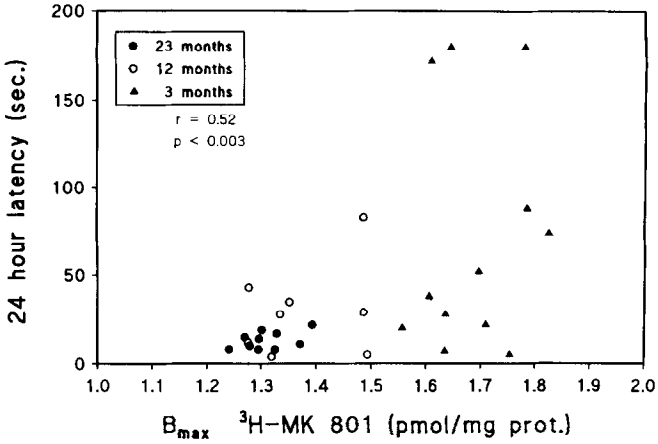

FIG. 2. Pearson correlation between 24-h latency of the passive avoidance aquisition task (Fig. 1) and B_{max} of specific [^{3}H]MK-801 binding in the forebrain for young, middle-aged, and aged NMRI mice. Each point represents an individual animal.

was significantly different between the young and the aged mice (Bonferroni t-test: $t = 3.11$, $p < 0.0125$). Even though the middle-aged mice did not exhibit a statistically significant decrease (Bonferroni t-test) in 24-h latency compared with young mice, the individual data given in Fig. 2 suggest an already-present impairment of memory capacity in several animals of the middle-aged group, which would fit the decrease of B_{max} values described earlier.

A correlation of the maximal number of binding sites (Table 1) and the passive avoidance latencies 24 h after the electrical stimulus (Fig. 1) reveals a highly significant relationship ($r = 0.52$, $p < 0.003$) (Fig. 2).

As a third parameter, we also determined the fluidity of the forebrain membranes used for the NMDA receptor density determination. Fluidity is given as anisotropy of the fluorescent probe DPH inside the membrane. The individual values are shown in Fig. 3. We found a highly significant age-related

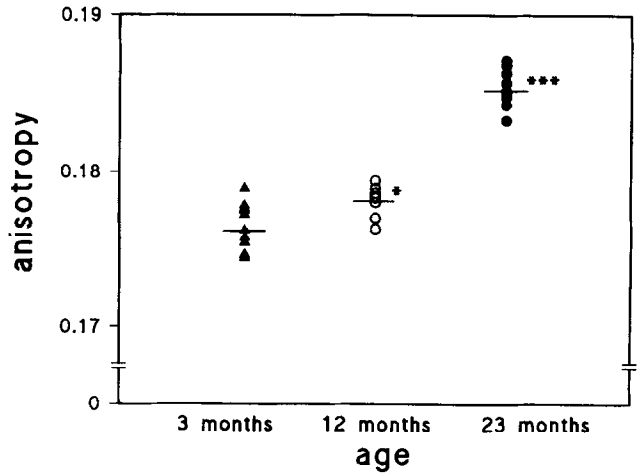

FIG. 3. Effects of age on DPH anisotropy in forebrain membranes of female NMRI mice. Membranes were prepared and measured as described in METHODS. ***$p < 0.001$; *$p < 0.05$. For the number of animals in each age group, see Table 1.

decrease ($p < 0.001$) in membrane fluidity (as indicated by the increased anisotropy) between young and aged, or middle-aged and aged animals, respectively. Even the middle-aged mice exhibited a significant decrease in membrane fluidity ($p < 0.05$) compared with young animals.

Because a remarkable relationship between B_{max} values and 24-h latencies was observed, we compared the anisotropy values with binding as well as behavioral data. As indicated in Figs. 4 and 5, we observed highly significant negative correlations between anisotropy and B_{max} (Fig. 4) and anisotropy and 24-h latency (Fig. 5). However, these correlations are mainly valid for the young and middle-aged animals, for which relatively small changes of anisotropy correlate significantly with both parameters. It appears that the pronounced additional decrease of membrane fluidity present in the aged group (increase of anisotropy) is not accompanied by similar changes of either NMDA receptor density or 24-h latency (Figs. 4 and 5).

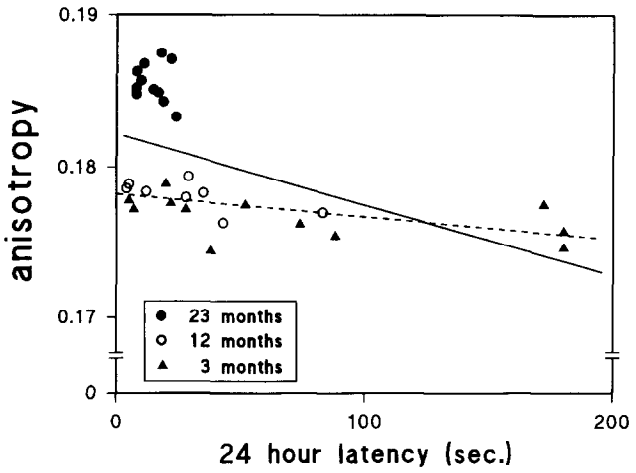

FIG. 5. Influence of age on the correlation between passive avoidance learning (24-h latency) and membrane fluidity. Correlation was done over all three age groups ($r = -0.52$, $p < 0.003$; solid line) as well as for young and middle-aged animals ($r = -0.58$, $p < 0.01$; dashed line). For details see Figs. 1 and 3.

DISCUSSION

This study confirms the age-related decline of NMDA receptor density in the forebrain of female NMRI mice (5). It is interesting that a major part of the age-related loss of NMDA receptors was already present in middle-aged (12-mo-old) animals. There was also a small but significant increase of K_d of [^{3}H]MK-801 binding with aging. Over all three groups, the K_d correlated significantly with anisotropy ($r = 0.47$, $p < 0.01$). This is consistent with the assumption that the access of the ligand to its binding site inside the NMDA gated channel is slightly hindered as a result of decreased fluidity, resulting in reduced affinity (31,32).

Deficits of passive avoidance learning are well documented for aged mice (9,11,34). This has also been confirmed by additional experiments with female NMRI mice (Stoll et al., in preparation), in which we have demonstrated a superiority of the passive avoidance paradigm over other learning models when demonstrating age-related cognitive deficits in this species. Our observation that long-term memory (24-h latency in our model) is more affected by aging than acquisition (60-s latency in our model) also parallels other findings in passive avoidance paradigms for aged rodents (11). Passive avoidance impairment of our female NMRI mice already seemed to be present at an age of 12 mo, with some further deterioration after one more year of age. Again, these observations agree with previous findings (11).

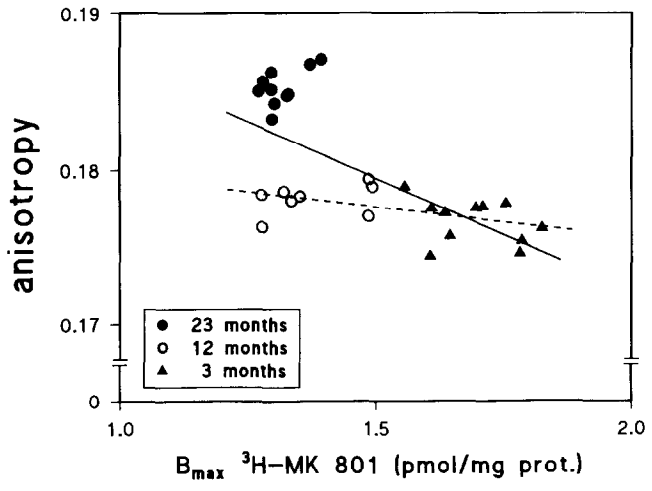

FIG. 4. Influence of age on the correlation between B_{max} of specific [^{3}H]MK-801 binding and membrane fluidity. Correlation was done over all three age groups ($r = -0.70$, $p < 0.001$; solid line) as well as for young and middle-aged animals ($r = -0.49$, $p < 0.03$; dashed line). For details see Table 1 and Fig. 3.

The significant decrease of 24-h latency in the middle-aged mice was paralleled by a pronounced decrease of NMDA receptor density. This is remarkable and contrasts with the work of Strong et al. (34), in which a significant deterioration of cognitive functions of 1-yr-old mice was not paralleled by a similar reduction of cholinergic markers. Cholinergic deficits were only present in this latter study in the very old age group (26 mo) (34). In our experiments, the impression that the deficit of passive avoidance performance and the decrease of NMDA receptor density are related is further supported by the highly significant relationship between 24-h latency and B_{max} of specific [^{3}H]MK-801 binding (Fig. 2) over all age groups investigated. Positive correlations between learning ability and NMDA receptor density have also been reported by Pelleymounter et al. (28) for aged rats and by Keller et al. (19) for rats with inborn high or low learning ability. By contrast, a negative relationship between learning performance and NMDA receptor number has been reported by others in aged rats (16), although there was also an age-related decrease of receptor number. However, in these other studies NMDA receptor binding was only determined using a single concentration of [^{3}H]L-glutamate. Some evidence exists that suggests an increase in the affinity of L-glutamate for the NMDA receptor in aging (2,5), probably as an adaptive response to the reduced receptor density (5). Thus, the results cited above (16), using only one radioligand concentration for receptor determination, might be explainable by the age-related decline of NMDA receptor density superimposed by the enhanced affinity of L-glutamate during aging. Nevertheless, taking all data together, the evidence suggests a direct relationship between NMDA receptor density and learning performance. However, we cannot completely discount the alternative explanation that aging affects NMDA density and learning per-

formance in a similar fashion, without both parameters being related.

The biochemical basis by which aging affects NMDA receptor number and cognitive functions is not known. Studies on the biochemical mechanisms of age-related changes of density and function of central α_1-adrenergic, β-adrenergic, dopaminergic, and muscarinic cholinergic receptors have suggested a crucial role of the fluidity of the neuronal membrane (4,14,18,25). Some preliminary findings suggest that NMDA receptor properties can be significantly altered by various fluidizing agents (7,32). This links the age-related loss of neuroreceptors with the "membrane hypothesis of aging," which assumes that a variety of changes of the composition of brain membranes, such enhanced lipid peroxidation, enhanced cholesterol to phospholipid ratios, and enhanced cross-linking of structural proteins, finally affect brain function by altering the properties of transmembrane proteins. Although all of these changes of membrane composition might alter fluidity, the specific mechanisms relevant for the receptor deficits are not yet specifically identified. Observations indicating that receptor deficits can easily be reversed in vitro by membrane fluidization (18,25) suggest that fluidity changes per se, rather than changes in the composition of membrane fractions of different fluidity, are involved.

The results of the present investigation support the conclusion that small changes in membrane fluidity correlate inversely with NMDA receptor density. This conclusion, however, probably only holds true for the small changes of membrane fluidity that are observed between young and middle-aged animals, which correlate highly significantly with receptor number. There is an additional large decrease of membrane fluidity in the aged animals, which, however, does not seem to have further effects on receptor density. Accordingly, the small changes of the overall membrane fluidity already present in the middle-aged animals could represent either the functionally relevant part of a continuum or a different alteration of membrane properties from the pronounced changes in membrane fluidity that are observed in the aged animals.

The 24-h latency similarly correlated with membrane fluidity when comparing the young and middle-aged animals. This could simply confirm our initial hypothesis that changes in membrane fluidity, by decreasing the number of NMDA receptors, affect passive avoidance performance. However, we cannot completely refute the alternate explanation that age-related alterations of membrane fluidity affect passive avoidance learning and receptor number independently.

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